

Development of a DNA vaccine inducing HIV-1 Gagp24-reactive T cells

by

Shuai Guo



Thesis for Master's Degree in Molecular Bioscience
60 study points

University of Oslo
November 2016

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Shuai Guo

Master Program in Molecular Biology
Department of Molecular Biosciences
The Faculty of Mathematics and Natural Sciences
University of Oslo

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Supervised by Inger Øynebråten

Performed at Department of Pathology
Oslo University Hospital, Rikshospitalet
November 2016



UiO : University of Oslo



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Shuai Guo

<http://www.duo.uio.no/>

Trykk: Reprosentralen, Universitetet i Oslo

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Acknowledgements

The work presented in this thesis was performed at the Department of Pathology (PAT), Oslo University Hospital in the period between January 2013 to December 2014.

I want to thank Alexandre Corthay for the opportunity to do my master thesis in the Tumor Immunology group. To my supervisor Inger Øynebråten: Thank you for giving me the opportunity to do this project and being a fabulous supervisor. Thank you for all your great guidance and quick feedback which keep helped me keep going with my work. Thank you for not only inspiring me with your enthusiasm and friendly teaching environment but also training me with your rigorous academic spirit. Thank you for supporting me in times of frustration and stress, and all the effort for making me more like a researcher.

Many thanks to Aram Nikolai Andersen for helping me with the experimental skills, for sharing his knowledge, for all the inspired discussions. Also thanks to Kahsai Beraki, the senior research technician for all the help and the technical experience he shared and taking care of the cells and other matters.

I am grateful to Inger Øynebråten for critically reading this thesis even on their busy schedule. Thanks to Aram Nikolai Andersen and Lars La Cour Poulsen for their help to give feedback on my thesis.

I am also grateful to all the members of Tumor Immunology Group for all the help I received and all the memorable time and thoughts we shared during the whole process. Thank you for all the presentations and birthday celebrations.

I wish to thank my friends for supporting me during my studying time. Thanks to my dear girlfriend Yingqian, who became my wife while I was working on this thesis, for all the patience, encouragement and understanding. Last but not least, I would like to thank my family for supporting me these years.

Abbreviations:

µg	Microgram
µl	Microlitter
aa	Amino acid
ACT	Tris-buffered ammonium chloride
APC	Antigen presenting cell
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CCL3	CC chemokine ligand 3
CCR	CC chemokine receptor
CLIPs	Class-II associated invariant-chain peptides
CTL	Cytotoxic T lymphocyte
Dept.	Department
DMEM	Dulbecco's modified Eagle's medium
DRiPs	Defective ribosomal products
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked immunospot
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
Esb	Murine methylcholanthrene-derived T cell lymphoma
FCS	Fetal calf serum
Foxp	Forkhead box
Gag	Group-specific antigen
GATA	GATA binding protein
HEK	Human embryonic kidney
HIV-1	Immunodeficiency Virus type 1
IFN-γ	Interferon-gamma
IgG3	Immunoglobulin gamma 3
kb	Kilobase
kDa	Kilodalton
LB	Lysogeny broth
LDS	Lithium dodecyl sulfate
MARCH	Membrane-associated Ring-CH
mg	Milli gram
MHC	Major histocompatibility complex
min	Minutes
MIP	Macrophage inflammatory protein
MIP-1α	Macrophage Inflammatory Protein 1 alpha

ml	Milli liter
mM	Milli molar
NaCl	Sodium chloride
NIP	5-iodo-4-hydroxy-3-nitrophenylacetyl
NK	Natural killer
nm	Nanometer
NO.	Number
OD	Optical density
PBST	Phosphate-buffered saline with Tween 20
pSTATs	Phosphorylated active stats
PVDF	Polyvinylidene difluoride
PVDF	Polyvinylidene difluoride
ROR	RAR-related orphan receptor'
rpm	Revolutions per minute
RT	Room temperature
scFv	Single chain fragment variable
SDS-PAGE	Sodium Dodecyl Sulphate polyacrylamide Gel Electrophoresis
STAT	Signal transducer and activator of transcription
TAP	Transportor associated antigen presentation
TBE	Tris/borate/edta,
TCR	T cell receptor
TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris(hydroxymethyl)aminomethane
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UiO	University of Oslo
UV	Ultraviolet
V	Voltage
X	Times
XCL1	C chemokine ligand 1
αNIP	Anti-4-hydroxy-3-iodo-5-nitrophenylacetic acid hapten

Abstract:

Antigen-specific CD8⁺ T cells can fight pathogens, and development of technologies that can improve T cell responses, in particular CD8⁺ T cell responses, is a major goal in vaccine design. DNA-based vaccines have several advantages over other vaccine strategies. DNA vaccines can easily be modified, they are stable and suitable for large-scale manufacturing, and show good safety. Many studies have shown that targeting of antigen to antigen presenting cells (APCs) can promote a T cell response. However, the application of DNA vaccine must be assessed on a case-by-case basis due to for example risks of protein modification-leded unfunctional vaccine.

Here we examined whether vaccibody, a homodimeric vaccine format which can target APCs, could increase the immune response to a group-specific antigen (Gag) p24 from Human Immunodeficiency Virus type 1 (HIV-1). Two molecules fused with the antigen HIV-1 Gagp24 were tested for their efficiency as targeting unit: CC chemokine ligand 3 (CCL3) and C chemokine ligand 1 (XCL1) derived from mice. Fusion to the anti-4-hydroxy-3-iodo-5-nitrophenylacetic acid hapten (α NIP) was utilized as non-targeting control. We found that functional vaccibodies are produced and secreted *in vitro*. Targeting of Gagp24 by fusion to CCL3 or XCL1 significantly increased the number of CD8⁺ T cells compared to vaccination with the non-targeted control α NIP-Gagp24.

The results show that the developed DNA vaccine successfully induced HIV-1 Gagp24-reactive CD8⁺ T cell responses in mice. Therefore, this study also suggest that chemokines are promising molecular candidates in DNA vaccine development because small amounts of them can promote immune responses.

1.Introduction

1.1 Immunity and vaccination

The word “immunity” derives from the Latin word *immunis*, meaning exemption from burdens, charges or other public services [1]. The concept of immunity has been extended as a medical term, meaning biological freedom or protection against infection or other unwanted biological invasion from either self-derived or non-self-derived organisms. The immunity is classified into innate and adaptive immunity. The innate immunity, known as non-specific immunity, is the first line of defense and consists of multiple immunological barriers (such as the skin barrier, mucosal barrier, blood-brain barrier, blood-thymus barrier), molecules (complement, lysozyme, cytokines) and cells (macrophages, dendritic cells, natural killer cells). The barriers have the function to resist the entry of pathogens. If the barriers are invaded by pathogens, other components of innate immunity will contribute to defense in different ways depending on whether it is an extracellular or intracellular located pathogen. Although the innate immunity efficiently keeps people healthy from pathogen infection, it is still a risk that some of the pathogens are able to resist the innate immunity. Under such circumstances, the innate immunity will activate adaptive immunity that is specifically more powerful in defending of each pathogen [2]. The adaptive immune system initiates highly specific responses which can be divided into: Humoral immunity which relies on B lymphocytes (B cells) and Cell-mediated immunity which relies on T lymphocytes (T cells).

In 1801, Edward Jenner described how inoculation with cowpox virus could provide protection against smallpox with less risk compared to previous methods. He called the procedure vaccination [3]. The word “vaccine” originates from the Latin word *vaccinus*, which means “pertaining to cows” [4]. Nowadays, vaccination is still used and extended to describe a procedure that protects individuals from infection by prior exposure to the disease-causing agent in a form that does not cause disease. The goal of vaccination is to generate long-lasting and protective adaptive immunity. The vaccine agent could be dead pathogens, live-attenuated pathogens, the subunit from protein or DNA sequence of the pathogens. A vaccinated individual should have pathogen-specific antibodies and memory cells due to the adaptive immunity

induced by the vaccine and should be protected in subsequent exposure to the pathogen more rapidly than the first exposure that is initiated by the vaccine.

1.2 Antigen presenting cells and antigen presentation

1.2.1 Major histocompatibility complex (MHC) molecules

The pathogens that invade human tissues are divided into two types: extracellular pathogens that live and proliferate in the spaces between human cells and intracellular pathogens that live and proliferate inside of human cells. To generate T cell responses to different pathogens, MHC molecules are used to present antigens that derive from pathogens. There are two types of antigens, MHC class I molecule presents antigens from intracellular pathogens and MHC class II presents antigens from extracellular pathogens with an exception called cross-presentation. The cross-presentation allows uptake of extracellular antigens through endocytosis (normally leading to presentation on MHC class II molecules) to the MHC class I molecules. MHC class I molecule is comprised of a transmembrane chain and a non-covalently associated protein named β_2 -microglobulin. The transmembrane chain is made up by three domains named α_1 , α_2 , and α_3 (Figure 1.2.1). Domain α_1 and α_2 form the peptide binding cleft which binds and presents antigen to the T-cell receptor and α_3 contains a transmembrane region. The β_2 -microglobulin is not attached to cell membrane. The MHC class II has transmembrane chain, α chain and β chain. Two domains, α_1 and β_1 form the peptide binding cleft whereas α_2 and β_2 contains a transmembrane region spanning the cell membrane (Figure 1.2.1).

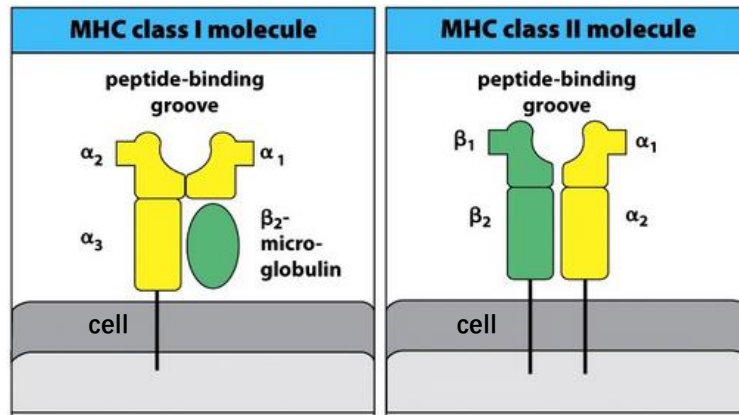


Figure 1.2.1 The structure of MHC molecules [5]: The two classes of MHC molecules differ in the structure. Left panel: An MHC class II molecule is made of two chains (α and β chain) that have a transmembrane structure on both chains and similar size. Right panel: MHC class I molecule is made of a larger chain (α chain) that has transmembrane structure and smaller peptide (β_2 -microglobulin).

The two classes of MHC molecules are expressed on different cells. MHC class I molecules are expressed on almost all nucleated cells except some erythrocytes whereas MHC class II molecules are expressed only on professional antigen presenting cells (APCs) and stromal epithelial cells in the thymus [6], [7]. Cells can increase the expression of both MHC class I and class II molecules in response to cytokines. In addition, interferon- γ (IFN- γ) can induce the expression of MHC class II molecules on some cell types which do not normally express them [8].

1.2.2 Antigen presenting cells

APCs are cells which are highly specialized to process antigens and display their peptide fragments on the cell surface. In addition, they can express co-stimulatory molecules required for activating naive T cells. There are three types of APCs: Dendritic cells, Macrophages and B cells.

