Development of an EBOV-GP virus entry system and investigation of the anti-EBOV activity of an extract of *Prunella vulgaris*

and

Investigation of the regulatory role of IL-34 in macrophages and CD4+ T lymphocytes during HIV-1 infection

by

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ABSTRACT

My aspiration to enter graduate studies was based on my interest to comprehend the proper molecular and immune mechanisms of HIV infection to explore new targets which can be developed for future anti-HIV therapies. Meanwhile, one area that I am also particularly interest in is applying HIV virological techniques and knowledge to explore the efficient way for controlling other serious infectious diseases, like Ebola. The research presented in this thesis aims to understand the role of a novel interlukin IL-34 in HIV infection, and develop an Ebola glycoprotein (gp)-pseudotyped HIV-based vector system to screen drugs, followed by characterizing its anti-Ebola properties.

Currently, no approved antiviral therapeutic is available for the treatment or prevention of Ebola virus (EBOV) infection. In this study, we developed an EBOV-glycoprotein (GP) pseudotyped HIV-1-based vector system for the screening of anti-EBOV-GP agent(s). Based on this system, we demonstrated that an aqueous extract (CHPV) from the Chinese herb *Prunella vulgaris* displayed a potent inhibitory effect on EBOV-GP pseudotyped virus (EBOV-GP-V)-mediated infection in HUVECs (Human Umbilical Vein Endothelial cells) and macrophage cell lines. In addition, our results indicated that CHPV was able to block the wild type Ebola virus infection in VeroE6 cells. The anti-EBOV activity of CHPV was exhibited in a dose-dependent manner. At a 12.5 μg/ml concentration, the compound showed a greater than 80% inhibition of EBOV-GP-V and EBOV infections. Likewise, our studies suggested that the inhibitory effect of CHPV occurred by binding directly to EBOV-GP-Vs and blocking the early viral events. Interestingly, our results have shown that CHPV was able to enhance the anti-EBOV activity of the monoclonal antibody MAb 2G4 against EBOV-GP. Overall, this study provides evidence that CHPV has anti-EBOV activity and may be developed as a novel antiviral approach against EBOV infection.

The another project of my study is focused on investigation of the stimulating effect of

IL-34 on HIV-1 infection in MDMs (monocytes-derived macrophages) and CD4 T lymphocytes. Our study has revealed that IL-34 was able to elevate the T-cell tropic HIV-1 replication in PBMCs, but IL-34 as well as M-CSF does not affect the susceptibility of CD4+ T cells directly. However, our study showed that IL-34 or M-CSF stimulated macrophages to release some unknown soluble factors, which are able to stimulate CD4+ T cells and render them more susceptible to HIV-1 infection. In conclusion, our study has provided evidence for a better understanding of the important role of IL-34 in both macrophages and CD4+ T lymphocytes during HIV-1 infection.

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LIST OF ABBREVIATIONS

aa	amino acid
AAV	adeno-associated virus
ADAM	adisintegrin and metalloproteinase
AIDS	acquired immunodeficiency syndrome
ALV	avian leukosis virus
APCs	Antigen Presenting cells
ART	antiretroviral treatment
ARV	AIDS-related virus
BCV	Brincidofovir
BSL-4	biosafety level 4 containment
C/EBPb	CCAAT/enhancer binding protein b
CA	capsid protein
CHPV	Chinese PV aqueous extracts
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CSF-1	colony stimulating factor 1
DCs	dendritic cells (DCs)
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DG	Diammonium glycyrrhizinate
DIS	dimer initiation site
DMEM	Dulbecco’s modified Eagle’s medium
EBOV	Ebola virus
EBOV-GP	EBOV-glycoprotein
EBOV-GP-Vs	EBOV-GP pseudovirions
EHF	Ebola hemorrhagic fever
ELISA	enzyme-linked immunosorbent assay
Env	envelope glycoproteins
EVD	Ebola virus disease
FBS	fetal bovine serum
FCS	fetal calf serum
Gluc	Gaussia luciferase
GP	glycoprotein
HAART	highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
HIV-D	HIV-associated dementia
HIV-Env-Vs	HIV-Env+ pseudovirions
hPBMCs	Human Peripheral Blood Mononuclear Cells
HSV-1	herpes simplex virus-1

HSV-2	herpes simplex virus-2
HTLV-1	human T-cell leukaemia virus
HUVECs	Human Umbilical Vein Endothelial cells
IFN	interferon
IN	Integrase
IRES	internal ribosome entry site
kDa	Kilodalton
LAV	lymphadenopathy-associated virus
LEDGF/p75	lens epithelium-derived growth factor
LTR	long terminal repeat
MA	matrix antigen
MAB	monoclonal antibody
MAPK	MAP kinase
M-CSF	Macrophage colony stimulating factor
MDMs	Monocytes-derived macrophages
MIP-1a	macrophages inflammatory protein 1 alpha
MLD	mucin-like domain
MTCT	mother-to-child-transmission
M-tropic	Macrophage-tropic
NC	nucleocapsid protein
Nef	negative factor
NHP	nonhuman primates

NPC1	Niemann-Pick type C1
ORFs	open reading frames
p.i.	post-infection
PBS	primer binding site
PC	positive controls
PIC	pre-integration complex
pis	packing signals
PMOs	phosphorodiamidate morpholino oligomers
PPT	polypurine tract
PR	protease
pre-GP	precursor molecule of GP
PTP-ζ	protein-tyrosine phosphatase ζ
PV	Prunella vulgaris
RBD	receptor-binding domain
Rev	regulator of expression of virion protein
rhIL-34	Recombinant Human IL-34
rhM-CSF	Recombinant Human M-CSF
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	reverse transcription complex
SAMHD1	SAM domain–and HD domain–containing protein 1
SD	standard deviations

sGP	secreted Ebola virus glycoprotein
ssGP	small, soluble Ebola virus glycoprotein
TACE	TNFa-converting enzyme
TAR	Tat-acting region
Tat	transactivator
TIM-1	T-cell immunoglobulin and mucin domain 1
TLR4	toll-like receptor 4
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
VR	virological synapses
VSV-G	vesicular stomatitis virus G
VSV-G–Vs	VSV-G pseudovirions
WB	Western blot
WST	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
ZEBOV	Zaire ebolavirus

CHAPTER 1

Introduction

1.1 Ebola virus

1.1.1 Epidemiology and clinical syndrome

Ebola virus (EBOV) is a 19 kb enveloped, negative-strand RNA virus belonging to the Filoviridae family that is the etiological agent of severe viral hemorrhagic fever in humans and non-human primates, commonly referred to as Ebola hemorrhagic fever (EHF) with case fatality rates as high as 90% [1]. EBOV was first identified in 1976 and resulted in approximately 430 deaths with a high fatality rapid spread illness in northern Zaire and southern Sudan, and since then over 20 reported natural Ebola virus outbreaks have occurred, primarily in the tropical regions of Saharan Africa [2]. That includes the 1995 outbreak that killed 245 individuals in Kikwit, and the outbreak in Uganda that happened in 2000 and caused 224 death, and *etc.* [3, 4]. Recently, the 2014-2015 Ebola outbreak in West Africa was the largest of all, causing over 11,000 deaths out of the over 28,000 people infected, in spite of the numbers are certainly underestimated because of the difficulties in epidemiological data reporting[5].

The primary transmission route of EBOV is direct contact with the blood or other bodily fluids of infected people or animals. As such, sexual practices, patient care, burial practices and infected bushmeat consumption, reuse or improper using medical equipment like syringes *et al*. can amplify the risk of transmission [6]. After 1-3 weeks of incubation, a flu-like illness is an initial symptom before severe EHF, like fever, fatigue and sore muscle [7]. However, after disease onset, the viral load may reach 108

copies/mL in patient blood, resulting in manifestations of EHF symptoms, like abdominal pain, vomiting, diarrhea, especially hemorrhages and coagulation abnormalities[8]. Eventually, the death usually was caused by multi-organ failure and hypovolemic shock that occurred within 6-16 days after initial symptom detection [7].

1.1.2 Disease pathogenesis

Based on previous pathogenesis studies, the productive infection of EBOV in its initial target cells, DCs, monocytes/macrophages is considered to responsible for causing the morbidity of EBOV infections, which contribute to the transportation of virus to lymph nodes via the lymphatic system, and to the spleen or liver through the blood circulation system [9]. Following the infection of macrophages and hepatocytes, the organ damage and hemorrhagic effects of EBOV will be contributed by the pro-inflammatory cytokines released from infected monocytes/macrophages, like TNF and IFN-γ, which can cause the disruption of vascular endothelium architecture. The loss of endothelial barrier and vascular dysfunction will lead to hemorrhagic effects and result in EBOV fatalities. In addition, the inflammation induced by infection of macrophages and neutrophils activation also is considered important contributor to facilitate the disease progression.

1.1.3 Treatment and prevention

The EVD outbreak has stimulated investigation of several different therapeutic strategies that target specific viral structures and mechanism of EBOV. Potential

therapeutic strategies include: 1) Nucleotide analog candidate therapies: T-705, a pyrazinecarboxamide derivative, has been shown to be efficacious against ZEBOV (Zaire Ebolavirus) *in vitro* and *in vivo*. Animal trials to evaluate the efficacy of T-705 against EBOV are underway [10]. Another potential therapy is brincidofovir (BCV), which was developed as an antiviral against double stranded DNA viruses and EBOV [11]. Unfortunately, the BCV human trial was stopped in Liberia because the manufacturer withdrew support in February of 2015. Two other novel antiviral compounds that act on RNA viral polymerase, JK-05 and BCX4430, also recently received attention as potential treatments for EBOV infections and animal studies have been completed or are ongoing [12]; 2) RNA-Silencing Molecules and antisense oligomers: A lipid nanoparticle/siRNA referred to as TKM-Ebola, is now entering human clinical evaluation in Guinea [13, 14]. Using antisense phosphorodiamidate morpholino oligomers (PMOs) to target viral mRNA sequences, two specific PMOs (AVI-6002 and AVI-6003) have undergone phase I clinical trials [13]; and 3) Immunotherapeutics: A novel immunotherapeutic (ZMapp) consists of a combination of monoclonal antibodies (c13C6, c2G4 and c4G7), optimized from two previous antibody cocktails (MB-003 and ZMAb) is able to rescue 100% of rhesus macaques when treatment is initiated up to 5 days post-challenge [15-18]. During 2014, ZMapp was also administered to seven patients with EVD in the United States and other countries, of these 2 died [19, 20]. The outcome is not considered to be statistically significant [21]. Moreover, very little of the ZMapp antibodies is currently available. Mapp Biopharmaceutic announced in September 2014, that supplies of ZMapp had

been exhausted [22]. The lack of drugs and unavailability of experimental treatment in the most affected regions of the West African Ebola virus outbreak have spurred some controversy. During the outbreak, several vaccine candidates were also evaluated in human trials [23]. Currently, two candidates employed in Phase II/III efficacy trials are the most promising clinically advanced vaccines, ChAd3-EBO Z vaccine and rVSV-ZEBOV vaccine, respectively. For ChAd3-EBO Z vaccine, it is a defective chimpanzee adenovirus type 3-vectored vaccine, engineered to express EBOV GP, that has been shown safety and immunogenicity in human Phase I trials [24, 25]. The rVSV-ZEBOV vaccine is derived from a glycoprotein deleted vesicular stomatitis virus, expressing a Zaire strain EBOV GP, which has demonstrated a 70-100% protection efficacy in preventing EBOV infection post-vaccination by its clinical trail in Guinea published in 2016 December [26]. However, several serious adverse events were reported recently, and how long the benefits of vaccination last is still unclear [27]. Therefore, even though several Ebola virus disease (EVD) therapeutic agents are now entering accelerated human trials in EVD-endemic countries, development of a new antiviral therapy that is available to populations that are at great risk for EVD is still an important global health need.

1.1.4 Ebola glycoprotein

1.1.4.1 Glycoprotein gene products

The EBOV glycoprotein (GP) gene normally contains seven consecutive adenosine nucleotides in a predicted hairpin loop, which encodes for the precursor of secreted GP

(sGP) (Figure 1). Due to the L polymerase stutters at the GP editing site during transcription, it can result in an additional adenosine residue insertion into mRNA at this site in 25% of transcripts, leading to the reading frame shift and producing a precursor molecule of GP (pre-GP). Additionally, a small soluble glycoprotein (ssGP) will be produced, while addition or deletion of an extra adenosine residue at the same site. Totally, the transcription chance result of pre-sGP, pre-GP and ssGP are 70%, 25%, and 5%, respectively [28]. In the present study, we focus on the GP that is cleavage of pre-GP by furin. GP as a 676-amino acid, structural transmembrane glycoprotein is inserted into the viral envelope, approximately 150-170 kDa in size depending on different glycosylation level. It consists of a surface unit GP1 (140 kDa) and a transmembrane unit GP2 (26 kDa). GP1 contains four different domains: base, receptor-binding domain (RBD), glycan cap and mucin-like domain (MLD). The mature GP is highly glycosylated and forms a trimer of GP1/GP2 heterodimers. The N- and O-linked glycans cover the surface of envelope GP, which play a role in virus adherence and it is also important for shielding the GP from neutralizing antibodies [29, 30].

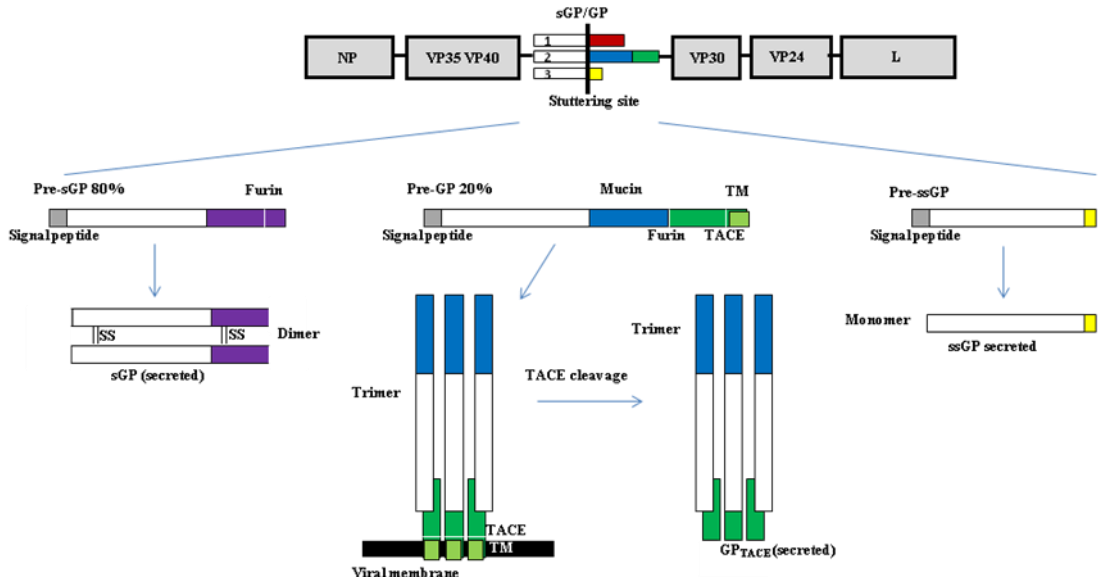

Figure 1: Transcription and processing of EBOV glycoproteins
sGP (shown as white and red rectangles) is encoded by the primary open reading frame of Ebolavirus GP gene. The produced pre-sGP is cleavage by furin results in the generation of a mature form of sGP and a Δ-peptide which is a small nonstructural fragment. The other two types glycoproteins: the production GP and ssGP are resulted by the GP gene co-transcriptional stuttering. GP as the virion-attached glycoprotein is matured via cleaving its precursor pre-GP. This cleavage results in the two subunits, GP1 and GP2 that is resulted by the cleavage, is linked by a disulfide bond and heterodimer trimerized as viral surface peplomer. Then, the soluble shed GP is released via cleaving the trimeric GP by TACE. ssGP as a small secreted glycoprotein is generated as a monomer. (Adapted from Lee JE *et al*., Future Virology 2009)

1.1.4.2 Ebola GP mediated viral entry

The EBOV glycoprotein (GP), which is a trimeric structure comprised of three monomers of GP1 and GP2, is an essential component of the viral envelope and plays a central role in viral entry [31] (Figure 2). EBOV GP1 is required for cell attachment and receptor interaction, while GP2 is required for membrane fusion. Some attachment factors on surface of target cells have been demonstrated to contribute to GP1 mediated 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 internalization of virus into cell endosomes via macropinocytosis [32-34]. Within a low pH environment of vesicles, the heavily glycosylated regions of GP1 are removed by cellular cysteine proteases, like cathepsins B and L, which induces the conformational change of GP to reveal binding domain to interact with the Niemann-Pick type C1 (NPC1) receptor [35]. Coincidently, the hydrophobic fusion loop of GP2 is exposed, which is responsible for viral and cellular membranes fusion to allow the entry of virus into the cytoplasm [36, 37]. Therefore, the EBOV-GP provides a potential target for therapeutic strategies to disrupt EBOV-GP-mediated virus entry and block the propagation of EBOV.

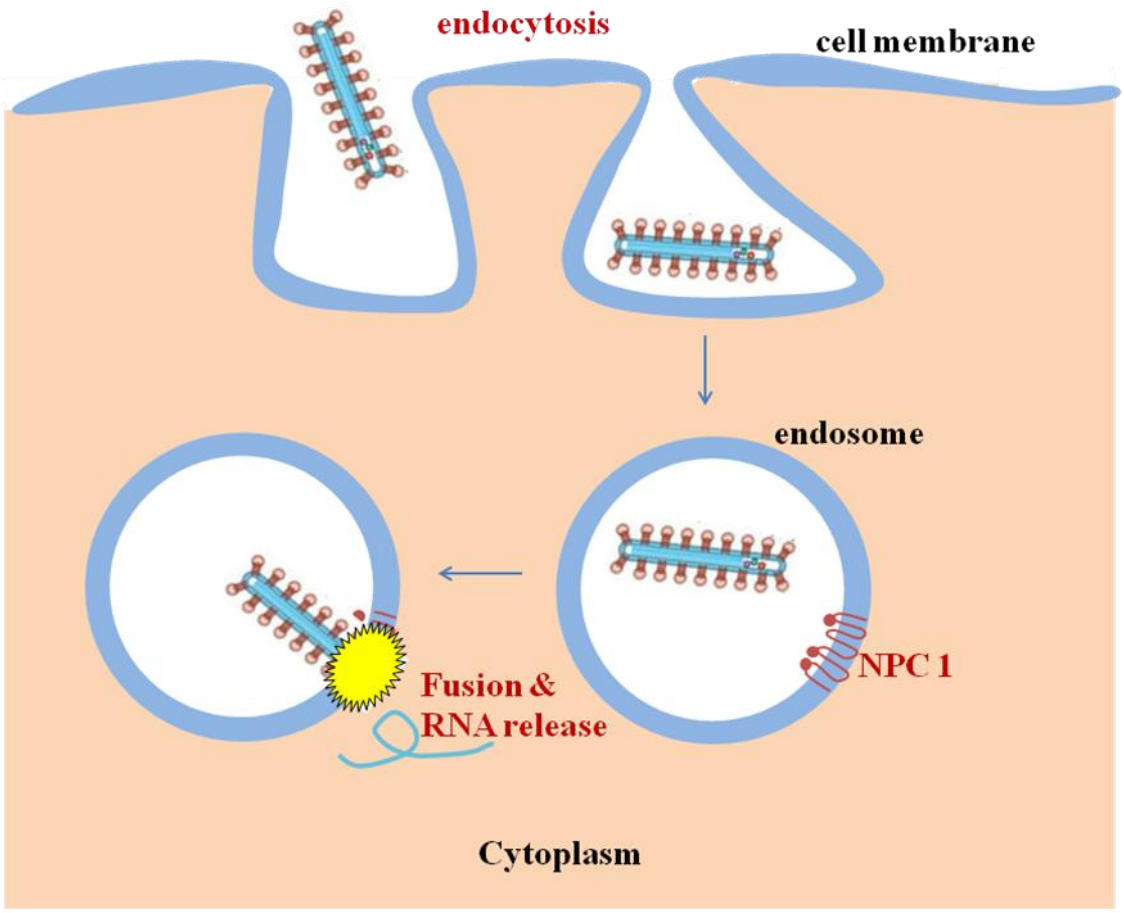

Figure 2: The model of Ebola virus entry
The tropism of EBOV for target cells occurs after attachment via the internalization of the EBOV virion into endosomes by macropinocytosis. Within the endosomes, GP is processed by cellular cysteine proteases, like cathepsin B, L, which results in exposing its receptor NPC1 binding sites on GP1, and inducing GP2 mediated membrane fusion and entry of virus into host cells. (Adapted from Carette JE *et al*., Nature 2011)

1.1.4.3 Ebola GP mediated Immune activation and increased vascular permeability

A unique feature of EBOV is that following infection, virus-encoded GPs are released from cells in soluble forms. High levels of soluble GP (sGP) and truncated surface GP are detected in the blood of patients and experimentally infected animals [38, 39]. In addition to GP, sGP and ssGP, the cleavage of surface GP at the membrane-proximal external region by the cellular metalloprotease TACE (TNFa-converting enzyme), a member of the ADAM (adisintegrin and metalloproteinase) proteinase family, also releases a shed GP that resembles virion GP1,2. [40]. Shed GP, sGP likely competes with virion-attached GP for antibody binding [41]. Most of the antibodies derived from EBOV survivors or macaques are directed towards sGP or/and shed GP rather than the virion-attached GP and those neutralizing antibodies that cross-react between sGP and /or shed GP and virion-GP may be absorbed by the much more abundant sGP [42, 43]. In a guinea pig model of EBOV infection, shed GP inhibits the neutralizing activity of EBOV antibodies [39].

Besides this antibody-neutralizing activity, the role of soluble GP in virus replication and pathogenicity has not yet been clearly defined. However, a recent study demonstrated that shed GP, but not sGP, activates human-derived DCs and macrophages and induces the secretion of pro- and anti-inflammatory cytokines. This activation can be diminished by anti-toll-like receptor 4 (TLR4) antibodies or by addition of sera lectin MBL, suggesting an interaction between shed GP and DCs and macrophages, and that TLR4 is likely to be an important cellular partner for this

binding. This study demonstrated the ability of shed GP to affect endothelial cell permeability both directly and indirectly through cytokine release. The above observations with shed GP provide a potential link between systemic inflammation and increased endothelial permeability that together contribute to the EVD disseminated intravascular coagulation syndrome[44]. Overall, soluble GP, especially shed GP, plays a particular role in high viral pathogenicity and should be combated with new therapeutic strategies.

1.1.5 Traditional Chinese medicine

Traditional Chinese medicine holds a unique position among all traditional medicines because of its many years of history. Many aqueous extracts of traditional Chinese medicinal herbs have been proven to have antiviral activities [45], and most of these are generally of low toxicity, cheap and readily accessible. Some Chinese herbs, including *P. vulgaris, L. Panaxginseng C.A. Meyer*, *Andrographis paniculata*, *Lentinus edodes* and *Spirulina platensis*, display inhibitory effects on the entry step of various viruses, such as HIV-1, Hepatitis B and HSV-1 [46-61]. However, whether these herbs could also have inhibitory activities against EBOV infection remains unknown.

1.1.6 *Prunella vulgaris*

1.1.6.1 General properties of *P. vulgaris*

Prunella vulgaris (PV), with almost 15 known individual species, is widely distributed in China, Europe, northwestern Africa and North America, and it is known as a

self-healing herb [62]. In China, PV has been used as an orally administered herbal medicine for treatment of various infections and/or a self-healing herb for at least hundreds years [63-70]. It has also been used to treat the patients with high blood pressure, headaches, lymphatic system disorder, goiter, tuberculosis and tumors [62]. It was popular in traditional European medicine in the 17th century for treating sore throat, reducing fever and accelerating wound healing.

1.1.6.2 Therapeutics Potential of *P. vulgaris* in Ebola disease

PV extracts are reported to have antiviral and anti-bacterial properties [71-74]. The Chinese PV aqueous extracts (CHPV) has been shown to contain highly pure anionic polysaccharides by HPLC analysis and exhibit potent antiviral activity against HIV-1 [71, 73, 75], herpes simplex virus-1 and -2 (HSV-1, HSV-2) [72, 74, 76]. Polysaccharides are known to affect the growth of animal viruses. The antiviral properties of anionic polysaccharides are expected to depend upon their complexation with virus proteins, which is likely to occur mainly through the formation of salt linkages or ion pairs between oppositely-charged groups on the polymers and virus proteins [77]. Polysaccharides can inhibit the viral life cycle at different stages or directly inactivate virions before virus infection, as follows: 1) polysaccharides might inhibit virus infection via direct actions on the virus surface by its negative charge, which makes the viruses lose the ability to infect cells, and thus effectively reduce virus multiplication [78, 79]; 2) some polysaccharides can interfere with the viral adsorption process either by interacting with virions or by interacting with virus receptors on the

host cell surface; and 3) polysaccharides can also interfere with virus internalization and uncoating by blocking the allosteric process of virus particles. The aqueous extracts of PV can decrease the replication of HSV by preventing the virus from binding to cells [72, 74, 76], and it inhibits HIV-1 infection through preventing viral attachment to the CD4 receptor and/or post-virion binding events [71, 73, 75]. This suggests that PV may have a potent antiviral activity against other enveloped virus, such as EBOV.

Therefore, the natural plant, PV, warrants further investigation as a promising antiviral, and because it is a natural and readily-available source, its cost is low, and it has low cytotoxicity, wide acceptability and a broad spectrum of antiviral properties.

1.2 Human Immunodeficiency Virus Type 1 (HIV-1)

1.2.1 The General Description of HIV-1

Human immunodeficiency virus (HIV) is an enveloped, (+)ssRNA virus, which belongs to the *Retroviridae* family and the genus *Lentivirus*, and causes the acquired immunodeficiency syndrome (AIDS). The most of *Retroviridae* family virus are most primitive and highly diverse. In 1908, avian leukosis virus (ALV) which can cause the chicken leukosis, was identified by a Danish veterinary term. After that, the other virus, called Rous sarcoma virus (RSV), that caused sarcoma in chicken was first identified by Peyton Rous in 1911 [80]. In the following decades, *Retroviridae* family virus were found in various other species, including mouse, cattle, cat and primates. The first human retrovirus was identified in 1980, called human T-cell leukemia virus (HTLV-1) [81, 82]. In the subsequent period, the acquired immunodeficiency syndrome (AIDS) was rapidly expanded in various parts of the world, like Western Europe, Australia,

United States and *et al.*. The hypothesis that infectious agent, most likely a virus, was responsible for the AIDS was suggested in 1982. A complex and unusual retrovirus, called HTLV-3 was initially identified by Dr. Luc Montagner in 1983[83]. At the same time, the same retrovirus was isolated and named by different groups, including lymphadenopathy-associated virus (LAV), AIDS-related virus (ARV) [84, 85]. HTLV-3 was renamed as HIV-1, and proved to be the etiologic virus responsible for AIDS disease. In total, there were two subtypes of HIV reported previously, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-2 spreading is restricted to a few western African countries. However, HIV-1 infection has been spread all over the world.

HIV-1 is predominantly acquired via sexual contact with infected individual [86]. Some other HIV-1 transmission routes have been identified, including exposure to blood, using contaminated injection needles and mother-to-child-transmission (MTCT) [87]. After initial infection, HIV-1 spreads into draining lymph nodes within few days, and then disseminates throughout the patient body [87, 88]. The active CD4+ T lymphocytes are preferential targets for HIV-1. Meanwhile, HIV-1 acute infection is able to induce the CD4+ T cells massive depletion. Besides, the prolonged chronic infection also induces the CD4+ T cells number decline and accelerates immune dysfunction. Then, the progressive immune deficiency leads towards to the high risk opportunistic infections and results in AIDS disease progression [89-91].

At moment, no approved protective vaccine and curative treatment are available for HIV-1 infection control. In spite of the antiviral therapies has been able to effectively control viral replication and greatly prolong life expectancy, especially highly active

antiretroviral therapy (HAART) [92]. While the rapid occurrence of drug-resistance strains and reservoir establishment have remained the most important challenges to viral eradication[93, 94]. Therefore, developing new anti-HIV-1 strategy is still urgent priority in the HIV-1 research field. Furthermore, knowledge of the HIV-1 associated immune regulation would greatly contribute to a better understanding of the mechanisms underlying AIDS disease.

1.2.2 The Epidemiology of HIV-1 Infection

Since the primary recognition of HIV-1, it has rapidly expanded throughout the world to become one of the major global health threats. Globally, since the beginning of the AIDS epidemic, 78 million people have been infected by HIV-1 and 35 million patients died because AIDS related illnesses. Meanwhile, the latest estimates of the epidemic indicated that 36.7 million people were still living with HIV−1 in 2016 (from Joint United Nations Program on HIV/AIDS). Although HIV-1 prevalence is variable across different countries or regions, the majority of patients were getting HIV-1 infection in low- and middle-income countries. Especially, sub-Saharan Africa is the most affected, with nearly 25.5 million people being HIV-1 positive. In 2015 alone, there were 2.1 million people who became newly infected by HIV-1 worldwide and roughly 150,000 were children. However, the number of HIV-1 positive people accessing antiretroviral treatment (ART) only is 17 million, which means only 46% of adults patients and 49% of children patients are receiving ART. Without the adequate preventive measures and curative therapies, HIV-1 still is a daunting global health challenge.

1.2.3 HIV-1 Virology

1.2.3.1 HIV-1 Classification

Based on the full length HIV-1 genome sequencing, HIV-1 is comprised of four highly divergent lineages, named as group M, O, N and P [95, 96]. Among all the known lineages, group M is the predominant pandemic group in every country and is responsible for most of HIV-1 infection cases [97]. Group M can be divided into 11 major clades, including A1, A2, B, C, D, F1, F2, G, H, J, and K. These subtypes or clades of HIV-1 have been recognized and distributed relatively evenly worldwide. Especially, the clade B predominant spreads in North America and Europe, but is barely circulates in Africa [98]. At moment, the clade C is the most prevalent HIV-1 group, and responsible for roughly 50% of infections.

Compared with HIV-1， HIV-2 also contains 50-60% nucleotide sequence difference with HIV-1 in genetic properties, except the geographically restriction of HIV-2. Besides, HIV-2 induced AIDS present slower disease progression and longer survival than HIV-1 infected patients. Furthermore, in both of vertical and horizontal transmission, HIV-2 shows greatly lower than that of HIV-1 does [99, 100]. Therefore, HIV-1 will be mainly discussed in the present study.

1.2.3.2 HIV-1 structure and genome

HIV-1 virion is around 100-120nm in diameter and spherical shape (Figure 3). It is coated by a lipid envelope, that contains the lipid bilayers derived from the cell

membrane and viral envelope glycoproteins (Env) [101]. On the surface of each mature HIV-1 virion, Env glycoproteins are formed from surface protein gp120 and the transmembrane protein gp41, and inserted into lipid bilayers and display in knobbed spike structure. Gp120 and gp41 bind to each other noncovalently and form the heterodimers [102]. The component of virion central core is Gag structural proteins, including p24, p17 and p7. The capsid protein(CA) p24 forms the cone-shaped core; the p17 constitutes the inner surface of the viral envelope; and the nucleocapsid protein(NC) p7 is located in the core as viral RNA binding partner [103]. Inside of the core, some other viral enzymes and accessory proteins are associated with the viral genome [104]. HIV-1 genome is a 9.8 kilobase pairs (kbp) positive single-stranded RNA molecule. As shown in (Figure 4), 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, Gag constitute the viral coat and inner core respectively. The Env precursor gp160 is encoded by Env gene, and 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). Besides, the remaining six non-structural proteins also are encoded by HIV-1, which contain four accessory proteins and two regulatory proteins, including 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 that are involved in regulating viral replication by affecting various steps of 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 polypurine tract (PPT) [106, 107].

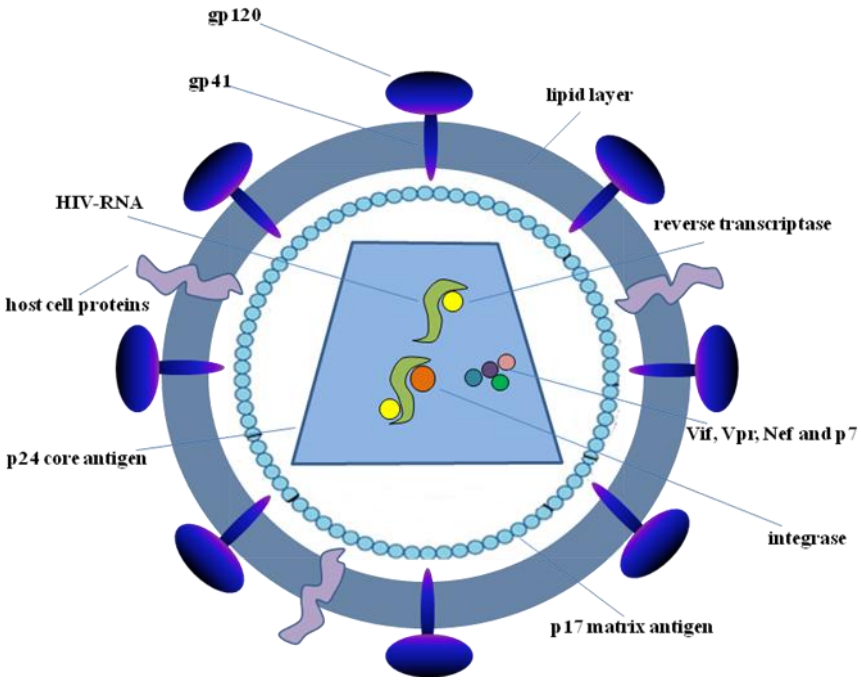

Figure 3: The structure of HIV-1
HIV-1 virus is enveloped by host cell proteins associated lipoprotein membrane that is embedded by Env glycoproteins complexes composed by two subunits, gp120 and gp41. The inner surface of HIV-1 envelope anchors into p17 matrix antigen (MA). Both of MA and capsid protein(CA) p24 compose the cone-shaped core of virion. The nucleocapsid protein(NC) p7 is located in the core as the binding partner of viral RNA which also interacts with RT and IN. Also within the core are the accessory proteins, Vif, Vpr, Nef, and p7. (Adapted from Hoffmann *et al*., HIV book 2012)

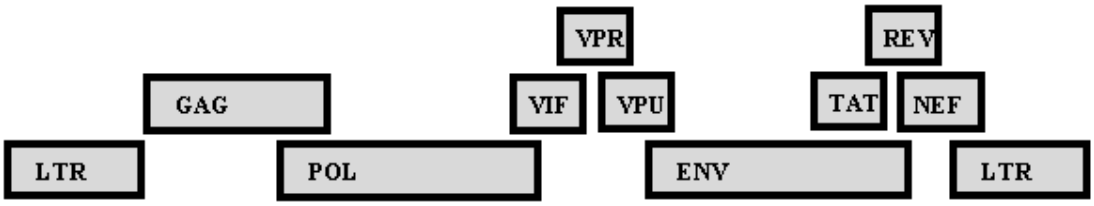

Figure 4: Genomic organization of HIV-1
HIV-1 genome organization and various viral protein synthesis, are showed at the schematic diagram. HIV-1 genome contains 9 ORFs regions with two flanked LTRs. The MA, CA, NC, PR, RT and IN, are generated via the cleavage process of Gag and Gag-pol polyproteins mediated by PR. The Env gene region encodes for the gp120 and gp41. In addition to the structural and enzymatic proteins, HIV-1 genome also encodes for Vpr, Vpu, Vif, Nef, Rev and Tat. (Adapted from Hoffmann *et al*., HIV book 2012)

1.2.3.3 HIV-1 proteins

HIV-1 structural proteins

HIV-1 Env glycoprotein is present as a gp120 and gp41 complex. Gp120 is responsible for the virus entry mediated by binding to the cell surface receptor [108]. As such, gp120 is a principle target for developing neutralizing antibody treatments or vaccine strategies. However, the variable region of gp120 trends to exhibit a high mutation rate, and therefore plays an important role for escape immune recognition. Gp41 as the transmembrane glycoprotein mediates the fusion between virus and cell membrane after viral attachment [109].

The 55 kDa precursor (Pr55-Gag) is encoded by Gag, and is further cleaved to generate MA, CA, p2, NC, p1 and p6. These structure proteins are very important for 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 [110], during the viral assembly. Meanwhile, due to MA located beneath the lipid bilayers, it as the important virion structure protein provides structural support to the envelope. Furthermore, because of MA as a part of reverse transcription complex (RTC) and pre-integration complex (PIC), it also involves in the nuclear migration, nuclear import and integration [111-113].

CA as a component of viral core is responsible for providing structural support to HIV-1 core and protects viral genome RNA. Additionally, the interactions between CA domains facilitate Gag multimerization and formation of the immature virion in the viral assembly process [114]. During the uncoating step, CA also has been known to

manipulate the capsid stability and disassembly to regulate the core uncoating.

NC as the nucleic acid binding protein, its major function is to select genomic RNA and deliver them into progeny virion by binding to the packaging signal (Ψ) of genomic RNA [115, 116].

HIV-1 enzymatic proteins

During the HIV-1 maturation, three enzymatic proteins PR, RT and IN are produced via auto-activating and cleaving 160kDa Gag-Pol polyprotein (Pr160-Gag-Pol) by viral PR. PR as a homodimer aspartic protease, its major activity is cleaving viral precursor proteins. In addition, PR also contributes to viral cytotoxicity effect by targeting some host cellular proteins, like cytoskeletal proteins, Bcl-2[[117, 118].

RT as an asymmetric heterodimer is consisted by two subunits 66kDa and 51kDa [119, 120]. The primary function of RT is to catalyze the reverse transcription of HIV-1 genomic RNA into cDNA. Besides, RT also has some other distinct functions, including DNA-dependent DNA polymerase, RNase H, strand transfer and strand displacement synthesis [121, 122].

IN as a tetramers enzymatic proteins is responsible for promoting the integration of the viral DNA into host chromosomal DNA [123]. In addition to integration, IN also involves another step of viral life cycle, including reverse transcription, nuclear import and post-integration steps[124].

HIV-1 accessary proteins

Accessory proteins are important for HIV-1 replication and pathogenesis of AIDS disease [125-128]. Vpr is a 14kDa protein that plays multifunctional roles in viral replication, such as HIV-1 nuclear import, cell cycle arrest, apoptosis. Firstly, Vpr makes cell cycle arrest to facilitate the viral transcription from long terminal repeat (LTR), which contributes to enhancing HIV-1 progeny virus production. Besides, the apoptosis of CD4+ T lymphocytes mediated by Vpr is through to contribute to patient immune suppression and progress AIDS disease pathogenesis.

Vpu has various important functions in favoring HIV-1 replication. Vpu degrades CD4 receptor synthesis and down-regulates MHC class I expression to contribute to immune escape. Meanwhile, Vpu contributes to enhance the virion detachment from the cell surface to promote the progeny virion release [129]. Additionally, some studies also found that Vpu down-regulated the expression of BST-2 to promote the viral release. Because BST-2 as an identified cellular antiviral factor inhibits progeny viruses release from cell membrane via β-TrCP and endo-lysosomal trafficking [130, 131].

Nef is a myristoylated protein 27kDa in size. Similar to Vpu, Nef also down-regulates CD4, CXCR4 and CCR5 receptor to avoid superinfecton [132, 133]. It also mediated the down-regulation of MHC-I expression to contribute to the immune evasion of virus reservoirs from cytotoxic T lymphocytes mediated cell lysis [134]. Moreover, studies found that Nef manipulates the cellular signal transduction of cytoskeletal remodeling and vesicular transport to facilitate viral replication.

Tat and Rev as two important regulatory proteins, play crucial roles in multiple viral replication steps. Tat and Rev are abundantly synthesized at the early stage of viral

infection, and facilitate the viral transcription and unspliced viral mRNAs nuclear export, respectively [135].

1.2.3.4 HIV-1 Life Cycle

The life cycle of HIV-1 replication is a complex process. Briefly, it can be categorized as two phases: the early stage from viral entry (receptor binding and membrane fusion) to viral reverse transcription and integration; and the late stage including transcription of integrated DNA to virus assembly, budding and progeny virus maturation (Figure 5). HIV-1 initiates infection by using gp120 to bind the CD4 receptor and CCR5/CXCR4 co-receptor at the cell surface to mediate successful entry step. The gp120 and CD4 interaction trigger the conformational change of gp120, resulting in exposure the binding sites of co-receptor CCR5/CXCR4. Following the CD4 and co-receptor binding, gp41 further facilitates the fusion of cell membrane with viral envelope lipid bilayers to release the viral core into the cytoplasm [136, 137]. Following the entry step, CA protein undergoes the disassembly process and is uncoated from the core to release the viral genomic RNA. Then, the released HIV-1 RNA associates with proteins to form reverse transcription complex (RTC). The reverse transcription occurs within RTC and is catalyzed by the RT to reverse transcribe viral RNA into complete double stranded cDNA. The newly synthesized viral DNA is transported into nucleus with the help of pre-integration complex (PIC), which is constituted by viral proteins, including IN, MA, NC, Vpr and CA; and some known cellular proteins, such as Ku70 HSP70, lens epithelium-derived growth factor (LEDGF/p75) and etc [138-140]. Following nuclear

import, the IN catalyzes the integration of HIV-1 cDNA into the cellular genome as provirus. This process is called integration, please refer to the following review for the details [141].

For the late stage of HIV-1 replication, it begins with integrated proviral DNA as the template to start transcription. RNA polymerase II catalyzes the transcription initiation by binding to the HIV-1 LTR. Meanwhile, Tat as the transcriptional activator promotes the transcription efficiency from LTR. The generated large amounts of various viral mRNAs are classified into three groups. First one is unspliced RNAs, which serve as progeny virion genomic RNA and mRNA template for Gag and Gag-Pol polyprotein synthesis [142, 143]. Following the full-length unspliced RNAs transcription, the incompletely spliced mRNAs encode for Env, Vpu, Vpr and Vif synthesis. Both of unspliced and partially spliced mRNAs are exported from nuclear to the cytoplasm with the help from Rev [142]. The third class is completely spliced short mRNAs, which encode Tat, Nef and Rev proteins. Following the nuclear export of transcribed viral mRNAs, all viral protein is synthesized, and initiates progeny virion assembly process. Gag plays important roles in multiple essential events during virus assembly. The association of Gag polyprotein initiates the HIV-1 assembles on the plasma membrane. Moreover, Gag recruits viral genomic RNA and other viral protein to encapsidate the progeny viral particles, and facilitates virus budding. Shortly after immature virus released from the plasma membrane, the further cleavage of Gag and Gag-Pol polyproteins are mediated by PR to generate completely mature progeny virion.