1.2.3 MHC class I antigen presentation pathway

The MHC class I molecules present either self- or pathogen-derived endogenous proteins that are at the end of their lives whose half-life varied greatly from minutes to days or a large portion (30-70%) of proteins which are degraded immediately before forming functional proteins [9], [10]. These degraded proteins are called defective ribosomal products (DRiPs) which are

defectively made because of transcriptional, translational mistakes and failed assembly [11]–[13]. The endogenous proteins and DRiPs are degraded by the proteasome in the nucleus and cytosol (Figure 1.2.3). Proteases not only cleave off protein fragments but also ligate two peptides resulting ligated peptides which cannot be predicted on the basis of the genomic sequence [14], [15]. The immunoproteasomes generate fragments for all of the different MHC I molecules. All kinds of peptides which are generated by proteasomes are substrates for cytosolic aminopeptidases. Most of peptides are trimmed and destroyed by cytosolic aminopeptidases. A small fraction of peptides escape terminal destruction by translation into endoplasmic reticulum (ER) lumen via transportor associated antigen presentation (TAP). Since TAP is not located in nuclear membrane, the nuclear peptides must first diffuse into cytosol in order to encounter TAP. In the ER, MHC class I molecules are partially folded and stabilized by additional chaperones, TAP, tapasin, ERp57, and calreticulin. The complex of these five molecules is called peptide-loading complex (PLC). Peptides may bind to MHC class I molecules directly or they may require further trimming by ER aminopeptidases associated with antigen presentation (ERAAP) before they are considered suitable for MHC class I binding inside or outside the PLC. Peptide-MHC class I complexes are then released from the PLC and then ER so that they are transported to the plasma membrane for antigen presentation to CD8⁺ T cells. Peptides and MHC class I molecules that fail to bind each other are degraded and transported back into the cytosol by ER-associated protein degradation (ERAD) system. These peptides are further trimmed or destroyed by cytosolic peptidases and proteasomes. A fraction of small cytosolic molecules or ions (such as ATP, Ca²⁺ and peptides) can enter the cytosol and MHC class I pathway in neighbouring cells by translocation through gap junctions [16]. It may cost some innocent bystander killing of non-infected neighbouring cells after MHC class I antigen presentation pathway appears to share antigenic peptides with its neighbour. Surface MHC class I molecules can be ubiquitinated by membrane-associated Ring-CH (MARCH) family proteins to promote internalization and lysosomal degradation. After MHC class I molecules are internalized into the endosomes, they enter the classical MHC class II presentation pathway. The peptides associated with MHC class I molecules can be released and then exchanged with new peptides generated by the endocytic pathway. Thereafter, a fraction of MHC class I and II molecules is transferred to the surface for presentation of endosomal antigen fragments [17]. This alternative pathway in MHC class I antigen presentation is more relevant to cross-presentation.

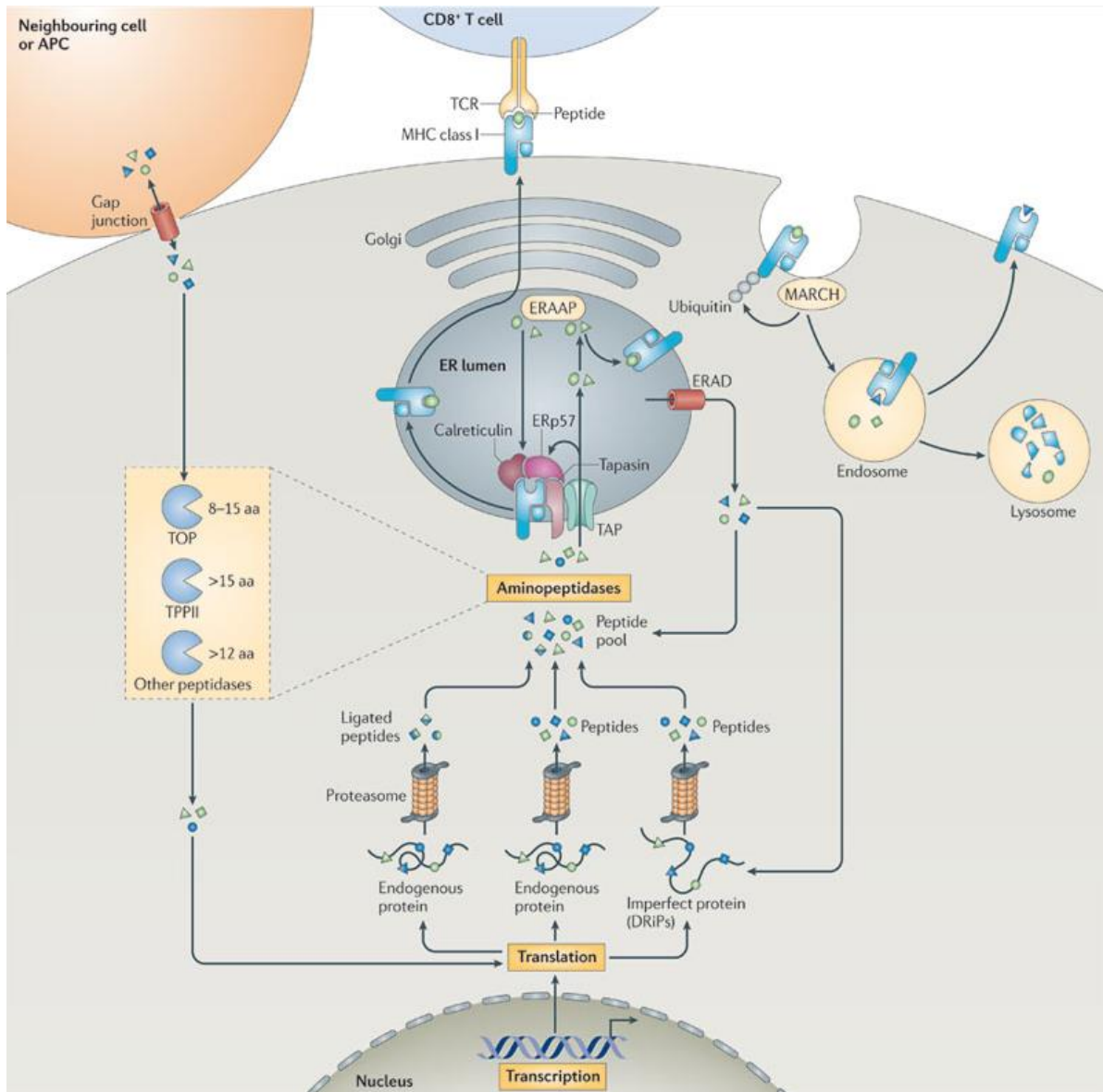


Figure 1.2.3 Schematic drawing of MHC class I antigen presentation pathway [18]. The DRiPs and endogenous proteins are degraded by the proteasomes in the nucleus and cytosol by similar manner. The proteasomes also generates new peptides by ligation. All peptides generated from proteasomes are substrates for cytosolic aminopeptidases such as TOP and TPPII which trim and destroy most of peptides whereas a small fraction of peptides escape the destruction by translocation into endoplasmic reticulum (ER) via TAP. Peptides may bind with MHC class I molecules after the trimming by peptide loading complex (which contains TAP, MHC class I molecules, ERp57, calreticulin and tapasin) or they may require further trimming by ER aminopeptidase associated with antigen processing (ERAAP). After the trimming process inside or outside of PLC, the peptides bind to MHC class I molecules and then released from the PLC and the ER so that they can be transported to plasma membrane for antigen presentation to CD8⁺ T cells. Peptides and MHC class I molecules that fail to bind each other are degraded and transported back into the cytosol by ER-associated protein degradation (ERAD) system. These peptides are further trimmed or destroyed by cytosolic peptidases and proteasomes. Peptides can enter MHC class I presentation pathway of neighbouring cell through gap junctions. Surface MHC class I molecules can be ubiquitylated by MARCH family proteins to promote internalization and lysosomal degradation. After MHC class I molecules are internalized into to endosome, a fraction of MHC class I molecules is recycled with MHC class II molecules to the plasma membrane for the presentation of endosomal antigen peptides. TOP = Thimet oligopeptidase; TPPII = Tripeptidyl peptidase II

1.2.4 MHC class II antigen presentation pathway

Expression of MHC II is controlled by master regulator MHC class II transactivator (CIITA), which is regulated by posttranslational modifications and factors that mainly are active in immune cells. Newly synthesized MHC II molecules are associated with a protein called invariant chain (Ii) in the ER (Figure 1.2.4). Ii blocks the MHC II peptide binding groove, thus preventing binding of other peptides in the ER. The cytoplasmic tail of Ii contains two sorting motifs that direct MHC II molecules to endosomal compartment. MHC II:Ii complex binds antigen peptide by a process directly catalyzed by interaction between MHC II:Ii complex and a non-peptide binding MHC II homolog, in human called HLA-DM [19]. HLA-DO and MHC II bind to same region of HLA-DM but HLA-DO binds much more tightly [20]. Therefore, HLA-DO inhibits functionally interacting between HLA-DM and MHC class II molecules by direct suppression of their binding. HLA-DM is always expressed at higher level than HLA-DO, thus resulting in a mix of free HLA-DM (active) and HLA-DM-HLA-DO molecule (inactive) [21]. Free HLA-DM is mainly localized to the internal vesicles of multivesicular endosome (MHC class II compartment or MIIC), whereas HLA-DM-HLA-DO molecule is mainly localized in the outer membrane [22]. In addition to stabilizing empty MHC II molecules, HLA-DM promote dissociation of weakly bound peptides in order to ensure accumulation of empty MHC II to high affinity peptide. Self-derived peptides are low stability protein, whereas pathogen-derived peptides are high stability protein. The pathogen-derived peptides may also be selectively targeted to the internal vesicles of endosomes which would promote the presentation of high-stability peptides because free HLA-DM is mainly localized in internal vesicles as mentioned before [22]. In the MHCIIIC, Ii is degraded by proteases and leaves only small fragments called class-II associated invariant-chain peptides (CLIPs) in the MHC II binding groove. HLA-DM in this step, catalyzes CLIPs removal and facilitates the binding of pathogen- and self- derived peptides to empty MHC II molecules. However, CLIPs removal does not have to be catalyzed by HLA-DM. Instead, CLIP may spontaneously dissociate from some MHCII-CLIP complexes and peptide-free MHC II molecules are then available in the peripheral membrane for binding to low affinity peptides derived from self-protein or become functionally inactive and degraded [22]. MHC class II transport from the MHCIIIC to plasma membrane after a specific time period which controlled by factors such as cholesterol, kinases, cytosolic pH but the molecular basis for this part is largely undefined [18]. The MHC II molecules at cell surfaces are finally degraded but the mechanisms that control degradation are not established (ubiquitylation maybe involved for half-life of MHC II molecules) [23].