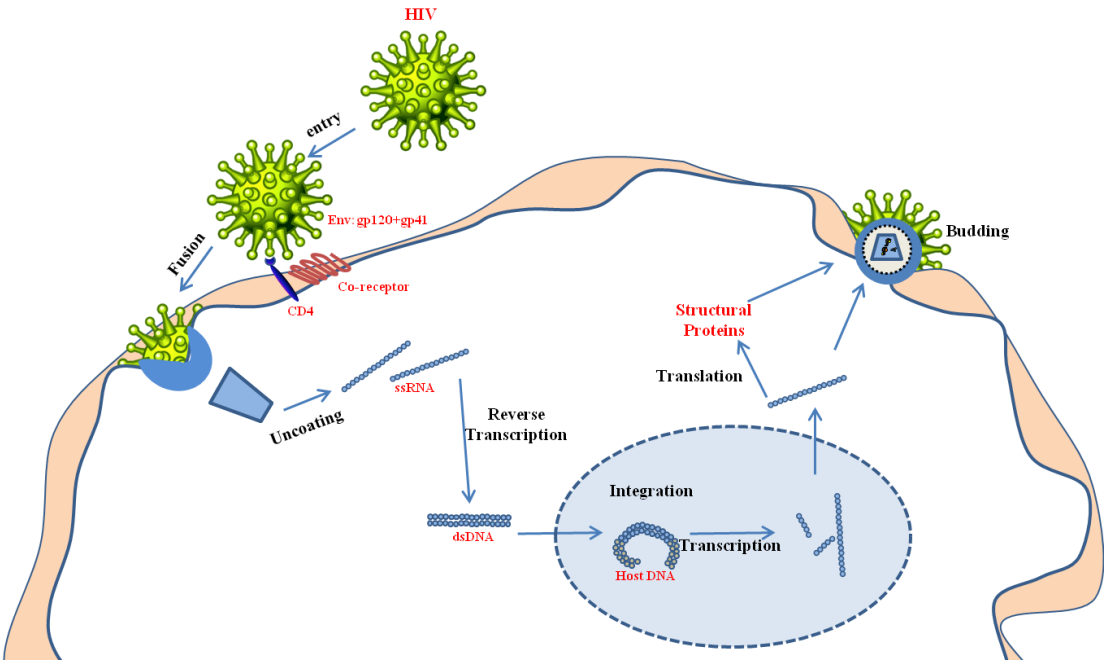

Figure 5: HIV-1 replication cycle

The HIV-1 replication steps are composed of early and late stage of viral life cycle. The early stage of replication includes receptor binding and membrane fusing mediated viral entry, uncoating, reverse transcription to double strand DNA, viral DNA nuclear import, and integration into host chromosome. The late stage replication steps are as follows: provirus transcription into HIV-1 RNA genome and mRNAs, RNA nuclear export into the cytoplasm, viral protein synthesis, HIV-1 assembly and release, and HIV-1 maturation. (Adapted from W.C. Greene *et al*., Antiviral Research 2008)

1.2.4 Macrophages in HIV-1 Infection

1.2.4.1 Macrophages

Monocytes as progenitors of dendritic cells (DCs) most tissue macrophages, are originated from myeloid progenitor cells in bone marrow. Under normal steady state conditions, circulating blood monocytes will random migrate into various peripheral organs where they differentiate into matured resident macrophage, like Kupffer cells in liver or microglial cells in brain *et al*. Based on the expression of major markers on monocytes surface CD14 and CD16, they can be classified into three major subsets (CD14+CD16-, CD14+CD16+, and CD14dimCD16+). However, it is unclear whether the distinct subsets monocytes are precommitted to differentiate into different tissue-macrophages or DCs. Macrophages are important component of immune system and also play crucial roles in organ development, tissue turnover and regeneration, like immune defense to pathogens, angiogenesis, would healing, and various chronic inflammation [144-146]. Macrophage as the "frontier soldiers" in innate immunity, they surveil and sense various types of antigen (i.e., microbes, immune complexes, and apoptotic or necrotic cells and so on) [147-149]. In response to the antigen stimulation, macrophages are being activated to modulate chemokines/cytokines secretion profile for recruiting more responding immune cells to inflammation site. Coincidently, macrophages as professional antigen presenting cells also contribute to the adoptive immune response [150].

1.2.4.2 M-tropic HIV-1 replication in Macrophages

The macrophages, as one of the major target cells of HIV-1, play a critical role in the establishment and pathogenesis of HIV-1 infection. The macrophage is believed as the initial cell type to be infected by HIV-1. Moreover, Macrophage-tropic (M-tropic) HIV-1 variants have been detected in the all stages of HIV-1 infection [151]. Based on the studies about viral dynamics in Monocytes/macrophages, in spite of monocytes are resistant to viral infection, however, monocytes derived macrophages are highly permissive to HIV-1. In addition to this, HIV-1 infected macrophages most likely play a principle role in sustaining high viral loads as suggested by the effect of infected macrophage reservoir on maintaining the high viral burden even after CD4+ T cells depletion *in vivo*[152]. Although other studies suggested that in naïve patients body, virus production in CD4+ T cells is responsible for 99% plasma viremia, and only 1% of the viral replication may be from macrophages[153]. But the half-life of viral DNA in infected macrophages is much longer than that in CD4+ T cells. Coincidently, previous studies indicated that, in the presence of ART treatment, macrophages become the primary source of plasma viremia when active HIV-1 replication in CD4+ T cells is blocked.

1.2.4.3 The Role of Macrophages in HIV-1 infection in CD4+ T cells

Although macrophage infection also induces serious symptoms, like HIV-associated dementia, HIV-associated nephropathy and osteoporosis, however, HIV-1 replication in T cells induced CD4+ helper T cells depletion is mainly responsible for the destruction of lymphoid organs, subsequent immune deficiencies, and fatal opportunistic infection.

However, infected macrophages as long-lived cellular HIV-1 reservoirs also play important role in facilitating virus dissemination to T cells, which lead towards to severe AIDS syndrome [154]. Additionally, tissue-resident macrophages present in every organ system, can contribute to HIV-1 dissemination throughout the patient body [155]. HIV-1 infected macrophages can facilitate the transmission and establishment of HIV-1 infection in the CD4+ T cells via multiple mechanisms [156, 157]. Firstly, the viral transmission from infected macrophages to uninfected CD4+ T cells is taking place through transient adhesive contacts. Following the property cell to cell contaction, macrophages fuse and transmit infectious progeny virion to CD4+ T cells via virological synapses (VR). Besides, some previous studies reported that the viral containing exosomes and microvesicles secreted from infected macrophages also contribute to facilitate the HIV-1 dissemination to CD4+ T cells. Secondly, macrophages as important immune cells have important role in fighting pathogens or cellular debris through phagocytosis. Meanwhile, macrophages also act as APCs and involve in adoptive immunity by presenting processed virus antigen peptides to CD4+ T cells through MHC II pathways. However, this interaction between infected macrophages and uninfected CD4+ T cells also promotes the HIV-1 transmission to T cells [158]. Furthermore, during the initial infection, monocytes/macrophages favor secreting inflammatory chemokines to recruit and activate a variety of responding immune cells, including monocytes, macrophages, DCs and T cells. It also has been reported that HIV-1 infected macrophages lead to the expression of CCL-2, CCL-3, CCL-4 and CCL-5 [159-161]. Thus, the recruitment creates an improved environment

for HIV-1 dissemination. Due to the effect of macrophages on facilitating the dissemination to T cells, infected macrophages also were proposed as the stable reservoir to harbor HIV-1 during antiretroviral drug treatment, and lead to the rebound of viral replication after drug suspension [162, 163]. As such, macrophages infection was suggested most likely to be responsible for the failure of HARRT to eradicate HIV-1 [164].

1.2.5 General information of IL-34 and M-CSF

CSF-1R is a receptor expressed by all types of macrophages, primarily controlling major phenotypes and functions, including survival, proliferation, and differentiation, through c-fms signaling [145, 165, 166]. Macrophage colony stimulating factor (M-CSF), also called as colony stimulating factor 1 (CSF-1), has been previously identified as the main agonist acting through CSF-1R [167-169]. By stimulating CSF-1R, it influences progenitor cells to differentiate into macrophages or other cell types. It is also involved in the development of the placenta. However, a new cytokine IL-34 was identified through comprehensive proteomic analyses by Lin *et al*. in 2008, which is a second ligand of CSF-1R [170]. Structurally, both of IL-34 and M-CSF are N-glycosylated processed proteins. However, they share no obvious sequence similarity with each other [171, 172] (Figure 6). Meanwhile, some other major structural differences of IL-34 and M-CSF are as follows. First, the mature IL-34 is composed of 222 amino acids, whereas the major isoform of M-CSF is 192 amino acids long [172]. Additionally, two monomers of M-CSF are linked by an intersubunit

disulfide at the top of dimer interface, whereas IL-34 assembles into a noncovalently linked dimer [173].

In fact, the existence of IL-34 as another CSF-1R ligand has been anticipated for a long time before its discovery. Although M-CSF is the major cytokine of CSF-1R, CSF-1R-/- mice displayed more severe developmental defects than the CSF-1 (op/op) mice [174, 175]. Moreover, the information gap between different phenotypes of mice lacking CSF-1 (CSF-1 op/op) compared to CSF-1R-/- mice can be explained by the phenotypes of IL-34-/- mice [170].

1.2.6 The distinct biological functions of IL-34 and M-CSF

Investigation into the possible interaction of IL-34 or M-CSF with CSF-1R and relative molecular events that follow led to puzzling scenarios of the two ligands inducing distinct signaling. In spite of the shared receptor of IL-34 and M-CSF, IL-34 displayed a stronger affinity for CSF-1R than M-CSF does [170]. In addition, IL-34 was demonstrated to interact with distinct regions of receptor different from M-CSF [176]. Moreover, the difference in the structural interaction of CSF-1R with IL-34 and M-CSF respectively induces distinct signal activation pathways, which suggest the complementary physiological functions of two ligands [177-181]. Additional studies have provided insight into the functional nonredundancy of IL-34 and M-CSF *in vivo*. Recently, the non-identical functions of IL-34 and M-CSF were discovered while studying the development of varying monocytes-macrophage lineage cells, such as; macrophages, DCs, LCs and microglia. For the macrophage, its development is only

partially controlled by IL-34, but principally regulated by M-CSF *in vivo* [180, 182, 183]

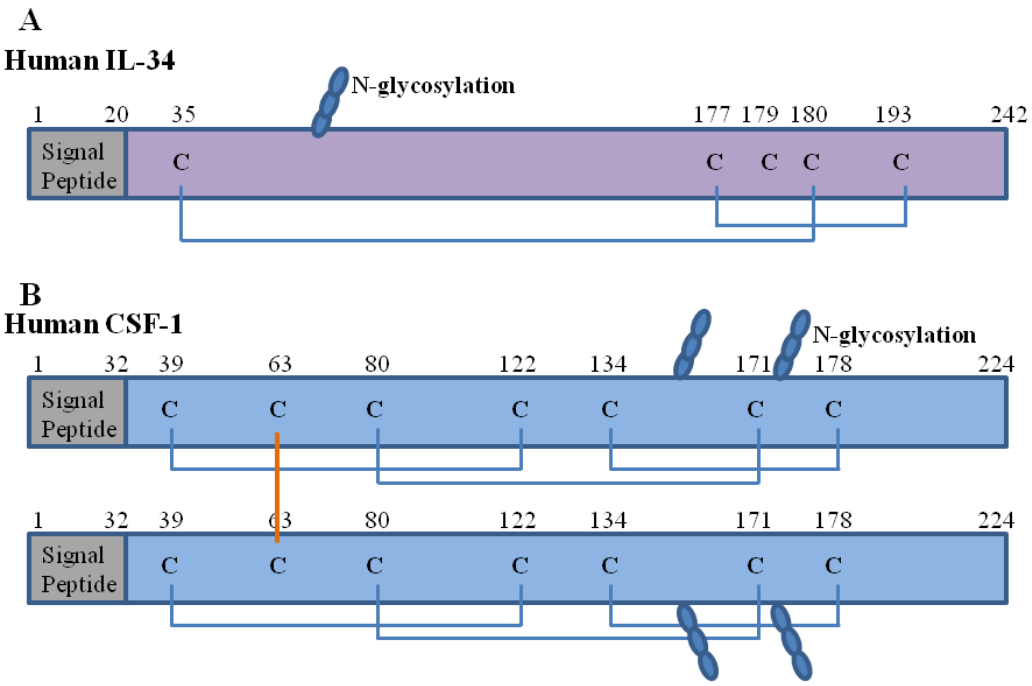

Figure 6: Structure of IL-34 and M-CSF

(A) The primary structure of IL-34 which is a noncovalently linked dimer. (B) The primary structure of M-CSF as a disulfide bond linked dimer. (Adapted from Nakamichi *et al*., Journal of Bone and Mineral Metabolism 2013)

CHAPTER 2

Materials and Methods

2.1 EBOV Project General Regents and Methods

2.1.1 Plasmid constructs

The EBOV-GP plasmid (pCAGGS-ZEBOV-GP) containing the gene of EBOV glycoprotein (GP) from the Mayinga strain was generated by cloning the full length of GP1,2 (nucleotides 142–2172; amino acid (aa) 1 to 676) into the eukaryotic expression vector pCAGGS [184]. The Lentiviral vector encoding for Gaussia luciferase gene (pLenti-Basic-Gluc) was purchased from Target system Inc. The helper packaging plasmid pCMVΔ8.2 encoding for the HIV-1 Gag-Pol, vesicular stomatitis virus G (VSV-G), and HIV-Envelope plasmids were described previously [185-187].

2.1.2 Cell culture, Antibodies and chemicals

The human cervical epithelial cell (HeLa), TZM-b1 cells, human lung carcinoma cell (A549), human embryonic kidney cells (HEK293T), and kidney epithelial cells extracted from African green monkey (VeroE6) were cultured in Dulbecco's modified Eagle's medium. Two CD4+ T-lymphoid cell lines, Jurkat and C8166 T cells, were cultured in RPMI-1640 medium. All cell lines were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin, in the exception of the Vero E6 cell line, which was cultured with 3% FBS. Human Umbilical Vein Endothelial cells (HUVECs) were cultured in EGM-2 Media (Lonza) containing FBS, hydrocortisone,

hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000 and heparin. HUVECs Cells were cultured for 3–4 days and were passaged for no more than 7 generations for the experiments described. Human PBMC derived macrophages were prepared by dispensing fresh PBMCs into 24-well plates at 37°C for 2 hrs. After gentle washing with DMEM, the adherent cells were cultured in DMEM containing 20% FBS and 10ng/ml macrophage colony-stimulating factor (M-CSF; R&D systems) for 7 days. DNA transfection in HEK293T cells was performed with a standard calcium phosphate precipitation method.

The mouse monoclonal antibody (2G4) against EBOV glycoprotein and anti-HIVp24 monoclonal antibody were described previously [188, 189]. The HIV-1 p24 ELISA Kit was obtained from the AIDS Vaccine Program of the Frederick Cancer Research and Development Center.

2.1.3 Preparation and purification of herb extracts

Ginsenoside, Spirulina polysaccharide, Lentinan and Diammonium glycyrrhizinate (DG) were obtained from Chinese companies (En Bang Biotech Co., Yuchang Biotech Inc., Luye Pharma Group Ltd. or China Tai-Tianqing Pharmaceutical Ltd.). Andrographolide was purchased from Sigma Inc. The dried fruitspikes of *P. vulgaris* L. (Labiatae) were purchased from Tong Ren Tang Health Pharmaceutical Co., Ltd. (Beijing, CN). The herb extraction process was based on methods described previously [47]. Briefly, dried herbs were soaked overnight in deionized water at room temperature and then boiled for 1.5 hrs. After centrifugation (3000 g, 30 min), the supernatant was

filtered through a 0.45 μm cellulose acetate membrane and lyophilized. The resulting dark brown residue was dissolved in deionized water and stored at -20°C. A single symmetrical peak corresponding to a molecular weight of polysaccharides (approximately 10 kDa) in the aqueous extract from PV was detected by HPLC analysis, as described previously [47].

2.1.4 Viruses production, infection and inhibition experiments

EBOV-GP pseudovirions (EBOV-GP-Vs) were produced by transfecting HEK293T cells with pCAGGS-ZEBOV-GP (5μg), pCMVΔ8.2 (7μg) and pLenti-Basic-Gluc plasmids (3μg). Meanwhile, VSV-G or HIV-1 Env pseudovirions were produced from 293T cells transfected with VSV-G or HIV-1 Env plasmid together with Δ8.2 and pLenti-Basic-Gluc plasmids. 48 hrs post-transfection, cell culture supernatants were collected and pseudovirions were purified from the supernatant by ultracentrifugation (32,000rpm) for 2 hrs. The pelleted viruses were resuspended into DMEM medium and virus titers were quantified using an HIV-1 p24 ELISA assay.

The pseudovirions were quantified by measurement of Viral-associated HIV-1 p24 level using ELISA assay. The same amounts of EBOV-GP-Vs, VSV-G or HIV-1 Env pseudovirions (as adjusted by p24 levels) were incubated with target cells (at 0.1 x 10^5 per well (96 well plate) or 0.4 x 10^5 cells per well (24 well plate) for 2 to 4 hrs and washed with medium. After 24 to 72 hrs, the supernatants were collected and the viral infection was evaluated by measuring Gaussia luciferase (Gluc) activity. Briefly, 50ul of Coelenterazine substrate (Nanolight Technology) was added to 20ul of supernatant,

mixed well and read in the luminometer (Promega, USA).

For testing of the anti-EBOV entry activity of Chinese herbs, various concentrations of each compound were mixed with EBOV-GP-Vs or directly added into target cells at different time points. After 2-4 hrs of infection at 37°C, the cells were washed twice with medium to remove excessive residue viruses and compound. The anti-EBOV activity was evaluated by measuring the Gluc activity in the culture supernatant at different time points after infection. Similarly, to analysis the inhibitory effect of MAb 2G4 and CHPV, a series of diluted 2G4 alone or the combination of 2G4 with varying concentrations of CHPV were mixed with EBOV-GP-Vs and added to HEK293T cells or macrophages. After 2 hrs of incubation, the cells were washed and the Gluc activity in the cell culture supernatants was detected at 24 and 48 hrs post-infection.

In the macrophage studies, a continuous exposure to CHPV/pseudovirion treatment also was performed. Briefly, CHPV was added 1 hr to cells prior to infection, followed by EBOV-GP-Vs infection with or without MAb 2G4. Then, infected cells were cultured in the same medium containing CHPV/ EBOV-GP-Vs ±MAb 2G4 without washing. The Gluc activity in the cell culture supernatants was monitored at 24 and 48 hrs post-infection.

The wild type EBOV virus preparation was described previously [190]. To test the inhibitory effect of CHPV on EBOV infection, we first added different amounts of CHPV or/and MAb 2G4 in VeroE6 cells for 10 min, then the cells were infected with approximately 100 PFU of EBOV-GFP in the presence of CHPV or MAb 2G4. After 12hrs of infection, the cells were washed and cultured with fresh medium without any

drug. At 48 hrs of infection, cells were fixed with 10% phosphate-buffered formalin and fluorescent plaques were counted using an AID Fluorescent plate reader, as described previously [191].

2.1.5 Binding Assay

For testing the binding of CHPV to EBOV-GP-Vs, EBOV-GP-Vs were mixed with CHPV, MAb 2G4 or medium alone and incubated for 10min at 4°C. The treated EBOV-GP-Vs were then collected by ultracentrifugation (Beckman SW41Ti rotor 22,000rpm, 2 hrs). The pelleted pseudovirions were re-suspended in fresh DMEM and used to infect HEK293T cells and their infectivity was monitored by measurement of the Gluc activity in the supernatants after 48 hrs of infection.

To detect the viral attachment to the cells in the presence of CHPV, HEK293T cells in 24 -well plates were first cooled on ice for 15 min and EBOV-GP-Vs (p24 0.51 ng) were added in the absence or presence of indicated concentrations of CHPV. After 1 hr incubation on ice, the cells were washed with cold PBS to remove unbound virus, lysed with RIPA lysis buffer and the virus attached to cells were detected with anti-p24 ELISA assay.

2.1.6 Cytotoxicity assay

The WST-1 cell proliferation assay (Roche) was used to determine the cytotoxicity of CHPV, as described earlier [192]. Briefly, HUVECs were cultured at 4×103 cells/well in a 96-well plate and treated with CHPV for 2 or 48 hrs. After 48 hrs, WST-1 was

added to the cultures at 10 µl/well and incubated at 37°C for 4 hrs. Absorbance was measured at 490 nm using a microplate reader.

2.2 HIV-1 Project General Regents and Methods

2.2.1 Recombinant proteins, antibodies and chemicals

Recombinant Human M-CSF (rhM-CSF), IL-34 (rhIL-34) and anti-human CSF1R antibody (MAB3291), also named Fms-Ab, were purchased from R&D Systems (Minneapolis, MN, USA). Human AB serum was purchased from Gemini Bio-products (Calabasas, CA). DEAE-dextran was purchased from Sigma.