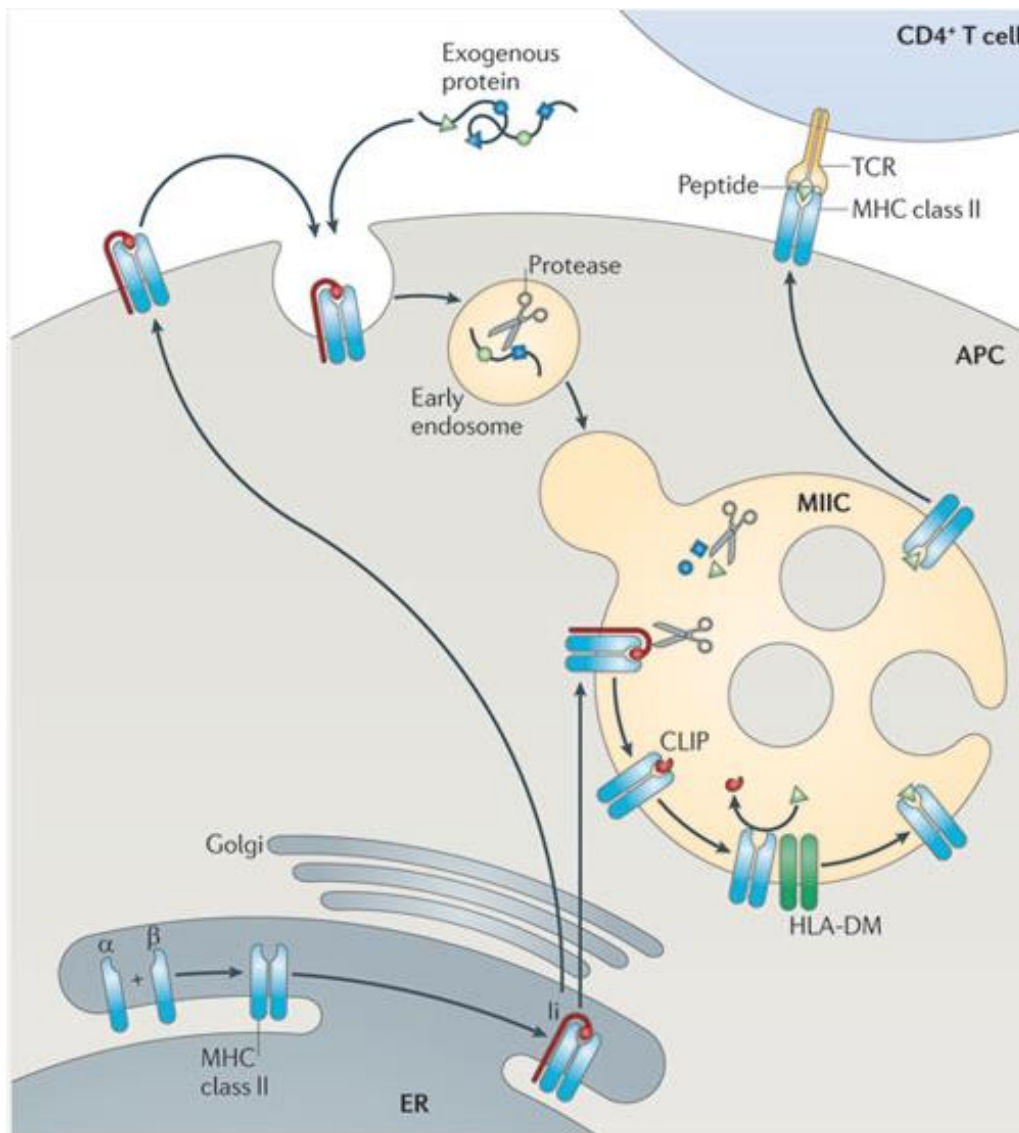


Figure 1.2.4: Schematic drawing of MHC class II antigen presentation pathway [18]. MHC class II α - and β -chains assemble in the endoplasmic reticulum (ER) to form an MHC class II molecule which is later bind with the invariant chain (Ii). The MHC II:Ii complex is then transported through the Golgi to the MHC class II compartment (MIIC), either directly and/or via the plasma membrane. Self- or pathogen-derived peptides are endocytosed and degraded by proteases in the MIIC. MHCII:Ii complex are degraded by proteases resulting in the forming of the class II-associated Ii peptide (CLIP) fragment in the peptide-binding groove of the MHC class II molecule and is exchanged for an antigenic peptide with the catalyzing of the HLA-DM. MHC class II molecules are then transported to the plasma membrane to present antigenic peptides to CD4⁺ T cells.

1.3 T cells

T cells precursors generated in the bone marrow migrate to the thymus where T-cell development occurs. T cells are involved in cell-mediated immunity. The antigen receptor on

T cell is referred as T cell receptor (TCR) which is generated by recombination of different gene segments. TCR only binds to antigens presented by different MHC molecules. Corresponding to the two classes of MHC molecules, there are two types of effector T cells, CD8⁺ cytotoxic T cell (CD8 T cell) and CD4⁺ helper T cell (CD4 T cell). Those two types of cells are identified by their expression of the co-receptors CD8 or CD4 on T cell surface. The co-receptors bind to conserved sites that are different from the sites recognized by TCR on MHC I or MHC II molecule surface. The initial activation of CD8/CD4 T cell requires interaction between MHC molecules and both TCR and co-receptor. CD4 T cell only can recognize antigen presented by MHC II molecules whereas CD8 T cell only can recognize antigen presented by MHC I molecules.

1.3.1 CD8 T cells and their functions

The CD8 T cells have an important role in directly destroying infected cells in adaptive immune responses to intracellular infections. At the point when the intracellular antigen has been presented by MHC I molecules as MHC: peptide complex on the surface of infected cells, the TCR and CD8 co-receptor on the surface of CD8 T cell recognize the complexes. The interaction triggers the CD8 T cell to deliver some toxic substances such as perforin, granzyme onto the surface of the infected cell, which induces its death by apoptosis. The CD8⁺ cytotoxic T cell has also other mechanisms for destroying cells such as: *i*) Fas/FasL-mediated apoptosis [24], [25]; *ii*) CD8 T cell can produce some cytokines which can also induce apoptosis. For example, Tumor necrosis factor alpha (TNF α)-mediated apoptosis. [26], [27]

1.3.2 CD4 T cells and their functions

CD4 T cells exists in several different functional subtypes and play critical roles for host defense and normal immune regulation by their ability to differentiate into specialized T cell subsets [28], [29]. The diversities of CD4 T cell function are determined by their cytokine secretion patterns and their tissue location and CD4 T cells can be divided into at least four subsets, namely, T helper (Th) 1 cell, Th 2 cell, Th 17 cell, regulatory T (Treg) cell [30]. There are four more potential Th cell subsets including Th3 cells [Transforming growth factor (TGF) β -producing CD4 cells] [31], Type 1 regulatory T (Tr1) cells (IL-10-producing CD4 cells) [32], [33], Th9 cells (IL-9-producing CD4 T cells) [32], [34] and T follicular helper (Tfh) cells [35]–[37].

Stimulation with IL-12 and IFN γ induce the expression of transcription factor, T-bet and signal transducer and activator of transcription (STAT) 4 in naïve T cells for Th1 differentiation. Th1 involved in protection against intracellular pathogen [38], [39] and mainly secrete IFN γ , IL-2 which are involved in macrophage activation, cytotoxic T lymphocyte (CTL) activation, production of opsonizing antibodies that enhance the phagocytosis of pathogens [40].

IL-4 induces the transcription of GATA binding protein (GATA) 3 and STAT6 in naïve T cells for Th2 differentiation. Th 2 cells involved in protection against extracellular pathogen [38], [39] and produce IL-4, IL-5, and IL-13 which lead to B cell activation and eosinophil recruitment [41], [42].

TGF β and IL-6 induce the transcription of RAR-related orphan receptor (ROR) γ t and STAT3 in naïve T cells for Th17 and IL-23 has been reported to promote the generation of Th17 cells [43]. Th17 cells produce many cytokines including IL-17A, IL-17F, IL-21, IL-25, IL-26 (human) and play critical roles during immune responses against extracellular bacteria and fungi in addition to the involvement in autoimmune diseases and chronic inflammation [44].

The stimulation of TGF β induces the transcription of forkhead box (Foxp) 3 and STAT5 in naïve T cells for Tregs. Tregs are required to maintain self-tolerance [45]. It is now known that various subsets of Tregs exist *in vivo* in immune system including natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs develop in the thymus whereas iTregs develop in the periphery environment from naïve (or in some conditions from differentiated) T cells [46]. It is demonstrated that nTregs and iTregs are involved in suppression of immune responses via various mechanisms and in promoting immune responses under certain circumstances [47], [48].

Therefore, the `signature` cytokines produces by Th3, Tr1, Th9, Tfh cells are not unique compared with the products of Th1/Th2/Th17/Treg cells and the transcription factors are not unique either, whether the four more potential Th cells represent new subsets from the known four T cell subsets is unclear and needs further investigation [30]. However, recent study indicates Tfh provide help to B cells for antibody production [49]. Th9 cells have been reported that they lead to allergic inflammation in lung and promote intestinal inflammation [32], [50]. Tr1 cells play a crucial role in promoting and maintaining tolerance and the main mechanisms to control immune responses are the secretion of IL-10, and the killing of myeloid cells through the release of Granzyme B [33]. Th3 cells provide help for IgA secretion, has suppressive

features for both Th1 and Th2 cells and may have a major role in many aspects of immune modulation and T-cell homeostasis [51], [52]. The relationships between Th1/Th2/Th17/Treg and Th3, Tr1, Th9, Tfh cells are shown in Figure 1.3.2.

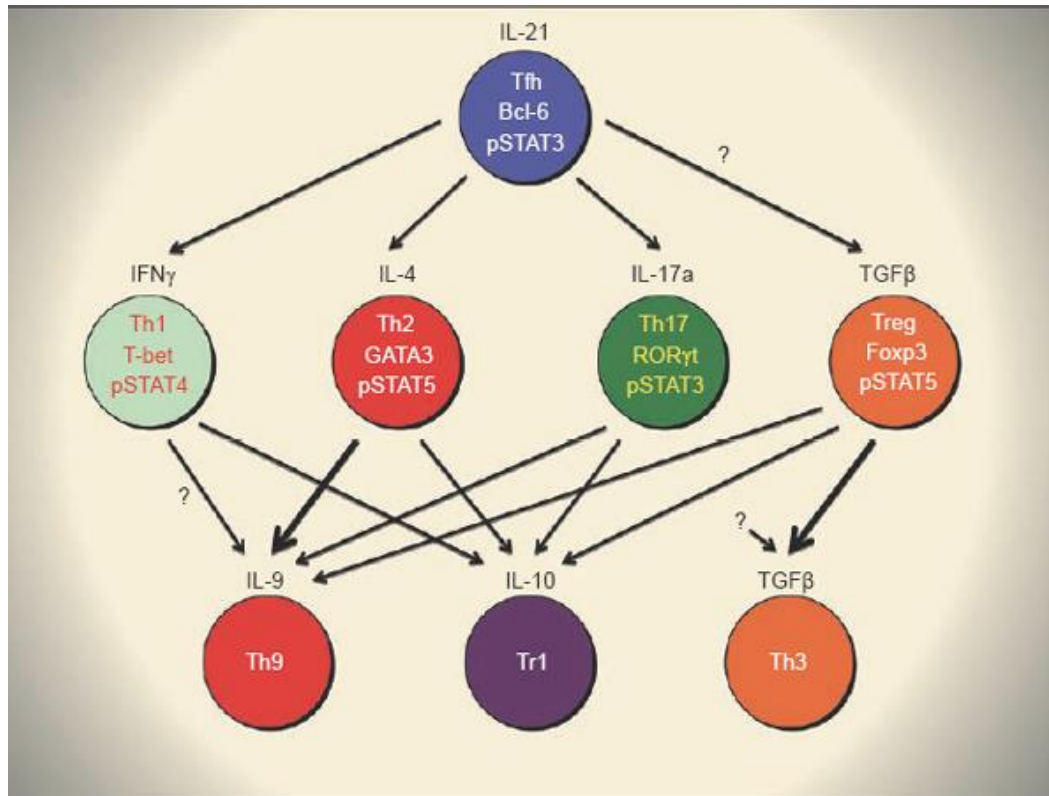


Figure 1.3.2 CD4 T cell subsets and their relationships [30]. CD4 T cells can be divided into at least four subsets, Th1, Th2, Th17 and Treg, with four more potential subsets including Th9, Tr1, Th3 and Tfh cells. The transcription factors that lead the differentiation of T cells are shown inside the cells and the secreted ‘signature’ cytokines are shown on the top of the cells. However, Tfh cells can secrete Th1, Th2, Th17, Treg cell’s ‘signature’ cytokines whereas Th1, Th2, Th17, Treg cells can also secrete Th9, Tr1, Th3 cell’s ‘signature’ cytokines as shown by arrows. The question marker is given if it is unknown whether Th cells can express that cytokines under certain circumstances. Therefore, whether Tfh, Th9, Tr1 and Th3 cells may represent subsets of Th1, Th2, Th17 and Treg subsets need to be further investigated.

pSTATs = phosphorylated active STATs

1.4 Human immunodeficiency virus (HIV) and HIV vaccine strategy

1.4.1 HIV biological characteristics and epidemic

Acquired Immunodeficiency Syndrome (AIDS) is caused by Human Immunodeficiency Virus, a member of the genus *Lentivirus* of the *Retroviridae* [53]. AIDS pathology is not clear so far

but numerous models have been suggested that the underlying loss of CD4⁺ cells in AIDS is crucial with following destruction of adaptive immunity when CD4⁺ cell number decline below 200 cells per mL of blood (normal count is 600 to 1000 per mL) [53], [54]. There are three types of HIV, HIV type I (HIV1), HIV type II (HIV2) and HIV type O (HIVO) have been identified as the primary cause of AIDS and HIV-1 is the major cause of AIDS in the world today [55], [56]. Since HIV-1 is the major cause of AIDS in the world today, our project will be primarily focus on HIV-1 infection.

Joint United Nations Programme on HIV and AIDS (UNAIDS) has reported approximately 35.3 million people were living with HIV in 2012 all over the world with 2.9 million new HIV infections globally and the overall growth of the epidemic has stabilized in recent years (Figure 1.4). Since the beginning of the HIV epidemic, nearly 30 million people have died from AIDS-related causes [57].

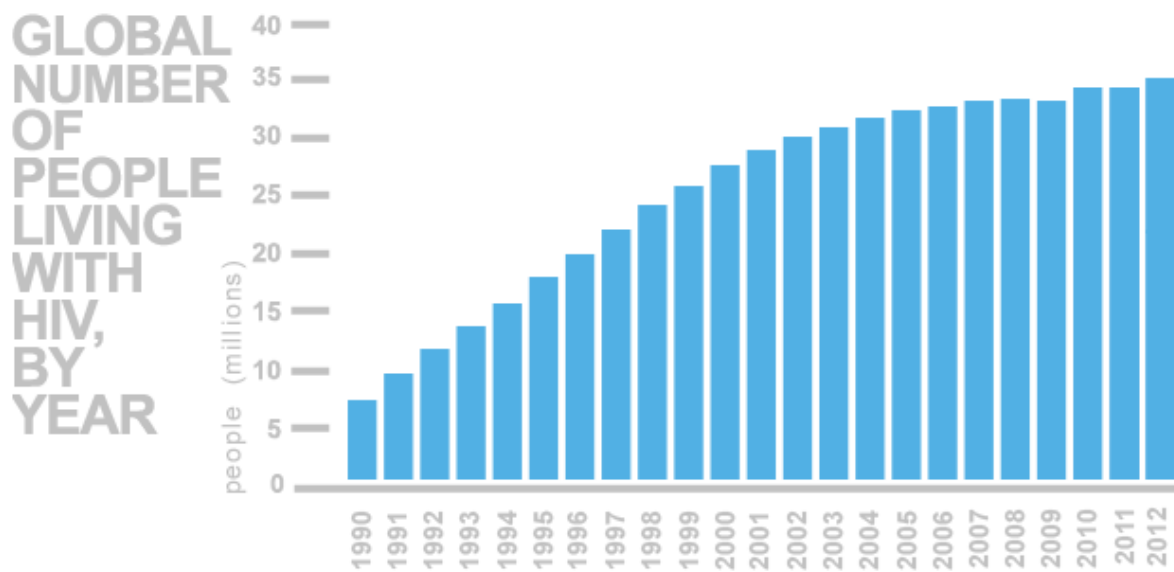


Figure 1.4 The HIV and AIDS Charity (AVERT) website statistics illustrating the number of HIV infected people worldwide in 1990-2012 [58], [59].

1.4.2 Structure of HIV-1

The diameter of HIV-1 viral particles is approximately 100 nm. The particle is surrounded by a lipoprotein membrane in which 72 spikes or glycoprotein complexes are integrated [60]. Each glycoprotein complexes is consisting of a trimer which contain an external glycoprotein gp120

and a transmembrane protein gp41 (Figure 1.4.2). The bonding between gp120 and gp41 is non-covalent so that it is possible that gp120 is separated spontaneously within the local environment. gp120 contains the binding sites of CD4 and seven transmembrane domain chemokine receptors that act as co-receptors for HIV-1. During the process of infection, the HIV-1 virus may also incorporate some host proteins from the membrane of the host cell into its lipid layer in order to facilitate adhesion to host cells. Group-specific antigen (gag) gene is the code for the core structural proteins of a retrovirus. The inner structural proteins, p17 (matrix), p24 (capsid) and p15, are derived from gag precursor protein, p55 [61], [62]. The p15 is modified into p7 (nucleocapsid) and p9 by proteolytic cleavage [62], [63]. The p24 core antigen encloses a protein-nucleic acid complex including two copies of HIV-1 RNA, the nucleoprotein p7, integrase and the reverse transcriptase. Since gag gene is conserved among HIV-1 strains, the amino acid sequence of p24 as its production is also highly conserved [64], [65].

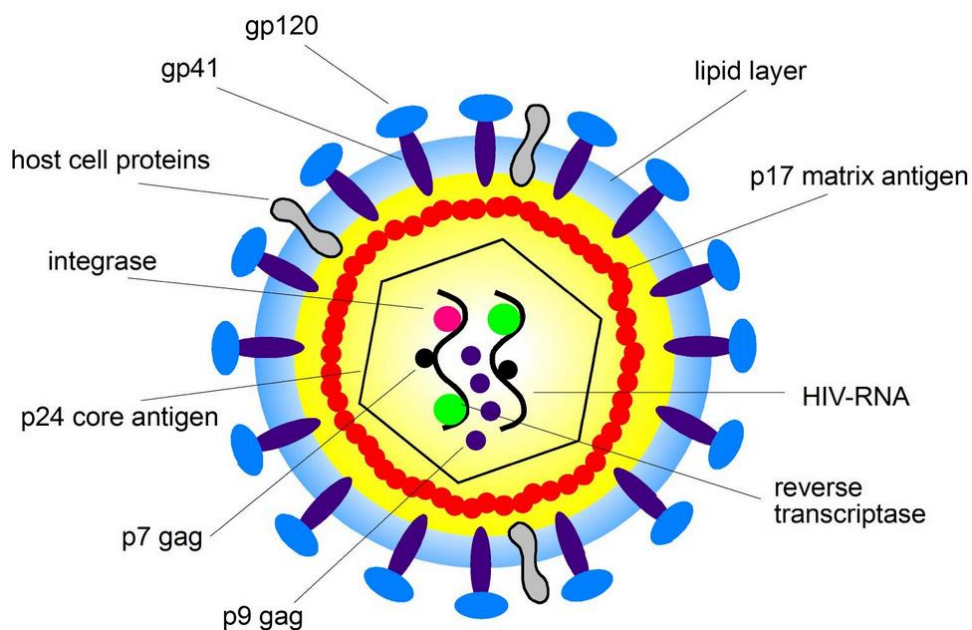


Figure 1.4.2 Structure of HIV-1 [66]. For detailed explanations see text.

1.5 Vaccination strategies towards HIV1

Vaccines can be used to protect individuals from diseases, but can also be used to treat existing diseases (therapeutic vaccines). The therapeutic vaccines have been studied for their efficiency

against cancer, HIV, hepatitis B [67], [68]. Vaccination started around the 10th century AD as a means of preventing smallpox. Different vaccine types modulate adaptive responses to provide further protection by either active or passive immunization. Active immunization involved administering of an immunogen that might be live/attenuated, killed/ inactivated or subunit in origin, whereas passive immunization involved giving pre-formed antibodies, usually to very recently exposed individuals [69]. The live attenuated vaccines which infect the host but do not cause disease and elicit strong and long-lasting immune responses, in the other hand, have the potential to revert to a pathogenic form [70], [71]. The killed or inactivated vaccines may be used, but side effects and low efficacy may still be a problem [72], [73]. The subunit-based vaccines which utilize pathogen-derived subunits such as protein or carbohydrate that induce a protective immune responses show good safety, for example in rare lipid disorders or haemophilia B [74], [75]. The subunit-based vaccines can be delivered as protein or DNA, DNA vaccines are particularly easy and fast to produce and are stable in terms of storage and temperature changes [76], [77]. Ideally, development of a vaccine which stimulates broadly neutralizing antibodies against HIV-1 is one strategy whereas the other one is development of a vaccine which elicits HIV-1-specific CD8⁺ cytotoxic T lymphocyte response [78], [79]. Since there is no identified immunogen yet that can induce neutralizing antibodies hindering an HIV-1 infection, T cell-based vaccine approach is a possible alternative to be used in order to control the viral load and thereby delay the onset of AIDS [79]. Such vaccines may not be fully protective against HIV infection but could be used therapeutically.

1.5.1 DNA vaccines

DNA vaccines are plasmids constructed to express one or more encoded proteins and subsequently are delivered into the cells. Initially, these plasmids were thought to function simply as a shuttle system for genes, resulting in transfection following the in situ production of antigen (for vaccines) or therapeutic protein (for gene therapy applications) [77]. DNA vaccine could result in both cellular [80], [81] and humoral immunity [82]. There are two working pathways of DNA vaccines but they are not well known yet. The first pathway is based upon that the DNA vaccine will be taken up by transfected local cells (such as myocytes) which will produce and secrete antigenic proteins. The antigenic proteins will then be taken up by APCs. The APCs migrate to the draining lymph nodes where they present peptide:MHC complex to T cells resulting in cellular and humoral immune response. The second pathway anticipates that DNA will be taken up by APCs directly and APCs will then produce and secret

antigenic proteins. Thereafter, antigen presentation to T cells will be initiated. Both pathways are involved in induction of adaptive immune responses [83].

1.5.2 Electroporation enhances the immunogenicity of DNA vaccine

One major factor that causes weak immunogenicity of DNA vaccines in humans is the relatively poor uptake of the vaccine by myocytes and APCs when injected intramuscularly [84]. The immunogenicity of DNA vaccines is increased by using electroporation which is a technique using co-administration of small, localized electrical fields to increase the transfection efficiency of the injected DNA [85], [86] and the recruitment of immune cells such as dendritic cells, T and B lymphocytes to the injection site [86]–[88]. Electroporation *in vivo* has been reported as an efficient method for increasing the immunogenicity of DNA vaccines encoding a number of antigens [89]–[91]. Electroporation of DNA vaccine is safe, tolerable and acceptable to healthy volunteers and the level of tolerability was independent of age, gender, body weight, skin fold thickness, vaccination in dominant versus non-dominant arm, or sequence of vaccination [92].