2.2.2 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. Human Peripheral Blood Mononuclear Cells (hPBMCs) were isolated from the blood of healthy adult donors by sedimentation on a Ficoll-Hypaque (Sigma-Aldrich, St. Louis, USA). Primary CD4+ T lymphocytes, CD14+ monocytes were purified from hPBMCs using the EasySep human CD4+ CD25+ T Cell or CD14+ cells enrichment Kit (Stemcell Technologies, Vancouver, Canada) respectively. The resting CD4+ T cells were cultured in complete RPMI-1640 medium overnight before experimentation. Isolated CD14+ monocytes were cultured in the complete DMEM and were either untreated or treated with 25ng/mL IL-34/M-CSF, or 10%

heat-inactivated human AB serum for 7 days in T25 or T75 flasks to allow the monocytes to differentiate into macrophages. Cells were fed every 2 days by replacing one-half of the cell culture medium with fresh medium. The matured macrophages as judged by morphology were harvested by detachment with 0.25% trypsin and plated in 24-well or 6-well plates with normal complete DMEM culturing for 24 hrs before subsequent experiments.

DNA transfection in HEK293T cells was performed with a standard calcium phosphate precipitation method.

2.2.3 Plasmid constructs

The HIV-1 proviral plasmid (ΔRI/ΔE/Gluc) was described previously [193]. Briefly, in the reverse transcriptase, integrase, and a part of envelope-deleted provirus HxBru, the nef gene was replaced with Gaussia luciferase (Gluc) gene to generate the HIV-1 tri-defective proviral plasmid ΔRI/ΔE/Gluc. The helper packaging plasmid pCMVΔ8.2 encoding for the HIV-1 Gag-Pol, vesicular stomatitis virus G (VSV-G) expressor were described previously [194]. The HIV-1 R5-trophic envelope glycoprotein plasmids (pLET-JRFL) were gifts from Dr. Vicente Planelles in the University of Utah [195].

2.2.4 p24 ELISA and Gaussia luciferase assay

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). As described before, 50ul of Coelenterazine substrate (Nanolight Technology) was added to 20ul of supernatant samples, mixed well and read in the luminometer (Promega, USA) [194].

2.2.5 Viruses production, and infection

For the single-cycle replication-competent virus production, we co-transfected VSV-G or HIV-1 Env(R5) expression plasmid together with ΔRI/ΔE/Gluc and Δ8.2 plasmids into 293T cells to produce trans-complemented VSV-G or HIV-1 M-tropic Env pseudovirions. 48hrs post-transfection, the pseudovirus was purified from the cell culture supernatants by ultracentrifugation (35,000 rpm for 2hrs at 4℃). The pelleted virions were quantified by p24 ELISA assay. To test single-cycle pseudotyped virus infectivity, the virus was incubated with prepared macrophages in normal DMEM for 2 hrs at 37℃ and washed with medium. After 48hrs of infection, the supernatants were collect to evaluate the viral infection by measuring HIV-1 p24 concentration and Gaussia luciferase (Gluc) activity.

Nevirapine-resistant strain HIV-1, was obtained from the NIH AIDS Research and Reference Reagent Program [196]. For evaluating the T-tropic virus replication in unstimulated primary T lymphocytes or hPBMCs, prepared $1.5x10^6$ CD4+ T cells or hPBMCs were infected with nevirapine-resistant HIV-1 via spinoculation at 1400 rpm for 30 mins at room temperature, and in the presence of 20mg/mL DEAE-dextran to increase the infection efficiency. After overnight infection, the DEAE-dextran and unabsorbed virus was washed away, and the cells were cultured in complete RMPI

without IL-34 or M-CSF. At the mentioned time points post infection, a portion of supernatant was collected to evaluate the viral replication by using p24 ELISA assay.

2.2.6 Real-time qPCR analysis

Before we carried out the infection, the virus was treated with 340IU/mL DNase (Roche Molecular Biochemicals) for 1 hr at 37℃ to avoid carryover plasmid DNA contamination. In parallel, heat-inactivated treated (30min at 80℃) virus was carried out as the negative control to exam the residual plasmid DNA removal. After 20 hrs infection, the well-washed cells were collected and genomic DNA was isolated using QIAamp blood DNA minikit (Qiagen). The integrated viral DNA was estimated by an Alu-LTR-nested PCR procedure by the Mx3000P real-time PCR system (Stratagene, CA). The detail procedure and primer used were described previously [197, 198]. The following primers were used for two rounds PCR. First round: Alu-Fr-5' (5'-TCCCAGCTACTCGGGAGGCTGAGG-3'), and Int-Gag Rv-3' (5'-GTCCAG AATGCT GGTAGGGCTATACA-3'); the second PCR was carried out using TD-Gag-Fr-5' (5'-ATCAAGCAGCCATGCAAATG-3'), TD-Gag-Rv-3' (5'-CTGAAGGGTACT AGTA GTTCC-3') [199].

To evaluate the effect of IL-34 or M-CSF on viral transcription in VSVG-Gluc-HIV infected macrophages, the treated cells were harvested and intracellular RNA was isolated by using High Pure RNA isolation (Roche) and carried out to reverse transcription process with MLV reverse transcriptase kit (Promega, Madison, USA). The viral transcription level was estimated by real-time PCR using the TD-Gag primers,

and normalized by the GAPDH gene amplification using following primers: GAPDH-Fr (5'-TGGGTGTGAACCATGAGAAG-3'); and GAPDH-Rv (5'-ATG GACTGTGGTCATGAGTC-3'), as described previously [200].

2.2.7 Trypan blue exclusion assay for cell viability

Trypan blue exclusion test was used to analyze the cell viability at the indicated days by counting the number of viable cells presented in a well mixed cell suspension. After trypan blue solution staining, cells were counted by TC 20 automated cell counter (Bio-RAD) according to the manufacturer's instructions.

CHAPTER 3

Characterization of the inhibitory effect of an extract of *Prunella vulgaris* on Ebola infection

3.1 Introduction

Traditional medicinal plants are new sources of agents with antiviral activities. They are easily accessible and have a low-cost natural, which makes them especially valuable in low-income countries. Some Chinese herbs, including *P. vulgaris*, L. Panaxginseng C.A. Meyer, Andrographis paniculata, Lentinus edodes and Spirulina platensis, display inhibitory effects on the entry step of various viruses, such as HIV-1, Hepatitis B and HSV-1 [46-61]. However, whether these herbs could also have inhibitory activities against EBOV infection remains unknown. In this study, we developed a sensitive EBOV-GP pseudotyped HIV-1-based vector system for screening anti-EBOV agent(s) and then investigated the antiviral mechanism of action. Based on this system, we identified an aqueous extract from the Chinese herb *Prunella vulgaris* (CHPV), which was able to inhibit EBOV-GP-V and eGFP-Ebola virus infections by binding to EBOV-GP and blocking viral entry. Interestingly, our results also showed that CHPV was able to enhance the anti-EBOV activity of the monoclonal antibody MAb 2G4 against EBOV-GP. Thus, this study provides evidence for the first time that CHPV, an aqueous extract from *Prunella vulgaris*, has potent anti-EBOV activity

3.2 Results

3.2.1 Generation of an EBOV-GP pseudotyped HIV-1-based vector system

To generate EBOV-GP pseudovirions (EBOV-GP-Vs), the EBOV-GP-expressing plasmid (pCAGGS-ZEBOV-GP) was co-transfected with an HIV-based lentiviral vector that encoded the Gaussia luciferase gene and a packaging plasmid (pCMVΔR8.2) in HEK293T cells (Fig. 7A). In parallel, HIV-1 Env+ pseudovirions or viruses without envelope glycoprotein were included as controls. The Gaussia luciferase (Gluc) is a bioluminescent enzyme that can be secreted into the cell culture media, enabling the analysis of viral replication by direct measurement of Gluc activity in the supernatant.

To examine the expression and incorporation of EBOV-GP in the cells and the virus, both virus-producing cell and virus lysates were analyzed by SDS-PAGE and Western blot with a mouse anti-EBOV-GP1, as indicated in Fig. 7B. The preGP (110 kDa) and GP1 (120-130 kDa) were clearly detected in EBOV-GP-transfected cells (Fig. 7B, lane 1), with molecular masses corresponding to those previously described for EBOV-GP [201-203]. As expected, abundant GP1 was detected in EBOV-GP-Vs, indicating that EBOV-GP can be efficiently incorporated into the pseudovirions (Fig. 7B, lane 3). Meanwhile, the HIV-1 capsid Gagp24 protein was detected in all of the cell lysates and the pelleted EBOV-GP-Vs and virions without envelope protein by rabbit anti-p24 antibodies (Fig. 7B, lower panel).

To test whether the EBOV-GP-V was able to infect HEK293T cells, equal amounts of EBOV-GP-Vs or viral particles without any envelope protein (as adjusted by HIV-1 Gagp24) were incubated with HEK293T cells for 4 hrs. The Gluc activities in the

supernatants were measured to evaluate the virus infectivity at 48 hrs post-infection. The results indicate that EBOV-GP-Vs were able to efficiently infect 293T cells, whereas viral particles without viral envelope protein did not produce any Gluc activity (Fig. 7C).

To further test whether the pseudovirion infection was mediated by EBOV-GP, a previously described monoclonal antibody (MAb), 2G4, which targets the Ebola virus glycoprotein [188, 204], was used to block the entry of EBOV-GP-V. Briefly, EBOV-GP-Vs were incubated with various dilutions of 2G4 for 30 mins and then added onto HEK293T cells. At 48 hrs post-infection (p.i.), the Gluc activities were measured and the results showed that the EBOV-GP-V infection of HEK293T cells could be efficiently blocked by 2G4 in a dose-dependent manner (Fig. 7D). A 50% inhibition of HIV-1 infection was achieved when 2G4 was used at a concentration of 1.72 μg/mL. These results clearly indicated that EBOV-GP was responsible for the entry of pseudovirions and their infection.

The ability of EBOV-GP-V to infect other cell types was further analyzed in different cell lines, including HEK293T cells, CD4+ TZMb1 cells, human cervical epithelial cells (HeLa), CD4+ T-lymphoid cells (C8166 and Jurkat cells), HUVECs and human lung carcinoma cells (A549) (Fig. 7E). In parallel, the same amount of HIV-Env+ pseudovirions (HIV-Env-Vs) and VSV-G pseudovirions (VSV-G–Vs) were used as controls. The Gluc activity was tested in the supernatant at 48 hrs p.i. As expected, the VSV-G–Vs efficiently infected all target cell lines, whereas HIV-Env-Vs only targeted the CD4+ T cells, including C8166, Jurkat and TZMb1 cells. In contrast,

EBOV-GP-V was unable to induce detectable levels of Gluc activity in both the Jurkat and C8166 T-lymphoid cells. In agreement with the previously described EBOV-GP tropism range [205], EBOV-GP-Vs were shown to be able to infect human HeLa cells, TZMb1 cells, human A549 cells and primary HUVECs. These results clearly distinguish the different tropisms of EBOV-GP from HIV-1 and VSV-G envelope proteins.

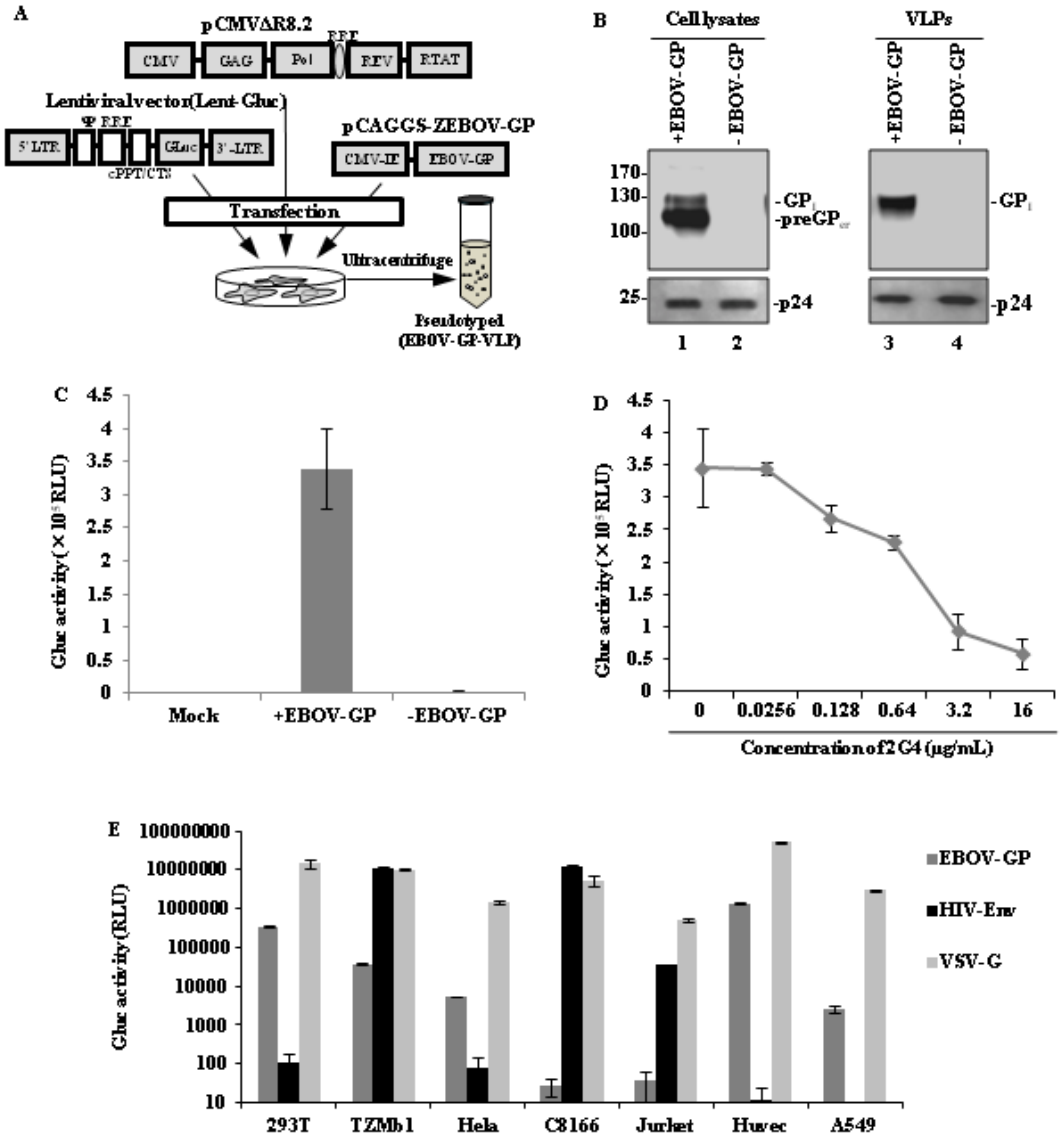

Figure 7: Generation of an EBOV-GP-V pseudotyped virus system. (A) Schematic representation of the 3 co-transfected plasmids, including the lentiviral vectors encoding for the Gaussia luciferase(Gluc) gene, the HIV-1 helper construct Δ8.2 and the pCAGGS-ZEBOV-GP plasmid. (B) Western blot showing the GP1, preGP and p24 protein expression in virions and transfected cells. (C) Equal amounts of GP+ or GP- virions (adjusted by p24) were used to infect HEK293T cells. After 48 hrs, the Gaussia Luciferase activity (Gluc) of the supernatants was measured and normalized as a percentage of the positive control (Gluc value of GP+ as 100%) (D) Dose-dependent 2G4 (MAb) inhibition of EBOV-GP-V transduction in HEK293T cells. The same amount of EBOV-GP-V was incubated with the indicated concentration of 2G4 for 30 min and added to HEK293T cells for 4 hrs. The cells were washed and cultured in complete DMEM without compounds. Gluc activities in the supernatant were tested after 48 hr of incubation. (E) Evaluating the transduction ability of EBOV-GP-V in different cell types. The results are the mean values ± standard deviations (SD) of three independent experiments.

3.2.2 Evaluation of different Chinese herbs as potential anti-EBOV-GP agents

The previous study demonstrated that EBOV-GP-Vs can mediate an EBOV-GP-specific viral entry and that viral replication can be easily detected by measuring Gluc activity in the supernatant. By using this system, we next tested the effect of different Chinese herbs on EBOV entry. A number of studies have suggested that the extracts of some Chinese herbs display a significant inhibitory effect on the entry and/or replication of different viruses (Table 1). Therefore, in our study, CHPV, ginsenoside, andrographolide, spirulina polysaccharide, lentinan and diammonium glycyrrhizinate extracted from *Prunella vulgaris*, as well as *Panaxginseng*, *Andrographis paniculata*, *Spirulina platensis*, *Lentinus edodes* and *Liquorices*, were assessed for potential anti-EBOV activity.

Our cytotoxicity study showed that none of these extracts had obvious cytotoxic effects on HEK293T cells up to concentrations of 40 μg/mL (data not shown). We therefore used serial dilutions of compounds from 0.625 μg/mL to 40 μg/ml. After 2 hrs of infection with EBOV-GP-Vs in the presence of each herb, the infected cells were washed to remove the residual pseudovirions and herb, after which the Gluc activity in the supernatant was measured at 48 hrs p.i. The results showed that in the presence of andrographolide or spirulina polysaccharide, EBOV-GP-V still replicated efficiently, suggesting that these herbs did not affect the replication of GP pseudotyped virus (Fig. 8). In contrast, CHPV showed a strong inhibitory effect on EBOV-GP-V infection, with an approximately 80% inhibition of EBOV-GP-V infection achieved when CHPV was

used at 10 μg/mL (Fig. 8). The ginsenoside also displayed a modest antiviral effect at a higher concentration of 40 μg/ml. Thus, we continued to characterize the anti-EBOV activity of CHPV and the mode of its action.

Table 1: Antiviral activities of different extracts or components from Chinese herbs.

Active extracts	Example of plant source	Antiviral actions	References
Aqueous extract of Prunella vulgaris	The purified bioactive extract from P. vulgaris L. fruitspikes.	Prevention of hiv attachment to CD4 receptors, and suppression of HIV-1 entry by disrupting the gp41 six-helix bundle formation. Inactivation of HSV-1 directly, blocked HSV-1 binding to Vero cells, and inhibited HSV-1 penetration into Vero cells.	Liu et al., 2002; Tabba et al., 1989; Yao et al., 1992; Zhang et al., 2007
Ginsenoside	Ginsenoside is a class of steroid glycosides, purified from Panaxginseng C.A. Meyer	Ginsenoside has effective anti-hepatitis B virus activity mediated by interrupting virus adsorption on host cell, DNA replication and secretion of hepatitis B surface antigen.	Kang et al., 2013; Lee et al., 2014; Li et al., 2001)
Andrographolide	A major bioactive chemical constituent of Andrographis paniculata	Andrographolide was reported for anti-HSV-1 and anti-hiv activity in vitro. The inhibitory effects the compound on viral entry and replication steps.	Chang et al., 1991; Seubsasana et al.,2011
Spirulina polysaccharide	A natural sulfated polysaccharide, isolated from Spirulina platensis	Spirulina polysaccharide was shown to target viral absorption, penetration stages and some replication stages after penetration into cells.	Hayashi et al., 1996a; Hayashi, 2008; Hayashi et al., 1996b
Lentinan	A polysaccharide isolated from a common edible mushroom, Lentinus edodes	The sulfated lentinan exhibits a potent anti-HIV activity by suppressing viral adsorption to the cells and reverse transcriptase.	Yoshida et al., 1988
Diammonium glycyrrhizinate(DG)	DG was extracted and purified from Liquorices (Radix glycyrrhizae)	Anti-hepatitis B virus by reducing transport to the membrane and sialylation of hepatitis B virus surface antigen. Anti-HIV by reduction of membrane fluidity leanding to inhibition of fusion. The antiviral activity against SARS related coronavirus, respiratory syncytial virus, arboviruses, vaccinia virus and vesicular stomatitis virus, also have been revealed in vitro studies.	Fiore et al., 2008; Harada, 2005; Sato et al., 1996

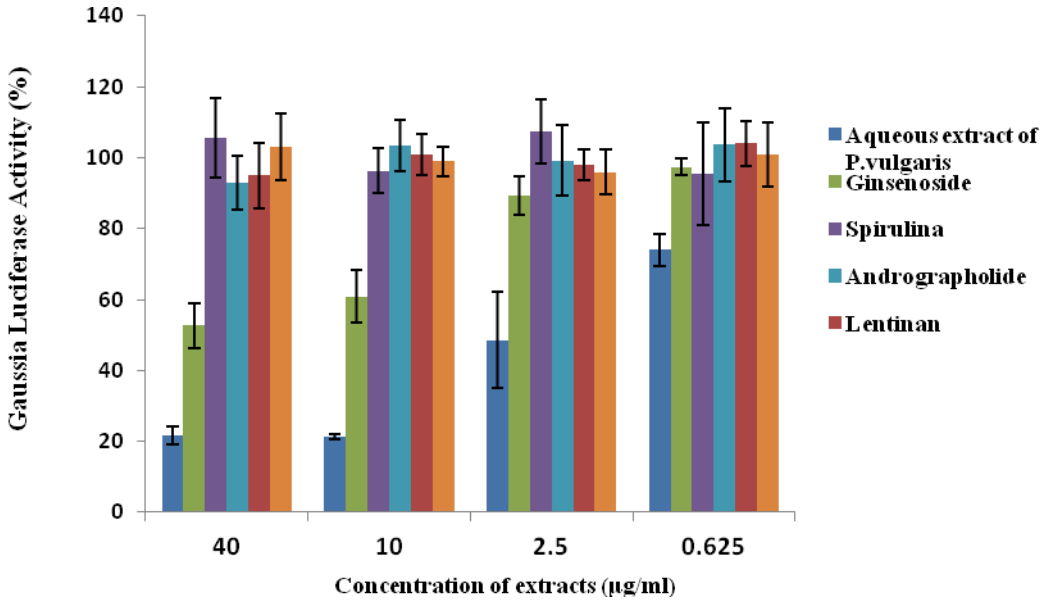

Figure 8: Anti-EBOV-GP activity of several extracts of different Chinese herbs. Serial dilutions of CHPV, *Ginsenoside*, *Andrographolide*, *Spirulina polysaccharide*, *Lentinan* or *Diammonium glycyrrhizinate* were mixed with the same amount of EBOV-GP-V and immediately added to HEK293T cells in 24-well plates. At 2 h post-infection, the cells were washed and cultured in complete DMEM without herb extract or compound. The Gluc activity in the 48 h supernatants is presented as a percentage of the control activity (%), a ratio of Gluc activity in the presence of herb versus the absence of any herb.