1.5.3 A new platform for enhancing DNA vaccine efficiency - vaccibody

Although DNA vaccines are safe, tolerable and efficient as mentioned above, sometimes the immune responses in humans have been disappointing with the help from electroporation and improved vector design [93]–[95]. There is a need for enhancing DNA vaccine efficiency. A well-known method to increase the immunogenicity of antigens is to chemically or genetically integrate antigenic proteins into antibody-like or antibody molecules that target APC [96]–[102]. This principle has been used in order to enhance DNA vaccine efficiency by constructing DNA plasmids that encode for APC-specific proteins. Therefore, a new type of DNA vaccine has been developed for secreting those APC-specific proteins that enhance delivery of antigen to APC, resulting in improved immune responses [103]–[106]. Moreover, some studies indicate a dimeric version was more immunogenic than the monomeric version so that the new DNA vaccine was designed as homodimer and named as vaccibody [107].

Vaccibody is a novel type of recombinant vaccine molecule that is antibody-based homodimer with two identical chains consisting of an N-terminal targeting unit, a dimerization unit and C-terminal antigenic unit (Figure 1.5.3 A) [91], [107]. The N-terminal targeting unit can be single-

chain variable fragment from APC-specific antibodies or natural ligands like chemokines for surface molecules on APC [91], [107], [108]. The dimerization unit consists of a shortened hinge region (h1 and h4 exons) and a C_H3 domain from human Immunoglobulin Gamma 3 (IgG3) chains [107]. The two chains are expected to homodimerize by hydrophobic interactions between C_H3 domains and disulfide bonds forming between cysteines in the hinge region [107]. The antigenic unit can be any antigenic protein. The mechanism for why vaccibody can improve T and B cell immune responses is hypothesized in a model described in Figure 1.5.3B.

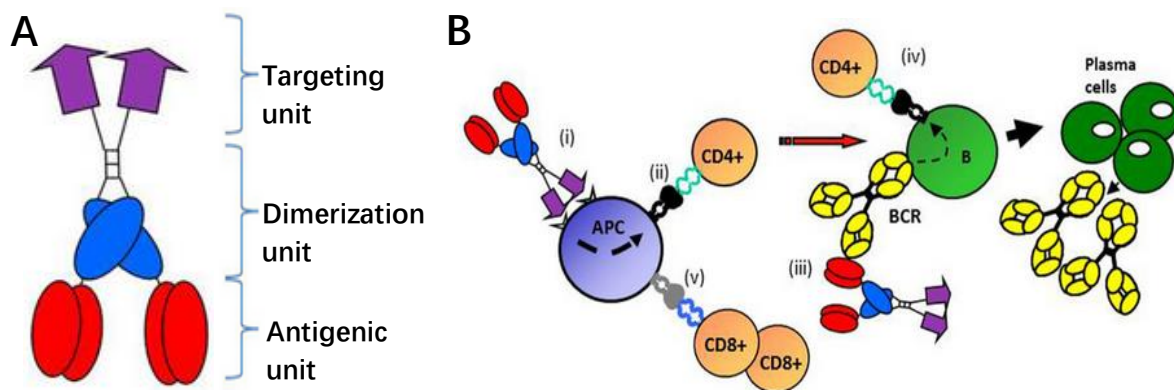


Figure 1.5.3 (A) A schematic drawing of vaccibody protein. The vaccibody consists of a targeting unit which may contain APC-specific protein, a dimerization unit derived from human IgG3 chain, an antigenic unit in where antigenic protein can be inserted. (B) The hypothesized mechanism of action of vaccibodies. (i) Secreted vaccibodies protein bind to surface molecules on APC and induce their maturation. (ii) Vaccibodies are internalized and processed and peptides from antigenic unit are presented on MHC class II molecules of APC to naïve CD4⁺ cells. Since APC is mature and express co-stimulatory molecules, the naïve CD4⁺ T cells can be induced into effector T cells. (iii) B cell with a B cell receptor (BCR) specific for conformational determinants on complete antigenic proteins internalize vaccibodies, process them and present antigenic peptide on MHC class II molecules to the effector CD4⁺ T cells. (iv) B cells receive help from the effector CD4⁺ T cells and develop into plasma cells that can secrete antibodies. (v) Vaccibodies with certain targeting units can induce presentation on MHC class I molecules and cross-priming of CD8⁺ T cells.

1.6 Targeting unit of vaccibodies

The vaccibodies, α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24, CCL3L1-Gagp24 have been used in this project.

Non-targeted vaccibodies, α NIP-Gagp24, contained a mouse scFv specific for the hapten 5-iodo-4-hydroxy-3-nitrophenylacetyl (NIP), which is not found in the body, as the N-terminal targeting unit [107].

mCCL3-Gagp24 use a mouse CCL3 protein as targeting unit which is known as MIP-1 α (Macrophage Inflammatory Protein 1 alpha). CCL3 is a ligand of the CC chemokine receptors CCR1 and CCR5, and is chemoattractant for monocytes, macrophages, and dendritic cells. CCR1 and CCR5 are also expressed by Natural killer (NK) cells, CD4⁺ and CD8⁺ T cells [109], [110]. The using of mCCL3 as targeting unit gave 80% protection in the tumor model which was significantly reduced when depleting CD8⁺ T cells in mouse model [108]. CCL3L1-Gagp24 which contain CCL3L1 protein is a human version of CCL3.

mXCL1-Gagp24 use a mouse XCL1 protein as targeting unit which is known as a small cytokine named as lymphotactin belonging to the XC chemokine family. It activates CD8⁺ T cell immune response [111]. The chemokine XCL1 attracts T cells and binding to a chemokine receptor called XCR1 [110], [112].

2. The aim of study:

Main goal: Develop vaccines that can improve T cell responses towards Gag p24 derived from HIV-1 and test their efficiency *in vitro* and *in vivo* (mice)

Sub goal:

* Examine the secretion and folding of designed vaccibody constructs (α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24, CCL3L1-Gagp24) after transfection *in vitro*

* Examine the efficiency of different designed vaccibody constructs (α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24) *in vivo* regarding inducing T and B cell responses

3. Materials and methods

3.1 Materials

3.1.1 Laboratory equipment

Name	Supplier, Catalog No.
2-Gel Tetra and Blotting Module with powerpac Basic Power Supply	BIO-RAD, 166-0828EDU
6.5mm Transwell® with 5.0µm Pore Polycarbonate Membrane Insert, Sterile	CORNING, 3421
Corning® 15 ml centrifuge tubes	SIGMA, 430791
Corning® 50 ml centrifuge tubes	SIGMA, 430829
Corning® 96 well Cell Culture Cluster	SIGMA, 3799
Corning® 96 well EIA/RIA plates	SIGMA, 3590
Countess® automated cell counter	Invitrogen, c10227
CTL-immunospot® S6 Micro Analyzer	CTL-Europe gmbh, Bonn, Germany
Elgen 1000 electroporation applications system	Inovio
Sunrise remote microplate reader	Tecan, a5002
Tissue Culture Plates, 6 wells	VWR, 734-2323
Trans-blot® turbo™ transfer system	Bio-rad, 170-4155
Trypan blue stain (0.4%)	Invitrogen, t10282

3.1.2 Reagents

Name	Supplier, Catalog No.
0.9% nacl solution	B. Braun, mtr7533
10% Mini-PROTEAN® TGX™ Gel	BIO-RAD, 456-1036
Agarose gel	LONZA, 50004
Amplicillin	Invitrogen, 11593-027
Anti-HIV1 p24 antibody [39/5.4A]	Abcam, ab9071
Anti-Mouse igg, Horseradish Peroxidase-Linked Antibody (from sheep)	GE Healthcare, NA931
Biotinylated polyclonal anti-mouse XCL1 antibody	R&D systems, BAF486
Bovine Serum Albumin 30%	BIO-RAD, 805095
BSA, Molecular Biology Grade	New England Biolabs, B9000S
DMEM with 4.5g/L glucose w/o L-Glutamine 500ml	LONZA, BE12-614F
DNA ladder (100 bp)	New England Biolabs, N3231S
Elefix PASTE for EEG	Nihon Kohden, Z-401CE
Ethidium bromide solution	SIGMA, E1385
Fetal Calf Serum	
Gentamicin 50 mg/ml 1 x 10 ml screw cap vial	LONZA, 17-518Z
Glycerol	SIGMA, G5516
HIV Gag Peptide (199-207)	Genscript, RP20251
HIV-1 gag (p24) antigen	VIROGEN, 00111-V
Human CCL3/MIP-1 alpha Biotinylated Antibody	R&D systems, BAF207
Human CCL3/MIP-1 alpha antibody (Clone 14215)	R&D systems, MAB670
LDS-sample buffer (4X), non-reducing	Alfa Aesar, J61894
L-Glutamine 200mm 100 ml	LONZA, BE17-605E
Lipofectamine 2000 Transfection Reagent	Invitrogen, 11668-027
Monoclonal Anti-Human igg (Fc specific) antibody	SIGMA, B3773
Mouse anti-human igg (CH3 domain) antibody	Bio-Rad, MCA878G

NaCl, Natriumklorid B.Braun 9 mg/ml	Braun, 12255203/1010
Neuraminidase	New England Biolabs, P0720
O-Glycosidase	New England Biolabs, P0733
Opti-MEM® I Reduced Serum Medium	Invitrogen, 31985-047
PBS	Invitrogen, 1491046
Phosphatase substrate	SIGMA, P4744
Restriction Endonucleases, bamhi	New England Biolabs, R0136S
Restriction Endonucleases, bsiwi	New England Biolabs, R0553S
RPMI 1640 Medium, glutamax™	Invitrogen, 61870-010
Seebblue® Pre-stained Protein Standard	Invitrogen, LC5625
Streptavidin-Alkaline Phosphatase Conjugate	GE Healthcare, RPN1234
Trans-Blot® Turbo™ Mini Transfer Packs	BIO-RAD, 170-4156
Tween20	SIGMA, MKBK1089V

3.1.3 Bacterial strains and plasmids

Bacterial strains and plasmids	Supplier
One Shot® TOP10 Chemically Competent <i>E. coli</i>	Invitrogen: Catalog Number-C4040
α NIP-gp120	Dr. Inger Øynebråten, Oslo University Hospital, Norway
mCCL3-Gagp24	
mXCL1-Gagp24	
CLL3L1-Gagp24	

3.1.4 Kits

Kit	Supplier and Catalog No.
Endofree Plasmid Mega Kit (5)	QIAGEN, 12381
Mouse IFN- γ elispotplus kit (ALP), pre-coated	MABTECH, 3321-4APT-4
Pierce ECL Plus Western Blotting Substrate Kit	Thermo, 32132
Quick Ligation™ Kit	New England Biolabs, M2200S
TMB Microwell Peroxidase Substrate System	KPL, 50-76-11
Wizard® Plus SV Minipreps DNA Purification System	Promega, A1460
Wizard® SV Gel and PCR Clean-Up System	Promega, A9281

3.1.5 Media

<p>Luria-Bertani (LB) Medium: 10 g Tryptone 5 g Yeast extract 10 g NaCl 1 L deionized water Autoclave to sterilize, mix with antibiotics before using</p>
<p>LB agar: LB medium with 15g/L agar Autoclave to sterilize, reheating and mix with antibiotics before using</p>
<p>SOC medium 0.5% Yeast Extract 2% Tryptone 10 mM NaCl 2.5 mM KCl 10 mM MgCl₂ 10 mM MgSO₄ 20 mM Glucose* *Note: add Glucose after autoclaving the solution with the remaining ingredients, and letting it cool down. Sterilize the final solution by passing it through a 0.2 μm filter.</p>

3.1.6 Software

Chemidoc™ MP System	BIO-RAD
Graphpad version 6.0	Graphpad Software, Inc.
Quantity One 1-D	BIO-RAD

3.2 Methods

3.2.1 Plasmid obtainment and storage

Vaccibody plasmids were provided by Dr. Inger Øynebråten. HIV-1 Gagp24-containing vaccibodies were prepared by subcloning Gagp24 cDNA into vaccibody-containing pLNOH2 vector via SfiI restriction sites, and by replacing the existing DNA fragment. In order to have sufficient amount of plasmids for further study and long-term storage of plasmids, the plasmids were transformed into *E.coli* and DNA isolation was conducted afterwards.

3.2.1.1 Transformation of *E.coli*

Transformation of TOP10 *E.coli* competent cells (Invitrogen) was done by using the heat shock method based on the manufacture's instruction. This involved incubating 25-50 µl of *E.coli* competent cells together with the target plasmid at 42°C for 30 seconds before the mixture was transferred onto ice for cooling for 2 minutes. Next, Lysogeny Broth (LB) medium (SIGMA) was added in the tube and incubated at 37°C with shaking (180-225 rpm) for 1 hour for growth. For selection of transformants, the cells (20 µl-200 µl) were spread on LB agar (Invitrogen) plates containing 50 µg/mL ampicillin (SIGMA) and incubated overnight at 37°C. The plasmids names, *E.coli* strains and antibiotic selections are concluded in Table 3.2.1.1.

Table 3.2.1.1 Summary of plasmids and *E.coli* strains used in the study

Plasmid	<i>E.coli</i> strain	Antibiotics resistance
mCCL3-Gagp24	TOP10	Ampicillin
mXCL1-Gagp24		
CLL3L1-Gagp24		
αNIP-gp120		

3.2.1.2 Growth of bacteria

The transformed *E.coli* strain (see table 3.2.1.1) was normally grown in 5 ml LB medium containing ampicillin (50-100 µg/mL) (Invitrogen) according to the antibiotic resistant selectable marker gene with shaking (200-225 rpm) for 12-16 hours at 37 °C. For mega-culture (500 ml), a pre-culture was prepared as described above and was transferred to the designed volume of LB medium containing ampicillin according to the manufacture's instruction.

3.2.1.3 Long-term storage of *E.coli*

Bacterial cultures transformed with the vaccine-containing plasmids were stored in glycerol (SIGMA) at -80 °C for long-term storage. Bacterial stock solutions were prepared by adding 150 µl 100% glycerol to 850 µl cell cultures. The suspension was mixed vigorously by vortexing and frozen immediately in liquid nitrogen before stored at -80 °C.

3.2.1.4 Plasmid isolation from *E.coli*

For the cloning procedures, the plasmids were isolated according to the protocol provided by Wizard® Plus SV Minipreps DNA Purification System (Promega). The plasmid DNA was eluted in 50 µl to 200 µl nuclease free water. For *in vivo* mice experiments, endotoxin free plasmids were isolated by using EndoFree Plasmid Mega Kit (QIAGEN).

3.2.2 Subcloning

3.2.2.1 Generation of vaccibody construct

All vaccibody plasmids were prepared by subcloning Gagp24 cDNA into pLNOH2 vector via

SfiI restriction sites as described in section 3.2.1. For construction of the α NIP-Gagp24-containing plasmid, plasmids α NIP-gp120 and mCCL3-Gagp24 were double digested for 2-4 hours at 37 °C (water bath) in order to release and recollect α NIP insert and Gagp24-containing construct. The reaction volume was 25 μ l consisting of NEBbuffer (New England Biolabs), the chosen restriction endonucleases BsiWI and BamHI (New England Biolabs), Bovine Serum Albumin (BSA) (New England Biolabs), water, and plasmid and enzymes which were added at the end. DNA fragments were isolated by 1.2% agarose gel in a TBE buffer consisting of tris (hydroxymethyl) aminomethane (Tris) base, boric acid, Ethylenediaminetetraacetic acid (EDTA) with ethidium bromide (SIGMA) according to the manufacture's instruction. Add 5 μ l of agarose gel loading buffer (loading buffer: digests=1:5) to the digests, and load 30 μ l mixture into a lane in the agarose gel (LONZA) with 5 μ l of 1 kb and 100 bp DNA ladder (New England Biolabs) in two separate lanes. Run the gel at 90 V for 1 hour.

The plasmid α NIP-gp120 (Figure 3.2.2.1) was double digested by the same enzymes and steps as described above.

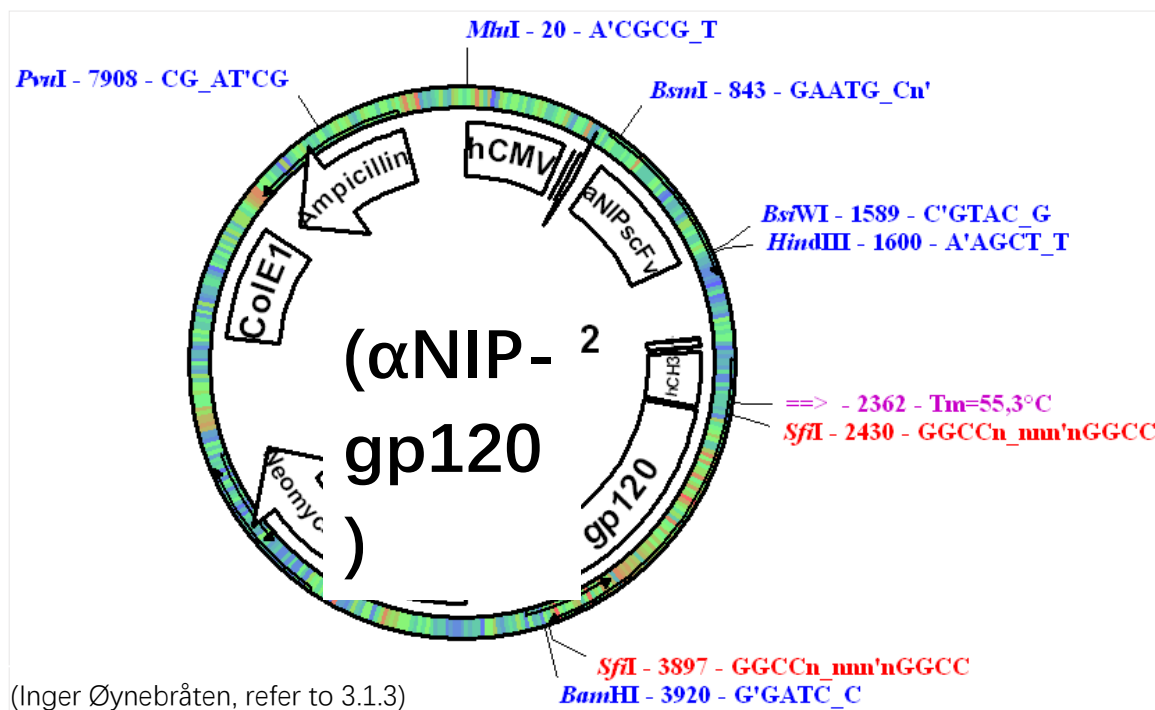


Figure 3.2.2.1 Schematic drawing of the pLNOH2 vector containing a vaccibody construct, here denoted α NIP-gp120. The pLNOH2 vector is derived from pcDNA3 [113]. The plasmid was used to generate α NIP-Gagp24 by restriction endonucleases BsiWI and BamHI. As the consequence that mCCL3-Gagp24 is constructed by pLNOH2 vector in the similar method in our lab, the mCCL3-Gagp24 construct is identical except α NIPscFV and gp120 fragments were exchanged to mCCL3 and Gagp24 fragments separately.

3.2.2.2 Agarose gel electrophoresis

Principle

Electrophoresis through agarose gels is the standard method for separation, identification, and purification of DNA and RNA fragments ranging in size from approximately 50 bp to 20 kb. The location of DNA within the gel can be determined directly by staining with low concentrations of intercalating fluorescent ethidium bromide dye and visualized under ultraviolet light. If necessary, these bands of DNA can be recovered from the gel and used for a variety of cloning purposes. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates towards the anode. The rate of migration depends on a number of parameters: molecular size of DNA, agarose concentration, conformation of DNA and composition of the electrophoresis buffer. The electrophoretic mobility of DNA is also affected by the composition and ionic strength of electrophoresis buffer. In the absence of ions, electrical conductivity is minimal and DNA migrates very slowly. In buffers of high ionic strength, electrical conductance is very efficient. Gel is stained, for example, with ethidium bromide and as little as 0.05 µg of DNA in one band can be detected.

Procedure

Agarose was mixed with TBE buffer to give the desired concentration of 0.8% or 1.2% and heated in the microwave oven until the agarose was dissolved completely and then cooled to approximately 60 °C. Ethidium bromide was added to achieve final concentration of 0.5µg/ml. The solution was transferred to a gel tray for solidification after placing one or two well-comb. The solidified gel was transferred to an electrophoresis chamber containing enough TBE buffer to cover the whole gel. DNA samples were mixed with 0.2 DNA-sample-volume 6x loading buffer (2 µl loading buffer at least for easy manipulation), before loading into the wells. The electrophoresis was run at 80-100 V until all fragments were sufficiently separated. The DNA was visualized under UV light and the result was saved in computer by Quantity One 1-D software (BIO-RAD).

3.2.2.3 Collection of digested plasmid DNAs from agarose gel

Gel visualization under UV light and the desired band from gel was removed by using clean blade as soon as possible in order to reduce the exposure time of gel under UV light and place it in a pre-weighed and labeled eppendorf tube. The DNA fragment in gel was purified by using

Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacture's instruction. At the end of the procedure, the column containing the DNA fragment was placed into an autoclaved 1.5 ml micro tube and the DNA was eluted by adding 35 µl Elution Buffer by centrifugation for 1 minute at 16,000 xg. The eluted DNA was stored at 4 °C or -20 °C.

3.2.2.4 DNA ligation, transformation of ligated product, plasmid isolation and long term storage

For ligation of the DNA construct into a vaccibody-containing vector, 50 ng of vector was mixed with a 3-fold molar excess of insert and the ligation was finished by using Quick Ligation™ Kit (New England Biolabs). Next, the ligation mixture was chilled on ice and transformed by adding 1-5 µl of the reaction into 50 µl competent cells or it was stored at -20 °C prior to transformation. The ligated plasmid was named as αNIP-Gagp24. Transformation of αNIP-Gagp24 and plasmid isolation was the same as described in sections 3.2.1.1 and 3.2.1.4. The long-term storage method is same as described in section 3.2.1.3.

3.2.3 Sequencing

DNA sequencing was conducted by GATC Biotech AB (Solna, Sweden) in order to verify that the sequences of vaccibodies were correct. Primers used for sequencing are listed in Table 3.2.3.

Table 3.2.3 Information of sequencing primers

Name	Supplier	Sequence
T7	GATC	5'-TAATACGACTCACTATAGGG-3'
BGH-Reverse	GATC	5'-TAGAAGGCACAGTCGAGG-3'

3.2.4 Mice and cell lines

BALB/c mice were purchased from Taconic (Ry, Denmark) and were 6 to 10 weeks of age when included in the experiments. All mice were delivered and nested to the animal department for at least 1 week before experiment. Before vaccination, the mice were anesthetized by subcutaneous injection of 200 µl of a mixture of Hypnorm (Fentanyl, 79 µg/ml; Fluanison, 2.5 mg/ml) and Dormicum (Midazolam 1.25 mg/ml) (ready-made product purchased from Dept. of Comparative Medicine, Oslo University Hospital, Rikshospitalet). The experiments were performed at the Dept. of Comparative Medicine, Oslo University Hospital, Rikshospitalet. The study was approved by the National Committee for Animal Experiments (Oslo, Norway).