3.2.3 CHPV interferes with the EBOV-GP-V entry step by acting on virus-like particles

To gain insight into the mechanism of how CHPV inhibits EBOV-GP-V infection, a consistent concentration of CHPV (10 μg/ml) was added to the HEK293T cell culture medium at various time points, including pre-, simultaneous and post-exposure of the cells to the EBOV-GP-pseudovirions. The results in Fig. 9A show that when cells were pretreated with CHPV for 2 and 4 hrs and then removed before adding pseudovirions, there was no inhibitory effect on virus infection (Fig. 9A), suggesting that the inhibitory activity of CHPV did not act directly on cells to render them resistant to infection. Interestingly, when the cells were incubated simultaneously with EBOV-GP-V and CHPV, a strong inhibitory effect was achieved, with no negative impact on viral infection when CHPV was added at 2 or more hrs post-infection (Fig. 9A). All of these results revealed that CHPV blocks EBOV-GP-V replication in the early steps of the infection. This also led us to believe that the major inhibitory effect of CHPV may be through its action on the virus itself. As shown in Fig. 9B, EBOV-GP-Vs were first incubated with CHPV (10 μg/mL) for 10 min at 4°C. The CHPV-treated viruses were pelleted by ultracentrifugation and washed once with media to remove residual unbound CHPV (Fig. 9B). As a control, EBOV-GP-Vs were incubated with the anti-EBOV-GP antibody 2G4 or media alone and were pelleted by ultracentrifugation. All pelleted viruses were used to infect HEK293T cells, and the Gluc activity was measured at 48 hrs post-infection. Similar to the pseudovirions incubated with 2G4, the pseudovirions pre-treated with CHPV also displayed a significant reduction of

infection ability, when compared with the pseudovirions that were incubated with medium alone (Fig. 9C).

To exclude the possibility that the free soluble active constituents of CHPV could be pelleted by ultracentrifugation, rather than being associated with virus, we performed the same ultracentrifugation procedure with CHPV alone. After centrifugation, the supernatant or re-suspended pellet sample was added to HEK293T cells, followed by EBOV-GP-V infection. As shown in Figure 9D, the CHPV in the supernatant after ultracentrifugation still showed strong antiviral activity compared with the EBOV-GP-Vs infection in the absence of CHPV. In contrast, the pellet portion did not exert any antiviral activity (Fig. 9D), indicating that the soluble active constituents of CHPV could not be pelleted. All of these results suggest that CHPV might be attached to the viral glycoproteins, consequently blocking viral entry. However, we could still not rule out the possibility that the presence of CHPV may also result in conformational changes of EBOV-GP.

To determine if CHPV directly interfered with the viral adsorption process by interaction with the virus, the cell attachment of EBOV GP-V was assessed by measurement of cell-associated pseudovirions by a p24 ELISA assay. Briefly, HEK293T cells were cooled on ice for 15 min and EBOV GP-V, in the presence or absence of CHPV (200, 20, 2 and 0.2 μg/mL), was added. After a 1 hr incubation on ice, the cells were washed with cold PBS to remove unbound virus and lysed with RIPA buffer. The cell-bound pseudovirions were measured by detection of the p24 levels in the cell lysate. The results show that as the pseudovirions were incubated continuously

with diluted CHPV, the amount of cell-associated p24 increased in a dose-dependent manner (Fig. 9E). At 20 ug/ml, CHPV could block approximately 80% of pseudovirion attachment. This result clearly indicates that CHPV could interfere with virus attachment to cells, consequently preventing virus entry.

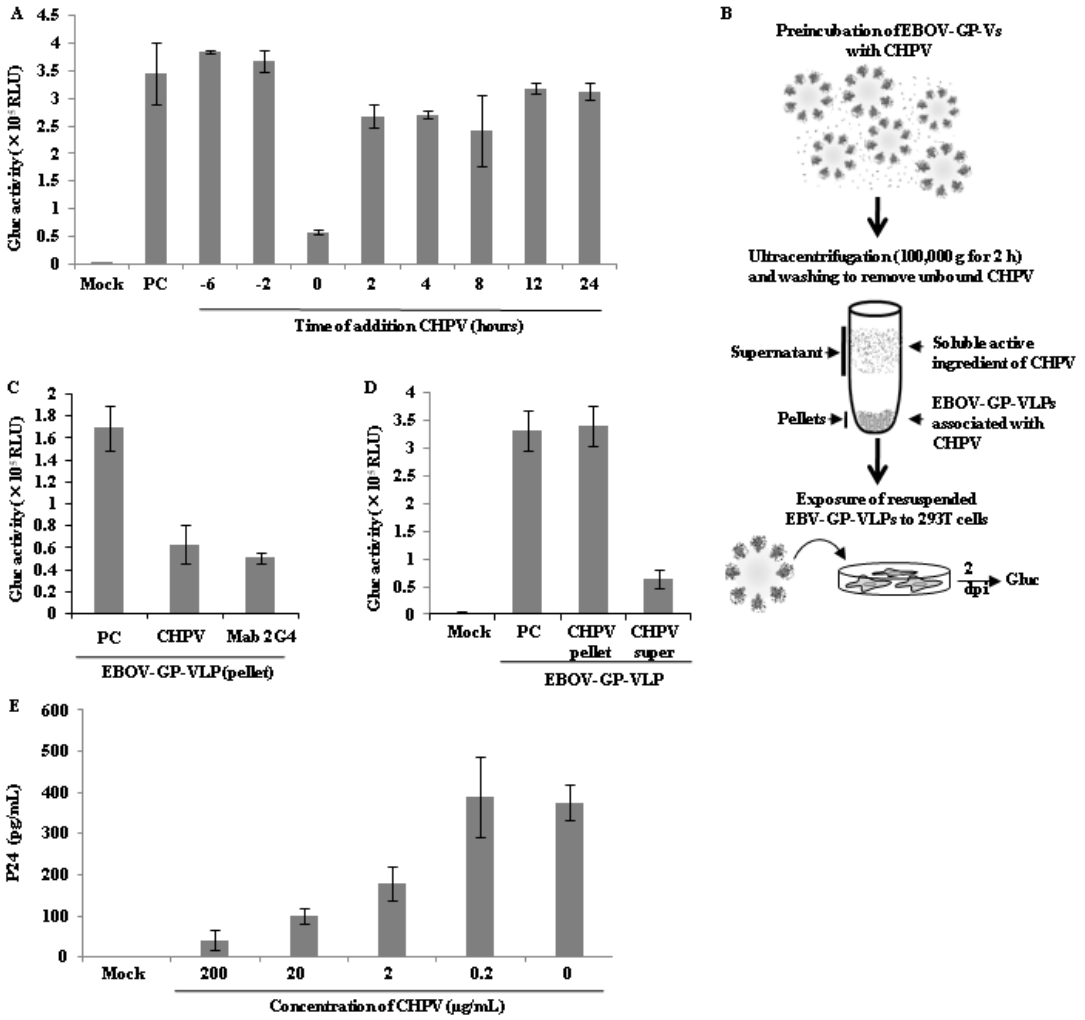

Figure 9: Characterization of the mechanism of CHPV anti-EBOV action. (A) A time-of-addition experiment was performed with EBOV-GP-V to identify the action target for CHPV. CHPV (10 μg/mL) was added at 6 hr and 2 hr prior to infection (washed away immediately before adding virus), during infection (0 hr), and at 2 hr, 4 hr, 8 hr, 12 hr and 24 hr post-infection. The mock-infected controls mean uninfected HEK293T cells, and the positive controls (PC) were HEK293T cells were infected with pseudovirions in the absence of CHPV. At 2 hrs post-infection, all of the cells were washed and cultured in complete DMEM. The Gluc activity was tested from the supernatant after a 48 hr incubation, and the data are shown as a percentage of the control activity (%), a ratio of Gluc activity in the presence of CHPV versus the absence of any compound. (B, C) Schematic diagram of the evaluation of infection of EBOV-GP-V-bound CHPV. EBOV-GP-V was incubated for 10 min with CHPV, MAb or DMEM at 4°C. The treated viruses were further concentrated by ultracentrifugation (100,000 g). The infectivity of the pelleted pseudovirions was tested by adding to HEK293T cells, and the Gluc activity after 48 hrs was shown as a relative ratio when compared with the virus incubated with DMEM. PC: EBOV-GP-V not treated with drug. (D) The same amount of CHPV was ultracentrifuged. The resulting supernatant and the pellet were incubated with EBOV-GP-V and added to HEK293T cells. The

Gluc activity after 48 hrs is shown as a percentage of control activity (%). The results are the mean values ± standard deviations (SD) of three independent experiments. (E) CHPV blocks the attachment of EBOV-GP-V. The same amount of EBOV-GP-Vs (p24, 0.51 ng) and various concentration of CHPV were added to HEK293T cells on ice for 1 hr. The sample without pseudovirions or CHPV treatment was used as a negative control. The cells were then washed with cold PBS to remove unbound virus and lysed by RIPA buffer. The viruses attached to cells were monitored by measuring the HIV-1 p24 antigen levels by anti-p24 ELISA.

3.2.4 Evaluation of the inhibitory effect of CHPV on EBOV-GP pseudovirion entry in HUVECs and human macrophages

As is well-documented, the dysregulation of endothelial cell functions in EBOV infection can induce a wide range of vascular effects that can cause changes in vascular permeability and hemorrhage [206, 207]. Some studies suggest that EBOV-GP is the main determinant of vascular cell injury [208, 209]. As we demonstrated in Fig. 7E, HUVECs were extremely sensitive to EBOV. Therefore, we choose primary HUVECs as target cells to evaluate if CHPV could also protect HUVECs from EBOV-GP infection. Briefly, various concentrations of CHPV were mixed with EBOV-GP-V and added immediately onto HUVECs. At 2 hrs post-infection, the cells were washed and cultured in medium without the herb. As expected, upon an increase in the concentration of CHPV, the Gluc activities of EBOV-GP-V-infected HUVECs decreased in a dose-dependent manner (Fig. 10A), indicating that the EBOV-GP-V infection in HUVECs was specifically blocked by CHPV. When 10 μg/mL of CHPV was used, less than 20% of Gluc activity could be detected. Meanwhile, Fig. 10B shows the cell viability after treatment with different concentrations of CHPV for 2 hrs or 48 hrs, as measured by the WST-1 cell proliferation assay. For the 2 hrs CHPV treatment group, we detected no cytotoxicity at a concentration of 320 μg/ml. For the 48 hrs continuous CHPV treatment group, the HUVECs grew normally at concentrations up to 40 μg/ml.

It is well known that macrophages are a major initial target cell for Ebola virus. Infected macrophages can attract more target cells, induce and increase vasodilatation,

vascular permeability and disseminated intravascular coagulation by producing proinflammatory cytokines and chemokines [210]. Therefore, it is necessary to evaluate the inhibitory effect of CHPV on EBOV-GP-V infection in macrophages. We first tested the effect of CHPV by incubating human primary macrophages with CHPV and EBOV-GP-V for 4 hrs. As shown in Figure 10C (the 4 bars on the right side), the Gluc activity of supernatant from infected macrophages is significantly decreased at 24 and 48 hrs in the presence of CHPV (20 μg/ml). We also infected macrophages in the presence of CHPV and maintained the macrophages in the medium containing EBOV-GP-Vs and CHPV for 48 hrs without washing. In this case, the EBOV-GP-Vs infection was inhibited by CHPV and the inhibition rate was as high as 95% compared with the positive control (Fig. 10C, left 4 bars), suggesting that the optimum inhibitory effect of CHPV could be obtained when there was a continuous presence of CHPV in the culture medium. Additionally, we observed no cytotoxic effect when 20 μg/ml of CHPV was present in the macrophages culture for 48 hrs (data not shown).

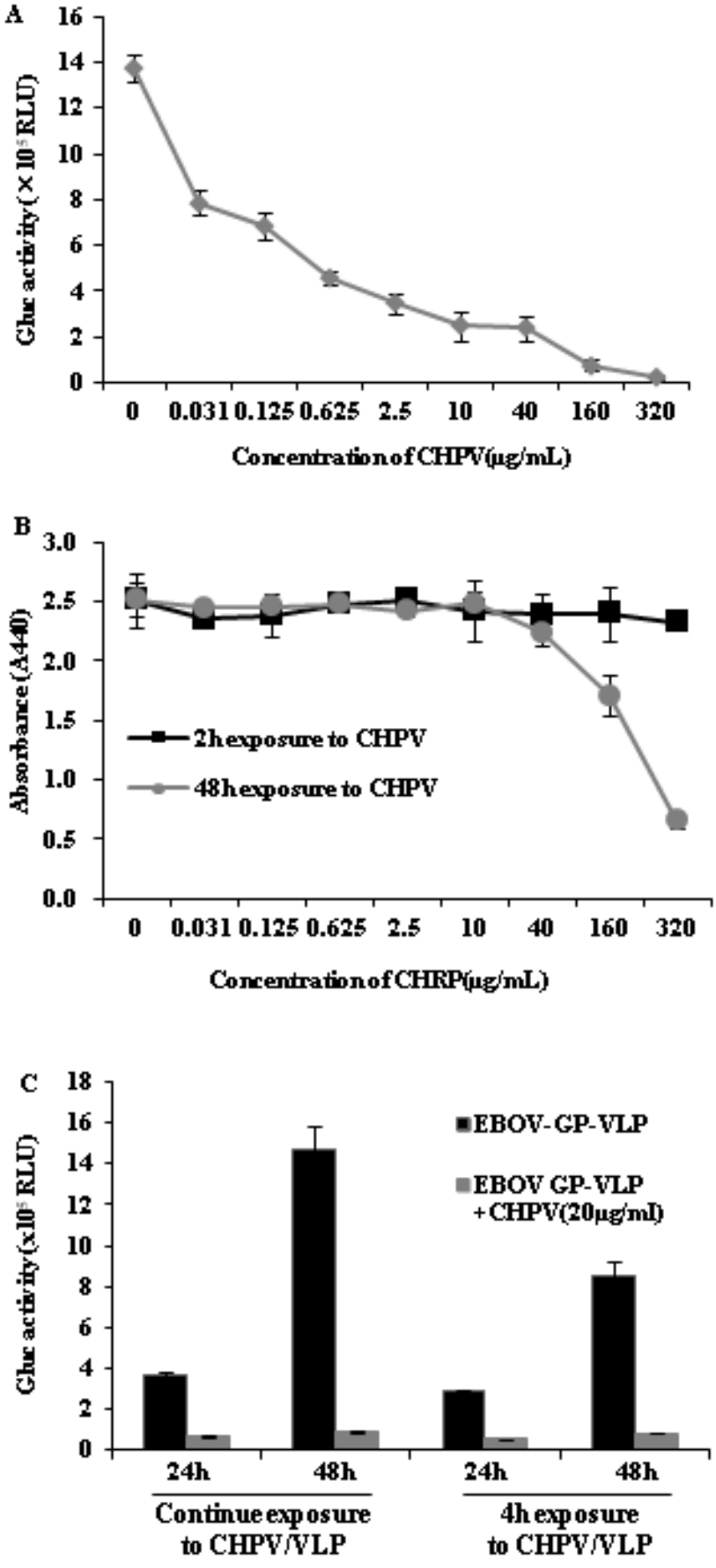
A
Gluc activity(×10⁵ RLU)
16
14
12
10
8
6
4
2
0
0
0.031
0.125
0.625
2.5
10
40
160
320
Concentration of CHPV(μg/mL)
B
Absorbance (A440)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h exposure to CHPV
48h exposure to CHPV
0
0.031
0.125
0.625
2.5
10
40
160
320
Concentration of CHRP(μg/mL)
C
Gluc activity(x10⁵ RLU)
18
16
14
12
10
8
6
4
2
0
EBOV-GP-VLP
EBOV GP-VLP
+CHPV(20μg/ml)
24h
48h
24h
48h
Continue exposure
to CHPV/VLP
4h exposure
to CHPV/VLP

Figure 10: Evaluating the anti-EBOV effect and cytotoxicity of CHPV in vascular endothelial cells (HUVECs) and its anti-EBOV effect in macrophages. (A) The same amount of pseudovirions mixed with the indicated varying concentrations of CHPV were immediately added onto HUVECs for 2 h, then the cells were washed and cultured in DMEM without CHPV. The Gluc activities were tested from the supernatants after 48 h of incubation. (B) The cells were treated with CHPV for 2 or 48 h, after which the WST- 1 cell proliferation assay was used to analyze cell proliferation. (C) Left 4 bars: CHPV was added into the macrophage culture 12 h before infection when EBOV-GP-V was added and continually cultured without washing; right 4 bars: EBOV-GP-V alone or mixed with CHPV (20 ug/ml) and immediately added to cell culture, followed by washing of the cells after 4 h of incubation. The cells were then cultured in medium without CHPV. The supernatants of all samples were collected at 24 or 48 h postinfection and used to detect Gluc activity.

3.2.5 Analysis the EBOV entry inhibitory effect of MAb 2G4 and CHPV

As described above, both EBOV-GP-specific monoclonal antibodies 2G4 (Fig. 7D) and CHPV (Fig. 8-9) could efficiently block the viral entry step. We next asked whether a stronger inhibitory effect could be achieved by combining CHPV and MAb 2G4 in relatively lower concentrations. In Fig. 11A, 2G4 was serially diluted from 16 μg/ml to 0.32 μg/ml, and each concentration was combined with the indicated concentrations of CHPV (10 and 0.625 μg/mL). The results show that the viral infection was reduced approximately 80% when the concentration of CHPV was 10 μg/ml. Interestingly, a greater than 90% inhibitory effect was achieved when CHPV (10 μg/ml) was combined with 2G4 at concentrations above 1.6 μg/ml. The additive effect was more evident when a lower concentration (0.625 μg/ml) of CHPV was combined with different doses of 2G4. For example, when 1.6 μg/ml 2G4 and 0.625 μg/mL CHPV were added alone, 56% and 26% inhibition were obtained, respectively. When these two drugs were added together at the same concentration, 81% inhibition was observed. This clearly indicates that a much stronger inhibition could be achieved through the combined use of 2G4 and CHPV, as opposed to using each treatment alone. It is obvious that to achieve the same level of inhibition, the MAb dosage could be reduced in the presence of CHPV. A similar phenomenon was observed in macrophages: at 4 hr post-infection with pseudovirions in the presence of CHPV, in the presence or absence of 2G4 in macrophages (Fig. 11B), the combination of CHPV (20 μg/ml) with 2G4 (1.6 μg/ml) could further decrease the infectivity of EBOV-GP-V compared with that of CHPV (20 μg/ml) or 2G4 (16 μg/ml) alone. Remarkably, when CHPV and 2G4 were added 1 hr

before infection, with the same amount of CHPV and 2G4 maintained during the course of infection, the infection of EBOV-GP-V was completely inhibited (Fig. 11C).

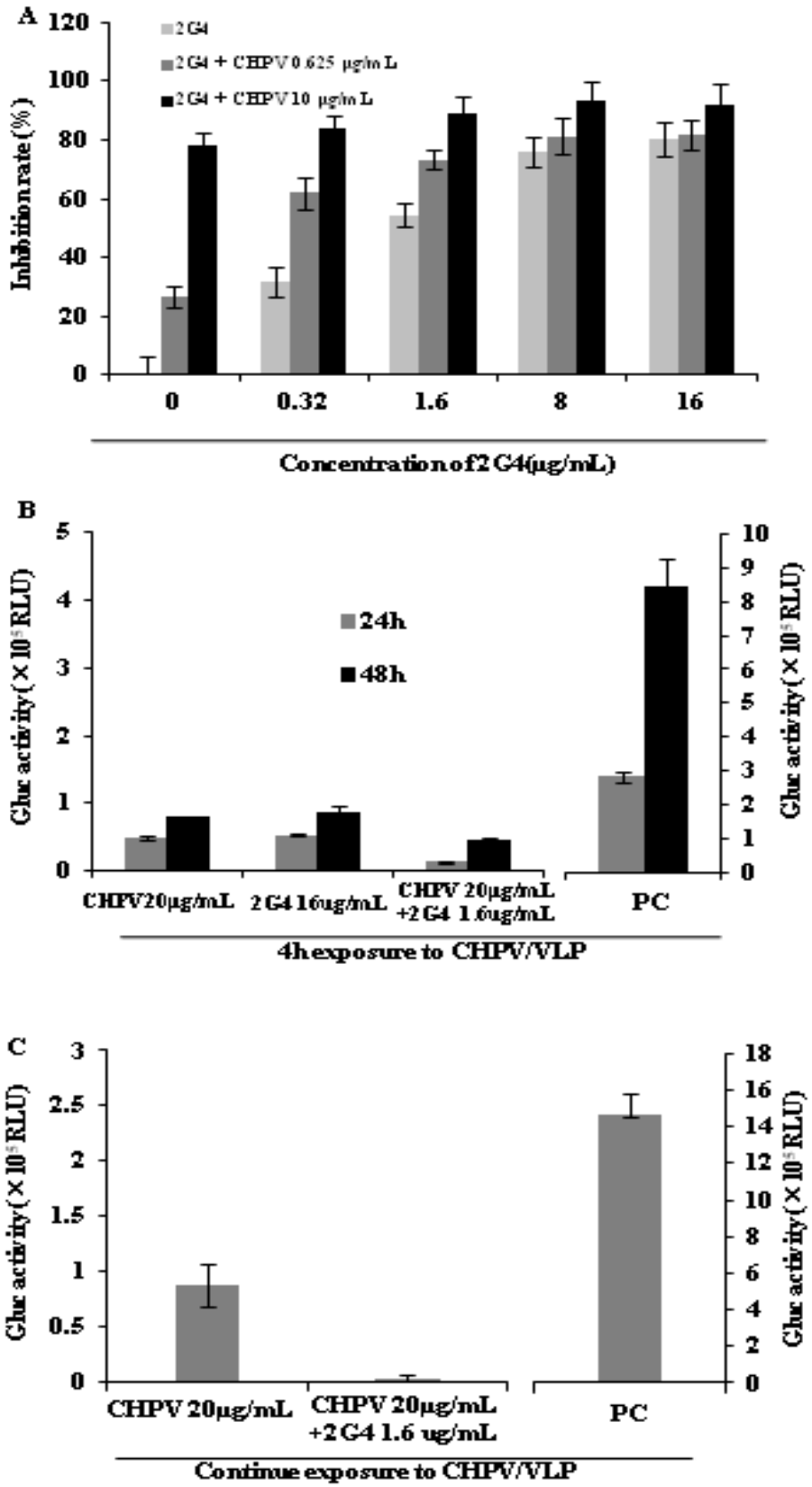

Figure 11: Analysis of the inhibitory effect of the combination of 2G4 and CHPV on EBOV entry. (A) Various concentrations of 2G4 alone or with CHPV (10 or 0.625 mg/ml) were added to HEK293T cells with the same amount of EBOV-GP-V. The

anti-EBOV effect was displayed as percent inhibition, a ratio of Gluc activity in the presence of 2G4 or/ and CHPV versus EBOV-GP-V alone. (B) The inhibitory effect of CHPV in the presence or absence of 2G4 in macrophages. The positive control (PC) was infected macrophages in the absence of CHPV or 2G4. EBOV-GP-V was mixed with CHPV (20 ug/ml) in the presence of absence of 2G4 (16 or 1.6 ug/ml) and immediately added onto the cell culture, followed by washing of the cells after 4 h of incubation. The cells were then cultured in medium without CHPV. The supernatants were collected at 24 and 48 h post-infection and used to detect Gluc activity. The major scales of the Y-axis on the left and on the right are different, with 0.5 units and 1 unit, respectively. (C) The same amounts of EBOV-GP-Vs in the presence or absence of 2G4 (1.6 ug/ml) were added onto CHPV (20 mg/mL)-preincubated (1 h) macrophages, and the same amount of CHPV with or without 1.6 ug/mL 2G4 was maintained during the course of infection. The positive control (PC) was infected macrophages in the absence of CHPV or 2G4. The supernatants were collected, and the Gluc activity was tested after 48 h of continuous culture without washing. The major scale base of the Y-axis is 0.5 units on the left and 2 units on the right.

3.2.6 Inhibition of CHPV against an eGFP-ZEBOV infection

Given that CHPV is able to block the EBOV-GP-mediated pseudovirion entry, the next

question we asked was whether CHPV could block the ZEBOV infection. To address this question, we performed an eGFP-ZEBOV infection in the presence of CHPV or 2G4 antibody in VeroE6 cells. Briefly, different concentrations of CHPV or 2G4 antibody were added to VeroE6 cell cultures for 10 min, then the cells were infected by 100 PFU of eGFP-ZEBOV in the presence of same concentrations of CHPV or 2G4. After 12 hrs of infection, the cells were washed and cultured with fresh medium without any drug. At 48 hrs post-infection, we did not observe any CPHV-induced toxic effect on the cells, and the cells were fixed with 10% phosphate-buffered formalin and fluorescent plaques were counted using an AID fluorescent plate reader. Remarkably, results revealed that the presence of 6.25 ug/ml of CHPV reduced more than 50% of eGFP-ZEBOV infection, while in the presence of 25 ug/ml of CHPV, eGFP-ZEBOV infection was inhibited by approximately 80% (Fig. 12A). In agreement with previous reports, 2G4 also showed a very strong blocking effect against Ebola virus infection [188, 211]. At 0.6μg/ml concentration of 2G4 antibody, approximately 80% of virus infection was blocked (Fig. 12A). These results provide strong evidence that CHPV is able to inhibit EBOV infection.

To further test whether the combined use of CHPV and 2G4 could enhance the anti-EBOV infection activity, we treated VeroE6 cells with 2G4 alone or 2G4 plus CHPV in VeroE6 cells during eGFP-ZEBOV infection, as described above. After 48 hrs of infection, the cells were fixed with 10% phosphate-buffered formalin and fluorescent plaques were counted using an AID fluorescent plate reader. Interestingly, the combined treatment of 2G4/CHPV potentiated inhibition against eGFP-ZEBOV

infection (Fig. 12B), as results revealed that while treatment with 0.31 or 1.2 ug/ml of 2G4 alone reduced eGFP-ZEBOV infection to approximately 25% or 17%, respectively, the presence of 2G4/CHPV (0.31 ug/6.25 μg or 1.2 μg /25 ug/ml) reduced eGFP-ZEBOV infection to approximately 17% or 6%, respectively, compared with eGFP-ZEBOV infection in the absence of antibody and CHPV (Fig. 12B). This indicates that combined treatment with 2G4 and CHPV during EBOV infection could achieve a better inhibitory effect against viral infection.

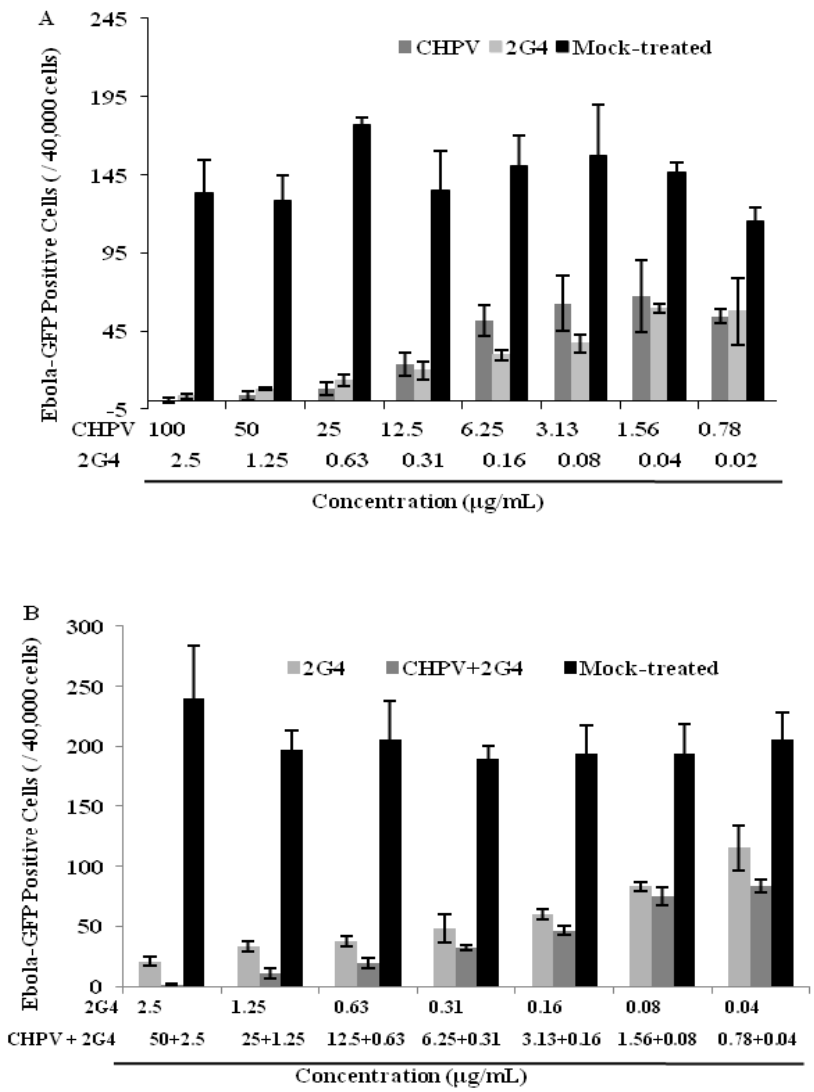

Figure 12: The inhibitory effect of CHPV on the eGFP-ZEBOV infection. The final treatment concentrations of CHPV were 2-fold dilutions beginning with 100 mg/ml down to 0.78 mg/ml, and 2G4 was started with 2.5 mg/ml followed by 2-fold dilutions down to 0.020 mg/ml. The indicated concentrations of CHPV or MAb alone were added to VeroE6 cells (A) or different concentrations of CHPV or MAb were mixed (as indicated) and added to VeroE6 cells (B) before exposure of the cells to 100 PFU of eGFP-ZEBOV. After incubating for12 h, the medium was changed with fresh medium. At 48 h later, cells were fixed with 10% phosphate-buffered formalin and fluorescent plaques were counted using an AID fluorescent plate reader. Error bars represent variation between triplicate samples, and the data of (A) and (B) are representative of results obtained in two independent experiments.

Discussion

Due to the fact EBOV is classified as a biosafety level 4 pathogen, the study of its replication, as well as the testing of anti-EBOV drugs, requires biosafety level 4 containment (BSL-4) and procedures, significantly limiting the EBOV-related research activities of many microbiology laboratories. To overcome these limitations, several previous studies have examined the use of EBOV-GP pseudotyped viral vectors in a BSL-2 environment, including replication-defective MLV and HIV, replication-competent vesicular stomatitis virus and Newcastle disease virus, for investigation of EBOV-GP-mediated virus entry mechanism and for the screening of new therapeutic agent(s) [205, 208, 212-217]. In these studies, the entry of pseudotyped virus-like particles were monitored by using enzymes or biological markers expressed when the pseudovirions gained entry into the cells. In this study, we used a sensitive Ebola-GP-mediated HIV-based vector system, which contains a Gaussia luciferase (Gluc) gene as a reporter (Fig. 7A). Because Gluc can be secreted into the supernatant after being expressed, this assay makes it more practical to evaluate and quantify the level of EBOV-GP-mediated virus entry and can be used for anti-Ebola-GP drug screening in a BSL-2 environment. Our results demonstrate that the infection of EBOV-GP-V required a functional EBOV-GP-mediated cell entry, which was neutralized by MAb 2G4, which is specific for EBOV-GP (Fig. 7D).

By using this EBOV-GP-mediated virus entry system, we have tested several Chinese herb extracts and shown that the aqueous extract of *P. vulgaris* (CHPV) exhibited the most potent inhibitory activity against EBOV-GP virus entry out of all of

the extracts tested (Fig. 8). Remarkably, CHPV could efficiently inhibit the infection of EBOV-GP pseudovirions in its primary target cell, the human macrophage. The inhibition rate reached as high as 99.5% when CHPV (20 ug/ml) was continually present in the culture medium (Fig. 11C). The same concentration of CHPV could inhibit approximately 80% of viral infectivity in HUVECs, even after cells were treated for only 2 hrs with CHPV and virus added soon after (Fig. 10). Moreover, our study has clearly shown that 1.25 ug/ml of CHPV reduced the wild type EBOV infection by 50%, while in the presence of 12.5 μg of CHPV, EBOV virus infection was inhibited by more than 80% (Fig. 12). These observations provide convincing evidence for CHPV as a potential agent against EBOV that deserves further investigation in animal studies.

To investigate the CHPV anti-EBOV mechanism of action, a "time-of-addition" study was performed. Here, CHPV was identified as having blocked EBOV-GP-V infection, possibly by suppressing virus attachment through targeting the viral GP protein (Fig. 9). Previous studies demonstrated that aqueous extracts of PV can decrease the replication of herpes simplex virus (HSV) by preventing virus binding to cells [48, 72] and inhibit HIV-1 infection through prevention of viral attachment to the $CD4^+$ T cell receptor [46, 47, 218], suggesting that CHPV targets an invariant component among these viruses. In fact, it was suggested that the antiviral activity of CHPV might be related to an anionic polysaccharide. Polysaccharide can form complexes with virus proteins, which is likely to occur mainly through the formation of salt linkages or ion pairs between oppositely charged groups on the polymers and the virus proteins, ultimately blocking virus entry [77]. For example, it is well established

that sulfated polysaccharide can selectively interact with the polycationic V3 loop, which is a highly basic region on gp120 [219], to interfere with the attachment of HIV-1 to cell-surface receptors [220-222]. Whether a polyanionic property is responsible for the viral entry inhibition mechanism of CHPV remains to be determined. However, at this point, we also could not rule out the possibility that the presence of CHPV might also result in conformational changes in EBOV-GP and render it unable to interact with its receptor, which requires further investigation. The questions of the specificity of the antiviral activity of CHPV and whether CHPV can act against all viruses or only for enveloped viruses still remain unanswered. A previous report has shown that a polysaccharide from *P. vulgaris* (at 100 ug/ml) was active against the HIV-1 and herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), but was unable to inhibit cytomegalovirus (CMV), human influenza virus types A and B, poliovirus type 1 or vesicular stomatitis virus (VSV) [223]. Consistently, our data also indicated that CHPV could not inhibit VSV-G pseudovirion infection (data not shown). Thus, CHPV action appears to be specific to certain virus species. In addition, our data also revealed that CHPV does not act on the cell surface or change its susceptibility to HIV-1 [47] or EBOV (Fig. 9A). Thus, all of these observations suggest that there is a specificity of the antiviral effect of CHPV. However, the underlying molecular mechanism(s) requires further investigation.

Another interesting observation in this study is that the combination of the anti-EBOV antibody (2G4) with CHPV could enhance their anti-EBOV activity. The 2G4 antibody was first reported by Qiu *et al*. [188], and it is a crucial component of the

two antibody cocktails, ZMAb and ZMapp. Mab 2G4 shows great potential in blocking EBOV infection *in vitro* and in animal studies [190, 211, 224, 225]. Recently, ZMapp was approved in February 2015 for a clinical trial in Liberia and the United States [226]. One disadvantage of using MAb as an anti-EBOV agent is its source limitation, especially for the resource-constrained geographic regions where the outbreaks of filovirus infection frequently occur. Therefore, the discovery of other agents capable of preventing EBOV virus infection or contributing to the enhancement of Mab-mediated anti-EBOV effects is needed. We investigated whether the presence of CHPV and 2G4 together would demonstrate a stronger anti-EBOV effect. The results showed that the combined use of lower concentrations of both 2G4 and CHPV could achieve the same anti-EBOV-GP efficiency as a high concentration of 2G4 alone (Fig. 11 and 12). The reason for this might be due to a combined effect through their different binding mechanisms. The 2G4 monoclonal antibody has been known to bind to the GP2 fusion loop, which is important for viral entry [188, 211]. In contrast, the action of CHPV is speculated to target the GP1, due to the mucin-like domain in GP1 that is responsible for viral attachment [32, 227-229] and the fact that CHPV exerts its effect during the attachment of virus to the cells. Additionally, we could not exclude the possibility that CHPV may also bind to the GP2 fusion region, which requires further investigation. This combined effect is significant, as (i) similar efficiencies would be achieved by using reduced amounts of antibody and CHPV, (ii) the combination of 2G4 and CHPV will reduce the likelihood of viral resistance and (iii) the therapeutic window could be extended by continuous use of CHPV in an infected individual. In addition, many

studies have demonstrated that the aqueous extract contains many components having immunomodulatory effects [63-70]. Thus, aside from directly having an anti-EBOV effect, CHPV might also have great potential as an immunomodulator to suppress the severe upset and disorder of the immune cell proliferation and contraction caused by EBOV infection, further extending the antibody therapeutic window.

Overall, we demonstrated that CHPV acted as an EBOV entry inhibitor and could enhance the inhibitory activity of EBOV MAb. This natural plant extract merits further investigation as a promising antiviral due to its rich source, low costs, low cytotoxicity, wide acceptability and strong antiviral properties. Additionally, further detailed chemical research to identify and purify the active ingredient(s) will facilitate its mechanistic analyses and applications.

3.3 Future Directions

3.3.1 Investigate the inhibitory effect of CHPV on EBOV GP-triggered immune activation and vascular permeability

Following infection, virus-encoded GPs are released from cells in soluble forms, such as sGP, delta peptide, GP1 and shed GP. High amounts of sGP and truncated surface GP are detected in the blood of patients and experimentally-infected animals [38, 39]. Unfortunately, Shed GP, sGP likely compete with virion attached GP for antibody binding [41]. Shed GP inhibits the neutralizing EBOV antibody activity in a guinea pig model [39]. Shed GP was also shown to activate human-derived non-infected dendritic cells (DCs) and macrophages by inducing the secretion of pre- and anti-inflammatory

cytokines through cellular toll-like receptor 4 (TLR4) [44]. In addition, this study demonstrated that shed GP affects endothelial cell permeability both directly and indirectly through cytokine release. Therefore, shed GP provides a potential link between systemic inflammation and increased endothelial permeability that together contribute to the disseminated intravascular coagulation syndrome in Ebola infection. Our findings that CHPV binds to EBOV surface GP and blocks viral entry lead to the hypothesis that CHPV can also bind to shed GP, which resembles the structure of virion GP1, 2, and would also inhibit the immune response and pathogenesis induced by EBOV GP. In this section, we will provide experimental evidence to support this hypothesis.

3.4.2 To investigate the inhibitory effect of CHPV on EBOV infection in vivo

3.4.2.1 Investigate the protective effect of CHPV in EBOV-infected mice and Guinea pigs

Our preliminary data demonstrated that the CHPV from natural medicine plant PV can efficiently inhibit Ebola virus infection by blocking viral entry in several cell types including primary macrophages and HUVECs. After testing the inhibitory effect of CHPV on wild type EBOV infection, we will further evaluate the possibility of using CHPV as anti-EBOV agent in an animal model.

Besides PV antiviral activity, it has been shown to have immunomodulatory properties. Its extract and chemical components can modulate various immune factors including histamine, TNF-α, IgG, IgG1, IgG2b,oxide, LTB4, IFN-gamma and IL-2 [63, 64, 67, 70]. It was shown that the methanol extract of PV with rosmarinic acid inhibited IL-2

gene expression by 50% in Jurket cells stimulated with anti-CD3 and anti-CD4 antibodies[66]. The aqueous extract of PV was found to be able to inhibit the release of histamine and TNF-alpha and results in a inhibition of immediate-type allergic reactions in rats [63]. Moreover, the polysaccharide fraction isolated from PV markedly stimulated the production of superoxide and nitrite in murine macrophage RAW264.7 and brine macrophage BV2 cells [68]. The immunomodulatory effect of PV might have significant meanings for controlling EBOV infection, whose virulence can be attributed to the immunopathogenesis and several immunoevasion mechanisms [230]. For example, EBOV infection of initial infection cells, such as monocytes and macrophages, leads to the uncontrolled release of proinflammatory cytokines and chemokines, including TNF-alpha, IL-1 beta and macrophages inflammatory protein 1 alpha (MIP-1a), as well as reactive oxygen and nitrogen radicals [231]. If PV can suppress the upregulation of inflammatory cytokines caused by EBOV infection together with its antivirial activity, it is possible to control the viral infection at early stage and relieve the condition of symptoms to extend the window of opportunity *in vivo*.

For mouse studies, we will use a mouse-adapted EBOV strain that is currently in use in filovirus laboratories because the wild type EBOV is non-lethal in mice. This mouse model of EBOV infection, which has been used for over 15 years since its generation, is a well-established model that leads to 95% lethality in mice [232]. This EBOV mouse model is an economical and accurate down selection tool because, historically, if a treatment has not conferred protection in mice, it also fails to provide protection in

higher animal models. The CHPV protective effect should also be tested in a standard guinea pig EBOV model [233]. This model results in 90% lethality with classic hallmarks of known human and nonhuman primates (NHP) EBOV pathologies.