The HEK293 cell line is originally derived from human embryonic kidney cells grown in tissue culture [114]. For the experiments included in this thesis, HEK293 cells were cultured in RPMI 1640 Medium GlutaMAX™ (Invitrogen) supplemented with 10% heat-inactivated FCS, Gentamicin (50 µg/mL) (LONZA) (hereafter referred to as complete RPMI). Esb-MP cell line (kindly provided by Dr. J. Van Damme, University of Leuven, Belgium) is an adherent subclone from Esb which is a highly malignant variant of a murine methylcholanthrene-derived T cell lymphoma, and Esb-MP cell line was selected based on its plastic adherence property [115]–[117]. ESb-MP cells were cultured in DMEM (LONZA) supplemented with 10% heat-inactivated FCS, L-Glutamine (4mM) (LONZA), Gentamicin (50 µg/mL) (hereafter referred to as complete DMEM). All mammalian cells were grown at 37 °C with 5% CO₂.

3.2.5 Transient transfection and harvesting of vaccibodies *in vitro*

Transient transfection was performed in HEK293 cells by using Lipofectamine 2000. Cells were seeded out into 24 well culture plates at concentration 1.5×10^5 cells/well (500 µl/well) the day before transfection with complete RPMI. The day after, 1.5 µl Lipofectamine 2000 and 0.5 µg plasmid DNA were added separately to 50 µl Opti-MEM® I Reduced Serum Medium (Invitrogen). Next, the two solutions were mixed and incubated at RT for 10 min in order to form liposome-DNA complexes. The complexes were then added to the wells. The cell culture was incubated at 37 °C with 5% CO₂. The supernatant was harvested on day 3 post transfection and was centrifuged in order to remove cells and cellular debris which may damage vaccibodies. The supernatant was stored at 4 °C for 5 days maximum before application to *in vitro* assays.

3.2.6 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed in Corning® 96 well EIA/RIA plates coated with antibodies or other reagents diluted in Phosphate-buffered saline (PBS) (Invitrogen) with 60 µl/well and incubated overnight at 4 °C. Plates were blocked with 10% BSA (BIO-RAD) at RT for 1 hour. Detection antibodies were incubated at RT for 2 hours. Different detection methods were used and are given below.

3.2.6.1. Sandwich ELISA for detection of vaccibody protein produced *in vitro*

For detection of secreted vaccibody molecules, ELISA plates were coated with NIP-BSA (1 µg/mL), anti-mouse CCL3 (4 µg/mL), mouse anti-human IgG antibody (CH3 domain, clone

A57H, 1 µg/mL) (Bio-Rad), Human CCL3/MIP-1 alpha antibody (Clone 14215, 1/100) (R&D systems). The biotinylated mouse monoclonal anti-human IgG (Fc, clone HP-6017) antibody (1:1000) (SIGMA), biotinylated polyclonal anti-mXCL1 antibody (1 µg/mL) (R&D systems), biotinylated goat polyclonal anti-human CCL3L1 (0.2 µg/mL) (R&D Systems) were used for detection. Samples were added as duplicates in 3-fold serial dilution with a start volume of 75 µl and incubated overnight at 4 °C.

Bound proteins were detected using Streptavidin–Alkaline Phosphatase (StreptAP) (1:3000) (GE Healthcare), followed by colorimetric reaction by use of Phosphatase substrate (1 mg/ml) (SIGMA). The OD (absorbance) at wavelength of 405 nm and reference wavelength of 620 nm were measured using Sunrise Remote Microplate Reader.

3.2.6.2. Sandwich ELISA for detection of serum antibodies in immunized mice

ELISA plates were coated with HIV-1 Gagp24 antigen (2 µg/ml) (VIROGEN). Serum samples were added in duplicates in 2-fold serial dilution starting with dilution 1:50. The plates were incubated overnight at 4 °C and bound antibodies were detected with anti-Mouse IgG conjugated with Horseradish Peroxidase (from sheep) (1:500) (GE Healthcare) followed by colorimetric reaction with TMB Microwell Peroxidase Substrate System Kit (KPL). The OD was measured at wavelength 450 nm with reference wavelength of 620 nm by use of Sunrise Remote Microplate Reader.

The results are given as Endpoint titers which were determined as the serum dilution that gave an OD value at 450 nm at least two times of the average OD value measured for serum samples obtained from mice given Sodium Chloride only (NaCl group) (Refer to 3.2.9).

3.2.7 Chemotaxis assay

Chemotactic activity of vaccibody protein was evaluated by using Esb-MP cells and 6.5mm Transwell® with 5.0µm Pore Polycarbonate Membrane Insert-Sterile (CORNING) (hereafter referred to as Transwell insert). Esb-MP cells were harvested and re-suspended by using RPMI 1640 with 1% BSA in order to have a concentration of 20,000,000 cells/ml. 600 µl complete RPMI containing vaccibody proteins (supernatant from transfected HEK293 with mCCL3-Gagp24 plasmid), positive control (supernatant from transfected HEK293 with mCCL3-gp120

plasmid) and negative control (supernatant from transfected HEK293 without plasmid) were added to the wells (Lower compartment, Figure 3.2.7) in a 24-well plate (Plate A). All supernatant come from transfections as described in 3.2.5.

Three 6.5 mm-Transwell inserts were transferred into another 24-well sterile plate (Plate B). 100 μ l of the Esb-MP cell suspension were added to Transwell inserts separately before the Transwell inserts were transferred into Plate A wells which contained 600 μ l of the samples. Plate A was incubated for 2 h at 37°C with 5% CO₂. Cells were harvested in 1.5 mL tube separately, then 200 μ l of 2mM EDTA was added to the lower compartment of the well to release the cells that have adhered to the bottom of the well and harvested into same 1.5-mL tube separately. The loss of cells that migrated through the microporous membrane but adhered to it was ignored. The Countess® Automated Cell Counter was used to count cells.

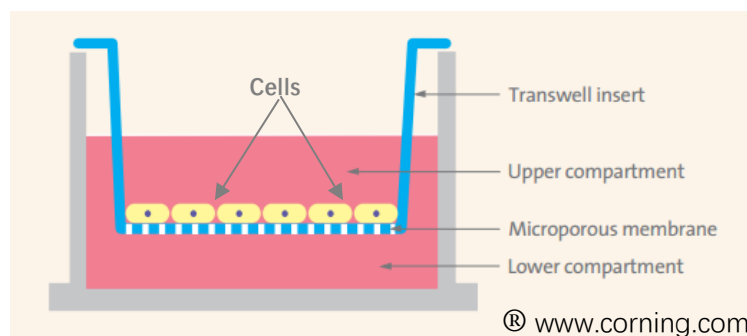


Figure 3.2.7 Schematic diagram of cell culture plate with Transwell insert

3.2.8 SDS-PAGE and Western blotting

Principle

Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a method in which proteins are run in a vertical SDS-polyacrylamide gel under denaturing conditions. The proteins are denatured in the presence of beta-mercaptoethanol or dithiothreitol (DTT) and SDS. Beta-mercaptoethanol or DTT (in our case, DTT) reduce disulfide bridges whereas SDS is a negatively charged detergent that dissolves hydrophobic molecules, leaving the proteins linearized with a negative net charge corresponding proportionally to their length. The proteins are separated in the polyacrylamide matrix as they migrate towards the anode. By using a protein standard, the molecular size of the proteins can be determined. To obtain good resolution, a discontinuous gel system is used. A stacking gel is layered on top of a resolving gel and it allows the proteins in an each loading well to be concentrated into a tight band during the first

few minutes of electrophoresis before entering the resolving portion of a gel in order to obtain optimal resolution. The resolving gel has a much smaller pore size compared to the stacking gel which leads to a sieving effect of pores in gel that determines the electrophoretic mobility of the proteins. The two gels differ in salt concentration, pH, acrylamide concentration [118].

Western blotting refers to blotting of electrophoresed protein bands from a polyacrylamide gel on to a nitrocellulose or Polyvinylidene difluoride (PVDF) membrane and their detection with antibody probes. The gel is overlaid with a nitrocellulose or PVDF membrane, and an electric field is applied so that proteins migrate from the gel to the membrane where they become bound. The membrane thus has an exact image of the pattern of proteins that was in the gel.

Procedure

For non-reducing samples, 3-6 μ l of supernatant from transfected cells (section 3.2.5) was mixed with non-reducing lithium dodecyl sulfate (LDS) sample loading buffer (4X) (Alfa Aesar). For reducing samples, 3-6 μ l of supernatant from transfected cells was mixed with DTT-added LDS loading buffer-(4X) (working concentration of DTT was 50 mM). The sample mixtures were incubated at 95 °C for 5 min, followed by cooling for 2 min on ice. Samples were then applied to 10% Mini-PROTEAN® TGX™ Gel (BIO-RAD) which were run at 125 V for approximately 1 hour. 12 μ l SeeBlue® Pre-stained Protein Standard (Invitrogen) was included. The membranes were incubated for 1 hour with a blocking solution consisting of 5% BSA and 0.05% Tween20 in PBS, before incubation with Anti-HIV1 p24 antibody (1:1000) (clone 39/5.4A, abcam) at 4 °C and 40-50 rpm overnight. The membrane was then washed 3-4 times in PBS with 0.05% Tween 20 (PBST) on the shaker for 5-10 min each time, followed by incubation with anti-Mouse IgG, Horseradish Peroxidase-Linked Antibody (1:10,000) (GE Healthcare) for 2 hours. All antibodies were diluted in PBST. After same washing step as before, antibody bound to the membrane were visualized by using Pierce ECL Plus Western Blotting Substrate Kit (Thermo) and imaged in ChemiDoc™ MP System (BIO-RAD).

3.2.9 DNA vaccination

The plasmids containing the vaccine constructs were prepared under sterile conditions and they were diluted to concentration 0.5 μ g/ μ l in 0.9% NaCl (B. Braun). Mice were anesthetized, their legs shaved, and conductive gel (Elefix PASTE) applied on the skin. Next, the DNA plasmids in 50 μ l solution were injected intramuscularly into each quadriceps femoris of the leg.

Immediately after injection, the electroporation was performed by using Elgen 1000 Electroporation Applications System equipped with a caliper electrode. The settings were: 100 mV (bipolar pulses) \times 0.2 ms (pulse sequence) and pulse sequence train being 10 and 1000. Mice were divided into four groups and were immunized with plasmids, α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24 or were given 0.9% NaCl solution (B. Braun).

3.2.10 Blood sampling from mice and isolation of cells from mouse spleens

After anesthetizing mice by the method mentioned in 3.2.4, the blood samples were obtained from heart. Hereafter, the BALB/c mice were sacrificed by cervical dislocation, dipped in 70% ethanol and placed on dissection board with its abdomen side up. The abdomen was opened up by scissor and the spleen was taken out and stored in complete RPMI on ice. The spleen was crushed through a steel mesh into a petri dish with approximately 15 ml complete RPMI in order to form a single cell suspension. The cell suspension was transferred to sterile Corning® 50 mL centrifuge tubes followed by 5 minutes Tris-Buffered Ammonium Chloride (ACT) treatment for red blood cell lysis. Next, the cells were washed and resuspended in complete medium, counted and re-diluted in complete RPMI to an appropriate concentration for Enzyme-Linked ImmunoSpot (ELISpot) assay.