3.4.2.2 Combine CHPV and MAb to enhance their protective efficacy in EBOV-infected animals

Qiu's previous studies have demonstrated that Ebola GP-specific MAbs protect mice and guinea pigs from lethal EBOV infection [234]. Moreover, 100% and 50% survival of EBOV-infected cynomologus macaques were observed when ZMAb, a combination of three EBOV-GP-specific mAbs (1H3, 2G4 and 4G7), was administered at 24 or 48 hours post-exposure [190]. However, the MAb-based treatment did not completely inhibit EBOV replication and there is only a limited time period in which to begin treatment after being infected. Interestingly, our data have shown that the natural medicine plant CHPV can efficiently inhibit Ebola GP-mediated viral entry in several cell types including primary macrophages and HUVECs (Fig 7-10). The inhibition effect was enhanced when a lower concentration of MAb 2G4 was used in combination with CHPV (Fig. 11A). Especially in macrophages, continually treated cells with CHPV inhibited 95% viral entry. When combined with lower dose of MAb (1.6ug/ml), the virus infection was completely blocked (Fig.11B). This combination treatment is significant because: A): we achieved similar efficiency using reduced amounts of antibody; B): we might be able to extend the therapeutic window; and C): the combination might reduce the occurrence viral resistance. In addition, many studies

have demonstrated that the CHPV has immunomodulatory effects [63-70]. Thus, in addition to a direct anti-EBOV effect, CHPV may also have great potential to reduce the pathogenesis or severity of the EBOV infection. Therefore, we will treat EBOV-infected animals with a CHPV/MAb combination and evaluate its protective effect.

CHAPTER 4

Investigation of the regulatory role of IL-34 during HIV-1 infection in macrophages and CD4+ T lymphocytes

4.1 Introduction

Although a significant role of macrophage in AIDS pathogenesis has been identified, how the HIV-1 life cycle was regulated by macrophage-associated components during macrophage infection remains elusive. Additionally, how soluble factors produced by macrophages manipulate the spread of virus to T cells remains unknown. Thus, it is necessary to adequately understand how the functions of macrophage in HIV-1 replication are regulated by macrophage-associated cellular factors.

The effect of CSF-1R ligand on HIV-1 replication in macrophage has been previously well described previously. Generally, there are two major effects of CSF-1R-mediated regulation on persistent HIV-1 infection and viral pathogenesis. A): sustaining the survival of HIV-1 infected macrophages, B): stimulating viral replication in macrophages by multiple mechanisms.

M-CSF, as the dominant CSF-1R ligand cytokine, plays an important role in sustaining monocyte/macrophage lineage cells survival and proliferation [235-237]. However, IL-34 is also a hematopoietic growth factor and binds CSF-1R on myeloid progenitor cells triggering relative signaling to promote the ontogeny and survival of macrophages similar to M-CSF [170, 238]. Thus, we can not discount the possibility that IL-34 and M-CSF contribute to enlarging the pool of HIV-1 susceptible macrophages and/or sustain the longevity of HIV-1 infected macrophages to benefit the virus production.

The reported effects of M-CSF and IL-34 on HIV-1 replication in macrophage are as follows. First, M-CSF can enhance HIV-1 replication in a dose-dependent manner [239]. Additionally, this significantly enhanced viral production can be blocked by treatment with specific M-CSF neutralizing antibody [240]. In a separate study, HIV-1 infected MDMs treated with M-CSF enhanced the kinetic of viral replication and shifted the viral load peak to an earlier time point [241]. Although IL-34 is a newly identified cytokine in recent years, the remarkable effect of IL-34 on M-tropic HIV-1 strain replication in macrophage lineage cells had been mentioned in several reports [242], [243].

Therefore, to further characterization of the effect of IL-34 on regulating the functions of macrophage in both of T-tropic and M-tropic HIV-1 replication, as well as exploration into the potential relationship of IL-34 and M-CSF in AIDS pathogenesis is crucial for the design of therapeutic strategies.

In the work presented here, the role of IL-34 in controlling early and late stage of M-tropic HIV-1 infection in MDMs is characterized, using M-CSF as control. We

found IL-34 and M-CSF can promote T-tropic HIV-1 replication in resting hPBMCs. Further investigation into the underlying mechanism proved that IL-34 and M-CSF stimulated monocytes differentiation such that they regulate unknown cellular factors expression profiles which in turn facilitate T-tropic HIV-1 replication efficiency in CD4+ T cells, rather than affecting the CD4+ T cells susceptibility to virus directly.

4.2 Results

4.2.1 IL-34 and M-CSF promote M-tropic HIV-1 replication through facilitation of combined early and late stages in the HIV-1 replication cycle.

In order to evaluate the effects of IL-34 or M-CSF on HIV-1 replication in macrophages, we treated the purified CD14+ monocytes from hPBMCs with IL-34 or M-CSF for 1 week before pNL4.3-Bal strains of M-tropic HIV-1 infection. At 9 or 12 days post-infection, the p24 Ag in culture fluids was detected by p24 ELISA, respectively. The Fig. 13A showed p24 Ag in IL-34 or M-CSF treated groups was significantly higher than the control, an equal number of macrophages cultured in medium alone. For further confirm the effect of IL-34 or M-CSF on viral replication in macrophage, we infected the same number of control, IL-34 or M-CSF induced macrophages with single-cycle replication-competent M-tropic-ENV pseudotyped Gluc reporter HIV-1 (R5ENV-Gluc-HIV), as described in Materials and Methods. At 48h p.i., we detected 3- to 4-fold-higher Gluc activity and p24 Ag concentration in both of IL-34 or M-CSF differentiated macrophages was seen in both investigated infection models. However, the effect of both IL-34 and M-CSF on the enhancement of Gluc activity and p24 Ag concentration was inhibited by a human CSF-1R specific neutralizing antibody (Fig.

13B). Similar results were also repeated by using single cycle virus. Nevertheless, in order to further analyzing and compare the effect of IL-34 and M-CSF on the susceptibility of MDMs to HIV, qPCR was used to examine the integrated DNA to rule out the post-integration steps of HIV-1 life cycle. Because integrated viral DNA was defined as the major feature of provirus, resulting from the early events of HIV-1 life cycle. Thus, it was always used to evaluate the susceptibility to HIV-1 [244]. R5Env-Gluc-HIV infected macrophages were collected to analyze the level of viral integrated DNA in the control, IL-34 or M-CSF. Meanwhile, IL-34 with either Fms-Ab, or M-CSF with Fms-Ab treated macrophages, were used to determine the involvement of IL-34 or M-CSF in early stage of HIV-1 infection. Interestingly, increased viral integrated DNA was detected by qPCR technique, which suggested that IL-34 might enhance early stage of viral replication (Fig. 13C).

Subsequently, the next question we posed was whether IL-34 and M-CSF also can promote post-integration steps to contribute to HIV-1 replication, even though their effect on facilitating early stage of viral infection was ruled out. Interestingly, a previous study indicated that the enhancement of HIV-1 replication mediated by M-CSF was not observed when monocytes were treated with M-CSF at the time of or 1 week after HIV-1 infection, which may suggest that there is no M-CSF-induced effect on increasing HIV-1 infection and replication besides finding that M-CSF induced MDMs differentiation and maturation lead to increased susceptibility to HIV-1 infection. However, there is more solid evidence to support our hypothesis. As the downstream signal transduction pathways of activated c-Fms in macrophages became

better understand, the activation of MAP kinase (MAPK) and JAK/STAT pathways were identified through stimulating the CSF-1R tyrosine phosphorylation by both of IL-34 and M-CSF [238, 242]. It is well documented that, by activating MAPK or JAK/STAT signaling pathway, the long terminal repeat (LTR) drived viral transcription is promoted in HIV-1 infected macrophages [245]. Thus, next we characterized and compared the effect of IL-34 and M-CSF on viral transcription in infected macrophages. In order to test whether IL-34, M-CSF are involved in post-integration steps (i.e., the late stage) of HIV-1 infection, the single cycle virus infected macrophages were treated by IL-34 or M-CSF, respectively (untreated group as control). Specifically, as the monocytes are resistant to HIV-1 infection, the monocytes purified from hPBMCs were cultured in DMEM supplemented with 10% human AB+ serum for 7 days before infection to enhance the monocytes adhesion and differentitation [246]. Then, in order to enhance the single cycle virus infection efficiency, the prepared cells were infected by VSV-G pseudotyped Gluc reporter HIV-1 single cycle virus(VSVG-Gluc-HIV). After checking the Gluc activity of the supernatant at 48 hrs p.i. to make sure the same infection extent, the cells were well washed and treated with normal medium, IL-34 or M-CSF, respectively. After 48 or 96 hs stimulation, the Gluc activities in the supernatant were increased over the control group, respectively. Similar results also were confirmed by p24 Ag concentration (Fig. 13D). The cells were then collected to further evaluate the effect of IL-34 or M-CSF on viral transcription in infected macrophages by RT-PCR. The data indicated both IL-34 and M-CSF induced 3-fold more viral transcription in corresponding treated VSVG-HIV-Gluc infected MDMs

than the control of same infection condition of cells cultured in normal medium (Fig. 13E). Interestingly, we found that M-CSF as a control treatment, similar efficiency was also identified, which coincides with a previous report that IL-34 and M-CSF induced similar MAPK phosphorylation and pathway activation in CSF-1R-expressing macrophages [238].

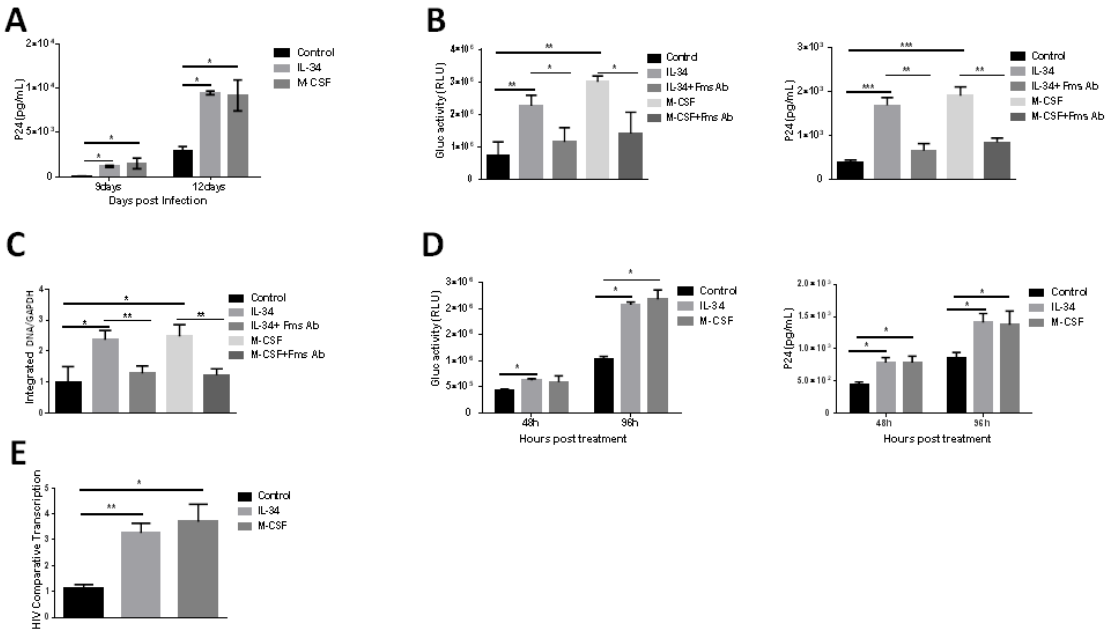

Figure 13: IL-34 and M-CSF promote M-tropic HIV-1 replication through facilitating combined early and late stages on the HIV-1 replication cycle. The IL-34, M-CSF treated or untreated CD14+ monocytes drived macrophages were cell number normalized and replated in 24-wells plate for next infection process. (A) The viral replication of 4.3-BAL HIV-1 in prepared macrophages was analyzed by p24 concentration ELISA. (B) The infectivity of single cycle pseudovirus R5ENV-Gluc-HIV in IL-34, IL-34 + fms-Ab, M-CSF and M-CSF + fms-Ab induced macrophages at 48 hrs post-infection were analyzed by p24 ELISA and Gluc activity, respectively. (C) After R5ENV-Gluc-HIV 20 hrs infection, the integrated HIV-1 DNA was tested by real-time PCR and normalized by GAPDH gene. (D) Purified CD14+ monocytes were differentiated by cultured with 10% AB serum. After 7 days AB serum treatment, macrophages were infected by single cycle VSVG-Gluc-HIV. The infected cells were treated with or without 25ng/mL IL-34/M-CSF at 48 hrs post-infection. After corresponding treatment hours, the Gluc activity and p24 concentration of supernatant were tested, respectively. (E) HIV-1 comparative transcription (GAG/GAPDH) of 48h treated group was detected by real-time PCR. Error bars in this figure represent the SD (standard deviations) of more than three separate samples. Results are representative of three independent experiments using primary cells from three different donors. (*, P<0.05; **, P<0.01; ***, P<0.001)

4.2.2 IL-34 and M-CSF enhanced T-tropic HIV-1 replication in PBMCs, rather than purified CD4+ T cells.

CD4+ T lymphocytes are the major target cells for HIV-1 infection, and thus it was necessary to evaluate whether IL-34 played a role on T-tropic virus replication in PBMCs and CD4 T cells, though cells of the macrophage lineage are the major effector cells for both IL-34 and M-CSF. First, primary CD4+ T cells and hPBMCs were isolated as described in methods. Briefly, PBMCs or CD4+ T cells isolated from three healthy donors were cultured with IL-34 or M-CSF for four days respectively. Then, the pretreated cells were cell number normalized and then infected by the nevirapine-resistant HIV-1 in the absence of IL-34/M-CSF. After overnight infection, the cells were washed and continuously cultured with fresh medium, IL-34 or M-CSF. At different days post infection, the supernatants were collected and analyzed for HIVp24 concentration by ELISA. For the hPBMCs group, the results showed that both of IL-34 and M-CSF treatment facilitate X4-tropic HIV-1 replication in hPBMCs from all three independent donors. Interestingly, both the IL-34 and M-CSF treated groups demonstrated a higher cell viability than untreated controls (data not shown). We want to know if the improvement of viral replication induced by IL-34 or M-CSF will be impaired when the viral replication was normalized by cell viability. However, the normalized data also reveals a significant effect of viral replication enhancement on IL-34 and M-CSF treated groups (Fig. 14A). For the primary CD4+ T cells that were obtained through the same treatment and infection process, there is no statistically

significant effect of HIVp24 increasing between IL-34 or M-CSF treated groups compared to control samples (Fig. 14B). In summary, T-tropic virus replication in hPBMCs was enhanced by IL-34 or M-CSF treatment, but not in purified resting CD4+ T cells.

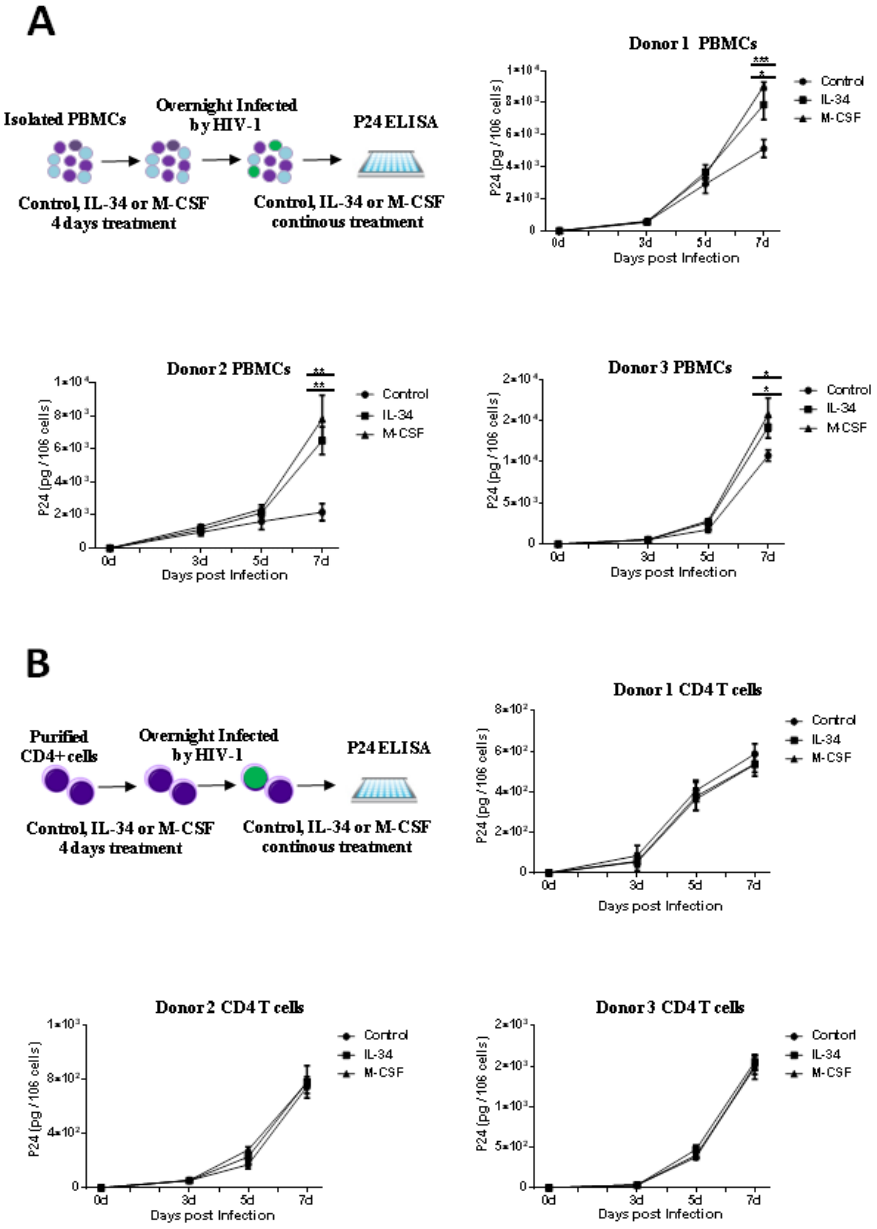

Figure 14: IL-34 and M-CSF enhanced T-tropic HIV-1 replication in PBMCs, rather than purified CD4+ T cells. (A) The PBMCs from heath donors were pretreated 25ng/mL IL-34 or M-CSF for 4 days, untreated sample as control. Then, the pretreated PBMCs were cell number normalized to replate in 24-wells plate, and cultured without IL-34/M-CSF before infection. The prepared PBMCs were infected by nevirapine-resistant HIV-1. After overnight infection, the cells were washed and continuously cultured with corresponding normal medium (control sample), IL-34(25ng/mL) or M-CSF (25ng/mL), respectively. The supernatants collected at 0, 3, 5, 7 d.p.i. were analyzed for the p24 concentration by ELISA. The p24 values were normalized by the cell numbers at indicated time point (analyzed by trypan blue exclusion test). (B) The primary CD4+ T cells went through the same pre-treatment, infection and p24 detection process. Error bars in this figure represent the SD (standard deviations) of three separate samples. (*, P<0.05; **, P<0.01; ***, P<0.001)

4.2.3 The presence of IL-34 and M-CSF acted on macrophages to release unknown cellular factors, which can facilitate T-tropic HIV-1 replication in CD4+ T cells.

Taken together, these results demonstrate that some other unknown mechanisms are responsible for IL-34 or M-CSF promoting T-tropic HIV-1 replication in PBMCs, rather than directly changing CD4+ T cells susceptibility. Thus, as the IL-34 and M-CSF are dominant ligands of CSF-1R, it is possible that IL-34 is able to indirectly affect T cell susceptibility by regulating macrophage function.

Notably, macrophages can be infected by T-tropic HIV-1 via selective capturing of HIV-1-infected CD4+ T cells [247]. Thus, considering the dominant role of IL-34 and M-CSF in facilitating HIV-1 replication in MDMs, we then set out to exclude the possibility that the enhancement of T-tropic HIV-1 replication in PBMCs is induced by IL-34 and M-CSF promoting viral infectivity and virion generation in MDMs. First, we found that even though IL-34 or M-CSF treatment enhanced transmission of T-tropic virus from infected T cells to macrophages to some extent. The viral production from these macrophages was far too limited to contribute to the increased T-tropic HIV-1 replication in PBMCs (data not shown). Importantly, we also evaluated the effect of IL-34 on the viral replication in CD4+ lymphocytes of T-tropic HIV-1 infected PBMCs. Briefly, the isolated PBMCs were infected by nevirapine-resistant HIV-1 for 3 days, then the cells were stimulated with IL-34, M-CSF or normal medium, respectively. After 6 days culturing and stimulation, the CD4+ cells were purified and continually cultured in normal medium to evaluate respective viral replication. Consistently, the

p24 ELISA results, shown in Fig. 15A, indicate that the HIV-1 replication was increased in the CD4+ cells that were purified from IL-34 or M-CSF treated, pre-infected, PBMCs. Thus, combined with previous results, we further confirmed the assumption that the replication efficiency of T cell was indirectly affected by IL-34 via regulating macrophages function.

One possible explanation of this phenomenon is the mediation, at least in part, by the promotion of systemic immune activation through the facilitation of macrophage and T cell interaction during HIV-1 infection. For instance, since macrophages are Antigen Presenting cells (APCs) during an adaptive immune response, which play an important role in inducing T cell activation, the CSF-1R ligands differentiated and activated macrophages were able to further activate resting T-cells, via MHC-peptide and costimulatory molecules [248]. There is a broad consensus that this robust immune activation and proliferation of effector CD4+ T cells causes further enhancement of susceptibility of CD4+ T cells to HIV-1 [249, 250]. In addition to well-known immune activation, we were eager to determine whether there are additional underling mechanisms involved in CSF-1R ligands mediated regulation of HIV-1 replication in T cells. To exclude the APCs dependent T cell activation, the PBMCs were pre-treated with IL-34 or M-CSF, before purifying corresponding CD4+ cell and exposure to HIV-1. Briefly, the isolated PBMCs were cultured with or without IL-34, M-CSF, respectively. After 6 days of culturing, the CD4+ cells were purified from the IL-34 or M-CSF treated PBMCs, and exposed to Nevirapine-resistant HIV-1. After 24 hrs infection in the absence of IL-34 or M-CSF, the infected cells were washed and cultured

in normal medium, after which the p24 concentration in the supernatant was measured by ELISA to evaluate the viral replication in the respective CD4+ T cells. Interestingly, the results showed that the viral replication was facilitated in the CD4+ T cells purified from the uninfected PBMCs and treated with IL-34 or M-CSF (Fig. 15B). That means that even in the absence of HIV-1 antigen stimulation, the susceptibility of CD4+ T cells were still increased when uninfected PBMCs were treated with IL-34 or M-CSF.

We further provided proof that the IL-34 was able to indirectly affect CD4+ T cell susceptibility by regulating cellular factors produced from MDMs. Briefly, CD14+ cells purified from PBMCs were stimulated by IL-34 or M-CSF to differentiate into macrophages for 7 days. The supernatant of IL-34 or M-CSF induced macrophages were proceeded by low-spin centrifugation to clear cell debris, and aliquoted for the continuous stimulation of homogeneous CD4+ T cells. After 6 days of treatment, the CD4+ T cells were cell number normalized and infected with HIV-1. Following overnight infection, the cells were washed and cultured in fresh medium without IL-34 or M-CSF. The p24 concentrations of supernatant were tested by ELISA following 3 days culture. The data in Fig. 15C indicates that the susceptibility of CD4+ T cell was enhanced, when the CD4+ T cells were continuously treated with the culturing supernatant from IL-34 or M-CSF derived homologous macrophages. Additionally, the results suggest that the production of unknown cellular factors from macrophages were potentially regulated by IL-34 or M-CSF to affect T cell susceptibility to HIV-1 and/or viral productivity.

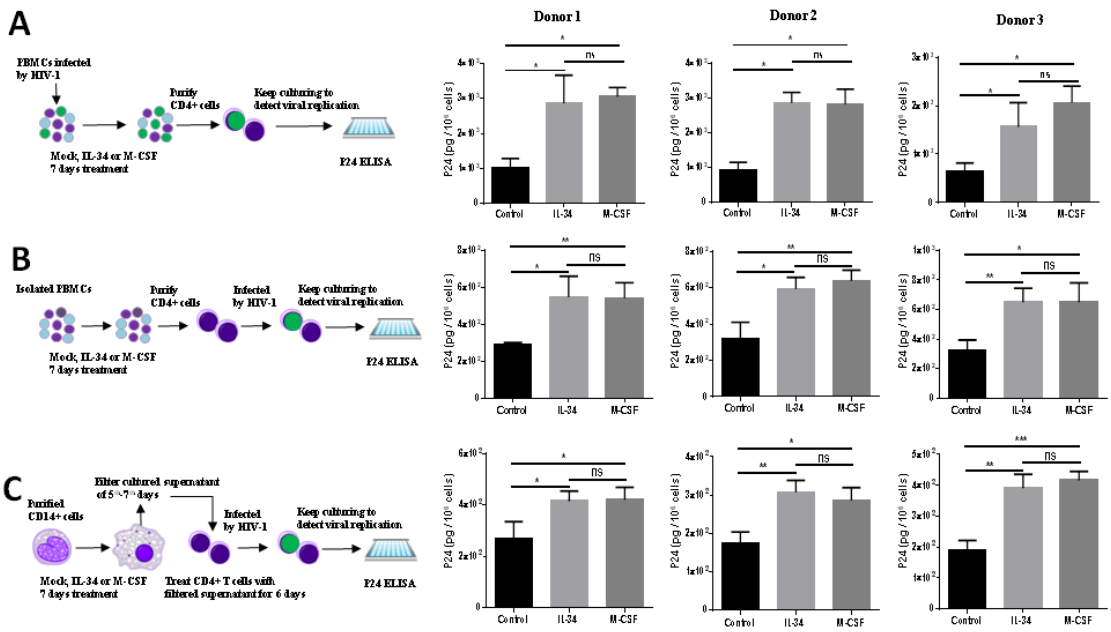

Figure 15: The presence of IL-34 and M-CSF acted on macrophages to release unknown cellular factors, which can facilitate T-tropic HIV-1 replication in CD4+ T cells. (A) The nevirapine-resistant HIV-1 infected PBMCs were plated in 6-wells plate ($1.5x10^7$ cells/well), and cultured with or without 25ng/mL IL-34/M-CSF. After 7 days treatment, the CD4+ T cells were purified, and cultured in 24-wells plate ($1.0 x10^6$ cells/well) with normal medium, respectively. Then, the p24 concentrations of supernatant were tested by ELISA after 3 days culture. (B) The fresh isolated PBMCs were cultured with or without 25ng/mL IL-34/M-CSF for 7days. The CD4+ T cells were respective purified and infected with nevirapine-resistant HIV-1 in the absent of IL-34 and M-CSF. Then, the CD4+ T cells were cell number normalized, and cultured with normal medium in 24-wells plate. After 3 days culturing, the supernatant was collected to analyze corresponding p24 concentrations. (C) CD14+ cells purified from PBMCs were stimulated with or without 25 ng/mL IL-34 or M-CSF for 7 days. The collected 5th-7th days supernatant were performed by low-spin centrifugation to clear cell debris, and aliquoted for continuously treating homogeneous CD4+ T cells, respectively. After 6 days treatment, the CD4+ T cells were infected by nevirapine-resistant HIV-1 and cultured in medium without IL-34 or M-CSF. The p24 concentrations of supernatant were tested by ELISA after 3 days culture. Error bars in this figure represent the SD (standard deviations) of three separate samples. (*, P<0.05; **, P<0.01; ***, P<0.001)

4.3 Discussion

In the present study, we proved IL-34 can enhance both the early and late stages of HIV-1 infection in MDMs. Furthermore, we found that IL-34 and M-CSF can enhance the X4 HIV-1 replication in PBMCs by sustaining host cell viability and facilitating the cells susceptibility to HIV-1. However, neither IL-34 or M-CSF affect the susceptibility of CD4+ T cells directly. Through more detailed analyses, we demonstrated that in the presence of IL-34 or M-CSF, macrophages were able to release unknown soluble cellular factors, which can stimulate CD4+ T cells and render them more susceptible to HIV-1 infection.

First, we confirmed the viral infection or replication of M-tropic HIV-1 was indeed significantly enhanced in IL-34 or M-CSF-induced MDMs compared with untreated group (Fig. 13A, B), which was consistent with previous reports [242]. Although the detailed mechanism on how IL-34 enhances the HIV-1 replication in macrophage is elusive, it is tempting to speculate the implied concept or mechanism based on the previous understanding of the effect of M-CSF on facilitating HIV-1 replication in macrophage.

Our initial belief is that the differentiation induced by IL-34 enhances the susceptibility of MDMs to HIV-1 to contribute to HIV-1 replication. Based on a previous report, 7 days of continuous M-CSF treatment increased the susceptibility of MDMs to HIV-1 400-fold over an equal number of untreated control cells. This suggested that the role of M-CSF in facilitating HIV-1 infection involves the ability of M-CSF to stimulate MDMs differentiation and/or activation, as well as contribute to the phenotype of

MDMs enhanced susceptibility to HIV-1 infection [239]. Specifically, SAM domain–and HD domain–containing protein 1 (SAMHD1) as one of the major cellular restriction factors acts by inhibiting HIV-1 infection through depleting the pool of nucleotides available to a reverse transcriptase for viral cDNA synthesis in human myeloid lineage cells, including MDMs [251, 252]. Additionally, the degradation of SAMHD1, which was reported to abrogate its anti-HIV-1 activity, was more prominent in M-CSF-differentiated MDMs than in undifferentiated monocytes [253]. These findings suggested that the high efficiency of M-CSF on SAMHD1 restriction to enhance the HIV-1 infection involves the ability of M-CSF to inducing monocyte differentiation to macrophages, potentially increasing the susceptibility of MDMs to HIV-1. Meanwhile, M-CSF has been found to improve cell surface expression of CD4 and CCR5, two crucial receptors involved in M-tropic HIV-1 envelope mediated entry, to promote the susceptibility of these cells to HIV-1 infection [254-257]. Interestingly, previous studies also indicated that the slightly higher HIV-1 replication in IL-34-macrophages is associated with a stronger macrophage differentiation phenotype and function compared to M-CSF-macrophages [242]. Therefore, although there was no critical evidence to show the effect of IL-34 on regulating the expression or function of CD4, CCR5 or SAMHD1, the strong effect of IL-34 on MDMs differentiation leads us to anticipate and evaluate the potential efficiency of IL-34 on modulating permissiveness of MDMs to HIV-1. As shown in Fig. 13C, our primary finding was that the integrated viral DNA was increased in IL-34-differentiated MDMs. That means the IL-34-differentiated MDMs can facilitate increased susceptibility to HIV-1. However,

IL-34 facilitated MDMs susceptibility to HIV at multiple steps in the early stage of HIV-1 life cycle, including viral entry, reverse transcription, nuclear import and integration. Thus, understanding which steps were responsible for the high permissiveness of MDMs to HIV-1 will be one of the aims of our future studies. Besides, as there was no statistical difference between IL-34 with M-CSF treatment, the next perplexing question is why IL-34 and M-CSF induced the similar effect on MDMs susceptibility to HIV-1, though we have an implied concept about the distinct biological ability of IL-34 and M-CSF to affect macrophage phenotype or function [242, 258]. In spite of IL-34 inducing a stronger affinity for CSF-1R than M-CSF, M-CSF compensates for its weaker affinity to the receptor, results in indistinguishable monocytes differentiation than IL-34 [170, 238]. Both of IL-34 and M-CSF induced CSF-1R tyrosine phosphorylation and ERK1/2 activation with similar kinetics, which suggest that once bound to the receptor, both ligands equivalently stimulated it to mediate responses [238]. While some studies have indicated that IL-34 and M-CSF may trigger non-overlapping downstream signaling pathways in monocytes to suggest a potential functional specialization between these two cytokines [181], regardless of different mechanisms, the consequence of stimulating differentiation by either IL-34 or M-CSF remained the same as reflected by similarly macrophage maturation and functions [170, 238].

In this report, we found that both of IL-34 and M-CSF was able to promote viral transcription from the single cycle HIV-1 virus infected macrophages by unknown mechanisms (Fig. 13D, E). C/EBPb, a transcription factor, was upregulated by M-CSF

treatment, which was previously proposed as one possible underlying molecular mechanism [259]. Briefly, CCAAT/enhancer binding protein b (C/EBPb), is a member of a family of transcription factors that regulate myeloid cell activation and function[260]. C/EBPb expression was upregulated by M-CSF treatment[261]. Interestingly, another study demonstrated that C/EBP proteins are required for HIV-1 LTR mediated viral transcription activity in infected macrophages [262]. We found a potent role of IL-34 in promoting viral transcription and production. Taken together, in addition to facilitating the susceptibility of macrophages to HIV-1 infection, IL-34 may also promote M-tropic HIV-1 replication in macrophage by enhancing viral transcription.

Although there is limited data for illustrating the role of IL-34 on M-tropic HIV-1 replication in macrophages, and none are published, to date, for the evaluation of T-tropic HIV-1 replication regulated by IL-34. Our next objective was to characterize and compare the efficiency of IL-34 and M-CSF on T-tropic HIV-1 replication in PBMCs and CD4+ T cells.

Fig. 14 showed that the replication of T-tropic HIV-1 in h-PBMCs from three independent donors was facilitated in both the IL-34 and M-CSF treatment. Moreover, we found that IL-34 can cause viable cell number growth. In order to exclude that the increased viral load was caused by cell proliferation, the p24 value of per million cells was calculated by normalizing cell proliferation and results showed that IL34 can enhance viral replication in resting PBMCs (Fig. 14A). However, because T-tropic virus replicate primarily in CD4+ T cells by using the co-receptor alpha-chemokine

receptor (CXCR4), for its entry [263], whether IL-34 or M-CSF facilitated nevirapine-resistant HIV-1 replication in purified CD4+ T cells directly was at first perplexing. In spite of no CSF-1R detection in T lymphocytes, the question remained whether a newly identified protein-tyrosine phosphatase ζ (PTP-ζ), an alternative receptor of IL-34, was responsible for stimulating nevirapine-resistant HIV-1 replication in CD4+ T cell [264]. We then set out to test this hypothesis. However, our data showed in Fig. 14B, discounted this possibility, leading to the conclusion that nevirapine-resistant HIV-1 replication in hPBMCs is enhanced by IL-34 or M-CSF treatment, but not in purified resting CD4+ T cells.

Our results in Fig. 15 proved that the susceptibility of CD4+ T cell to HIV-1 was enhanced, when the CD4+ T cells were continuously treated with the culturing supernatant from either IL-34 or M-CSF derived homologous macrophages. As such, we further proposed that the enhanced susceptibility of CD4+ T cell was regulated by unknown cellular factors released from the IL-34 or M-CSF derived homologous macrophages. In the present study, we found IL-34 and M-CSF not only directly enhance the replication of M-tropic HIV-1 in macrophages, but also facilitate the T-tropic HIV-1 replication efficiency in CD4+ T lymphocytes indirectly. To the best of our knowledge, this represents first characterized role of IL-34, as a newly identified cytokine in promoting HIV-1 replication, in both of macrophages and CD4+ T lymphocytes. We also emphasize the contribution of IL-34 and M-CSF on HIV-1 productive propagation in T lymphocytes by acting on macrophages to release cellular stimulating factors.

4.4 Future Directions

4.4.1 Analysis of the cytokines/chemokines production profile of IL-34/M-CSF treated macrophages.

Our data in Fig. 15 has shown that the presence of IL-34 or M-CSF acts on macrophages to release unknown cellular factors, which can stimulate CD4+ T cells and lead them more susceptible to HIV-1 replication. Actually, a number of cellular factors produced from macrophages have been characterized and determined to regulate the HIV-1 replication efficiency in CD4+ T cells, as cytokines, chemokines or metabolic enzymes are able to.

Apart from regulating viral proteins, the cytokine and chemokines network is the major manipulator of plastic capacity of HIV-1 to infect productively or spread progressively. Additionally, some cytokines and their corresponding receptors are considered as useful markers to monitor disease progression and have large impacts on regulating patient immune systems. During recent decades, the role of cytokine, chemokines and other relevant molecules in HIV-1 replication and propagation in CD4+ T lymphocytes and macrophages has been reviewed [265-267].

Due to the crucial role of cytokines and chemokines in immune and inflammatory reactions to tightly regulate T cells functions. Moving forward we want to determine how IL-34 and M-CSF are involved in those reactions to change HIV-1 infection efficiency in T cells. We focus our attention on a number of macrophages produced cytokines and chemokines that have been previously reported to affect HIV-1 infection

and replication in CD4+ T cells, to characterize and compare their expression levels between IL-34 or M-CSF stimulation (Table 2, 3). Based on this study, we will have a better understanding as to how IL-34 and M-CSF regulate immune factors production profiles of macrophages to profoundly manipulate the immune environment to favor increased HIV-1 replication. Furthermore, this work will also emphasize the therapeutic value of cytokines/chemokines during immune reconstitution, and develop new therapies by targeting HIV-1 induced immune dysregulation, ultimately curtailing HIV-1 replication and propagation.

Table 2: The cytokines that can be produced from macrophages, and have been discussed their role in HIV-1 infection and replication to T cells, are enlisted.

Cytokines	Effect on HIV-1 infection/replication	References
IFN-γ	Bimodal: IFN-γ enhances viral transcription, and expression of IFN-γ is increased in blood mononuclear cells during HIV-1 infection. But IFN-γ blocks R5 virus entry and virion release	[268-270]
IL-1β	IL-1β inhibits HIV-1 replication in Jurkat cells and PBMCs.	[271]
IL-8	IL-8 level enhance in HIV-1 infection patients. Meanwhile, IL-8 can increase replication and transmission of HIV-1 in cervical explant tissues	[272, 273]
IL-10	Bimodal: IL-10 responses are associated with sustained CD4 T cell counts in treated HIV1 infection. Meanwhile, IL-10 is upregulated during HIV-1 infection. But IL-10 also can trigger PD-1 and CTLA-4 pathway to inhibit HIV-specific T cells activation	[274-277]
IL-12	IL-12 enhances HIV-1 replication in PBMCs.	[278, 279]

Table 3: The chemokines that can be produced from macrophages, and have been discussed their role in HIV-1 infection and replication to T cells, are enlisted.

Chemokines	Effect on HIV-1 infection/replication	References
CCL-2	Enhancement of HIV-1 replication in PBMCs.	[280, 281]
CCL-4	CCL-4 can increase the adsorption and replication of T-tropic HIV-1 in C8166 cells and PBMCs.	[282]
CXCL-9	Enhancement of HIV-1 replication. CXCL-9 also can induce mucosal CD4+ T cell activation.	[283]
CXCL-10	Enhancement of HIV-1 replication to mediate neuronal injury	[284]
CCL-19	CCL-19 increases permissiveness of resting memory CD4+ T cells to HIV-1 infection	[285]
CCL-22	Inhibition of HIV-1 replication in CD4+ T cells.	[286]

4.4.2 Investigation of the role of IL-34 in HIV-1-associated dementia.

The crucial role of CSF-1R in AIDS pathogenesis has been introduced in the previous section. The therapeutic strategies developed based on the blocking of CSF-1R have been well shown in various diseases [287-289]. The M-CSF serum concentration was significantly enhanced, which was caused by decreasing receptor-mediated internalization of M-CSF [174, 287]. As such, targeting the ligands will be more efficient to limit HIV-1 replication and relative disease than a pure receptor blockade. Moreover, IL-34 as an alternative ligand has a narrower spatiotemporal range and less effect on controlling tissues development, than M-CSF does [173]. Therefore, developing new therapeutic approach by targeting IL-34 is a more sophisticated strategy to limit HIV-1 reservoir formation and macrophage infection associated symptoms.

HIV-associated dementia (HIV-D) is a subcortical dementia symptom commonly observed in some AIDS patients. The productive HIV-1 infection in the cerebrospinal fluid (CSF) and central nervous system (CNS) of patients is considered to be responsible for causing the HIV-D development [290, 291]. Coincidently, Glass *et al*. indicated that the macrophages/microglia cells activation is a correlate for the HIV-D severity. This finding emphasizes the crucial roles of macrophage/microglia cell on the viral replication in CSF and CNS in contributing to the disease progress [292]. Interestingly, several studies also proved the contribution of macrophages infection in disease development via various mechanisms. First, macrophages/microglia cells were identified as the only cell types that were permissive for productive HIV-1 infection

[293-295]. Infected macrophages also were believed to be responsible for trafficking HIV-1 entry into CNS [296]. Additionally, during massive HIV-1 infection in brain, various regulatory factors, such as cytokines, chemokines and toxic metabolites, produced by monocytes/macrophages, may be involved in neuronal injury and apoptosis either directly or indirectly [297-300].

M-CSF as the dominant CSF-1R ligand cytokine, plays an important role in monocytes/macrophages circulating in CSF and microglia cells maturation and activation, which should contribute to the HIV-1 encephalopathy progress. Correspondingly, Gallo *et al*. demonstrated that M-CSF was enhanced in the CSF of patients with HIV-D, and the enhanced M-CSF may promote the matured and activated macrophages generation to contribute to cells circulating and trafficking into the CNS [301, 302]. Taken together, M-CSF is the master cytokine of CSF-1R and significantly augments the role of monocytes/macrophages in the HIV-1 associated encephalopathy pathogenesis via stimulation of the monocytes/macrophages phenotype. However, whether IL-34 as the other alternative cytokines are also able to induce a similar effect in progressing HIV-1 encephalopathy like M-CSF dose, is a perplexing question we wish to answer in future studies.

Although some studies reported that IL-34 was found in only a limited number of cells in lymph nodes, spleen and the kidney *et al*., the neurons in brain were the predominantly subset cells to contribute to IL-34 secretion [182, 183, 303]. Due to this distribution, a predominant function of IL-34 in brain diseases was suggested. More importantly, based on previous understanding and our results as to the role of IL-34 in

monocytes/macrophages phenotype activation and HIV-1 infection in macrophages, we strongly believe that IL-34 plays a significant role in HIV-1 associated encephalopathy. In addition, according to studies regarding physiological phenotypes of IL-34-deficient in (IL-34-/-) mice, they found that brain microglia cells development and proliferation were independent of IL-34, comparatively a reduction of microglia cells was observed in the brain of mice lacking M-CSF [182, 183]. This means targeting IL-34 should have more clinical potential for limiting HIV-1 infection in macrophages and controlling associated cerebral diseases.

Therefore, to investigate the potential role of IL-34 in HIV-associated organ diseases, especially HIV-1 induced neuropathy, is crucial future study direction. IL-34 also displays great promise for the development of new therapeutic antagonists by targeting IL-34 or relative pathways for treatment of HIV-1-associated symptoms.

These two major parts of my thesis are much different. The reason of I integrated them together is because each of these projects respectively present my interest in expanding our understanding on molecular and cellular modulatory factors that are essential for virus infection, and translating these basic scientific discovery on retroviruses or other wildly used virus, like VSV and AAV, into other fields clinical applications, such as controlling other infectious disease, the development of oncolytic virus against cancer or novel gene transfer tools.

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