3.2.11 IFN- γ ELISpot

After DNA vaccination and splenocyte preparation as described in 3.2.9 and 3.2.10, respectively, the pre-coated Mouse ELISpotPLUS kit (ALP) was used for evaluation of IFN- γ -secreting antigen-specific T cells. The protocol according to the manufacture's instruction was followed. Splenocytes were transferred in numbers 5×10^5 , 2.5×10^5 , 1.25×10^5 cells/well in duplicates, and re-stimulated with synthetic peptides diluted to a concentration of 4 μ g/ml in complete RPMI. 57 synthetic 15-mer peptides spanning HIV-1 Gag overlapping by 4 amino acids were obtained from the NIH AIDS Reference Reagent Program (Catalog no. 8117) and they were divided into 5 pools. Each pool contained 9 to 12 peptides in order to represent the entire HIV1 Gagp24 polypeptide [119]. HIV-1 Gagp24 peptide pools span from aa 125-183 (pool 1), aa173-231 (pool 2), aa221-279 (pool 3), aa269-327 (pool 4), aa317-363 (pool 5) of HIV Gag p24 protein. The HIV-1 Gagp24 peptide (199-207) which binds to MHC class I molecule H-2 Kd in BALB/c mice was used to re-stimulate CD8⁺ T cells [120]. The splenocytes from the NaCl group incubated without peptides were used as negative controls. The ELISpot plates were incubated for approximately 24 hours at 37 °C with 5% CO₂. The spots were counted by CTL-

ImmunoSpot® S6 Micro Analyzer.

3.2.12 Bioinformatics and Statistics

The sequence analysis was conducted in Serial Clone 2.6 and pDRAW32 1.0. The statistical analysis was performed in GraphPad Prism 6. Significance was accepted at $p < 0.05$.

4. Results:

Many studies have shown that targeting of antigens to APCs-specific receptors may not only reduce the vaccine dose that is required to elicit T cell responses but also reduce the proportion of the vaccine dose that ends up in non-target cells which reduce the potential adverse effects such as “vector backbone toxicity”, “off-target responses” [121], [122]. Fredriksen *et al.* developed a targeted, bivalent vaccine format denoted to vaccibody, which showed enhanced induction of specific T and B cell immune responses against multiple myelomas, B cell lymphomas in mouse models [107], [108], [123]. Therefore, we engineered vaccibody constructs and tested them aiming to induce Gagp24-specific T and B cell immune responses against HIV-1. The vaccibody molecules are homodimers consisting of a targeting unit, a dimerization unit and an antigenic unit in each protein chain (Figure 4). The Gagp24 was cloned into pLNOH2 vectors encoding vaccibody constructs with different targeting units (murine CCL3, murine XCL1 and human CCL3L1). The vaccibody construct which contains single chain fragment variable (scFv) specific for 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) as targeting unit was used as non-targeted control and is denoted by α NIP.

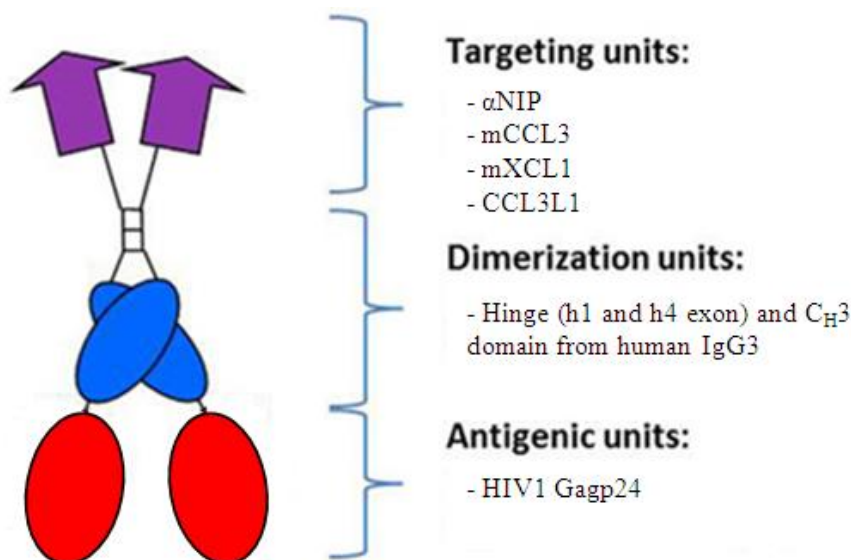


Figure 4: Schematic drawing of vaccibody protein. Modified from [107]. The vaccibodies are homodimers. Each vaccibody consists of two N-terminal targeting units (violet part) in the form of a chemokine (mCCL3, mXCL1, CCL3L1) that bind to surface receptors on APCs, or a scFv (α NIP) used as non-targeted control. A dimerization unit (blue part) consists of a hinge region (h1 and h4 exon) and C_H3 domain derived from human IgG3. The antigenic units (red part) composed of two identical C-terminal HIV1 Gagp24 antigens.

4.1 Detection of secretion of homodimeric HIV-1 Gagp24-containing vaccibodies

In order to examine whether the Gagp24-containing vaccibody constructs were produced and secreted, HEK293 cells were transfected with DNA plasmids encoding the vaccibodies. After 3 days, cell media were harvested and analyzed by different ELISAs. Because the dimerization unit is identical for all the vaccine constructs, an ELISA with anti-C_H3 antibody as coat and anti-Fc as detection antibody was utilized to compare the secreted levels of the vaccibodies (Figure 4.1A). All the vaccibody constructs were produced and secreted. Moreover, these results confirmed that the dimerization unit of different vaccibodies was detectable, meaning that it was presented and exposed when combined with Gagp24 in the vaccibody format. However, based on the ELISA, the levels of the vaccibodies appeared to vary. Highest levels were found for CCL3L1-Gagp24 and mCCL3-Gagp24 whereas the same dilutions resulted in lower OD values and levels for α NIP-Gagp24 and mXCL1-Gagp24 (Fig. 4.1A).

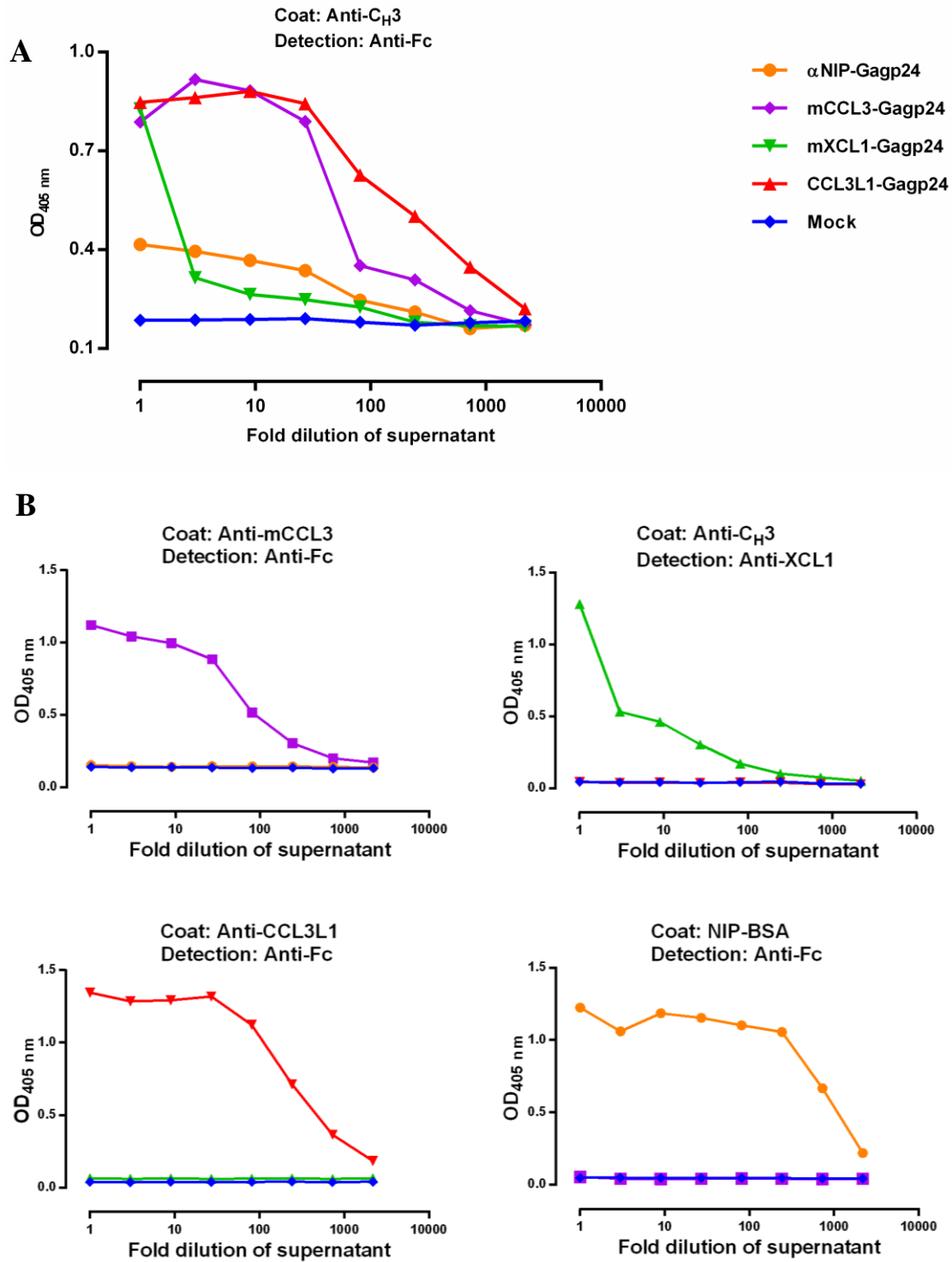


Figure 4.1: Detection of secreted vaccibody constructs. HEK293 cells were transfected with plasmids encoding α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24, CCL3L1-Gagp24 or were left untreated (Mock). Three days after transfection, the supernatants were harvested and analyzed by ELISAs combining different coating and detection antibodies as indicated above each graph. All experiments were repeated at least three times and data from one representative experiment are presented in each graph.

To confirm the presence of the unique targeting units of the vaccibodies, antibodies or a ligand specific for the various targeting units were used either for coating or detection in ELISA (Figure 4.1B). Anti-mCCL3, anti-mXCL1, anti-CCL3L1 or NIP-BSA was utilized in combination with antibodies towards the dimerization unit. According to the result, it is confirmed that different targeting unit of each vaccibody was detectable, meaning that they were presented and exposed when combined with Gagp24 in the vaccibody format (Fig. 4.1B).

Taken together, these data confirmed that all Gagp24-containing vaccibodies were produced and secreted from transfected HEK293 cells.

4.2 Size, stability and degradation of vaccibodies produced *in vitro*

To investigate the sizes of the secreted vaccibodies, their ability to form dimers and to evaluate the secretion of vaccibodies by another method than ELISA, western blotting was used. Supernatants from transfected HEK293 cells were harvested three days post transfection and analyzed by SDS-PAGE under non-reducing condition (SDS) and reducing condition (SDS+DTT). The negative control (Mock) was supernatant from non-transfected cells. Proteins were detected by western blotting using a monoclonal antibody against HIV-1 Gag p24.

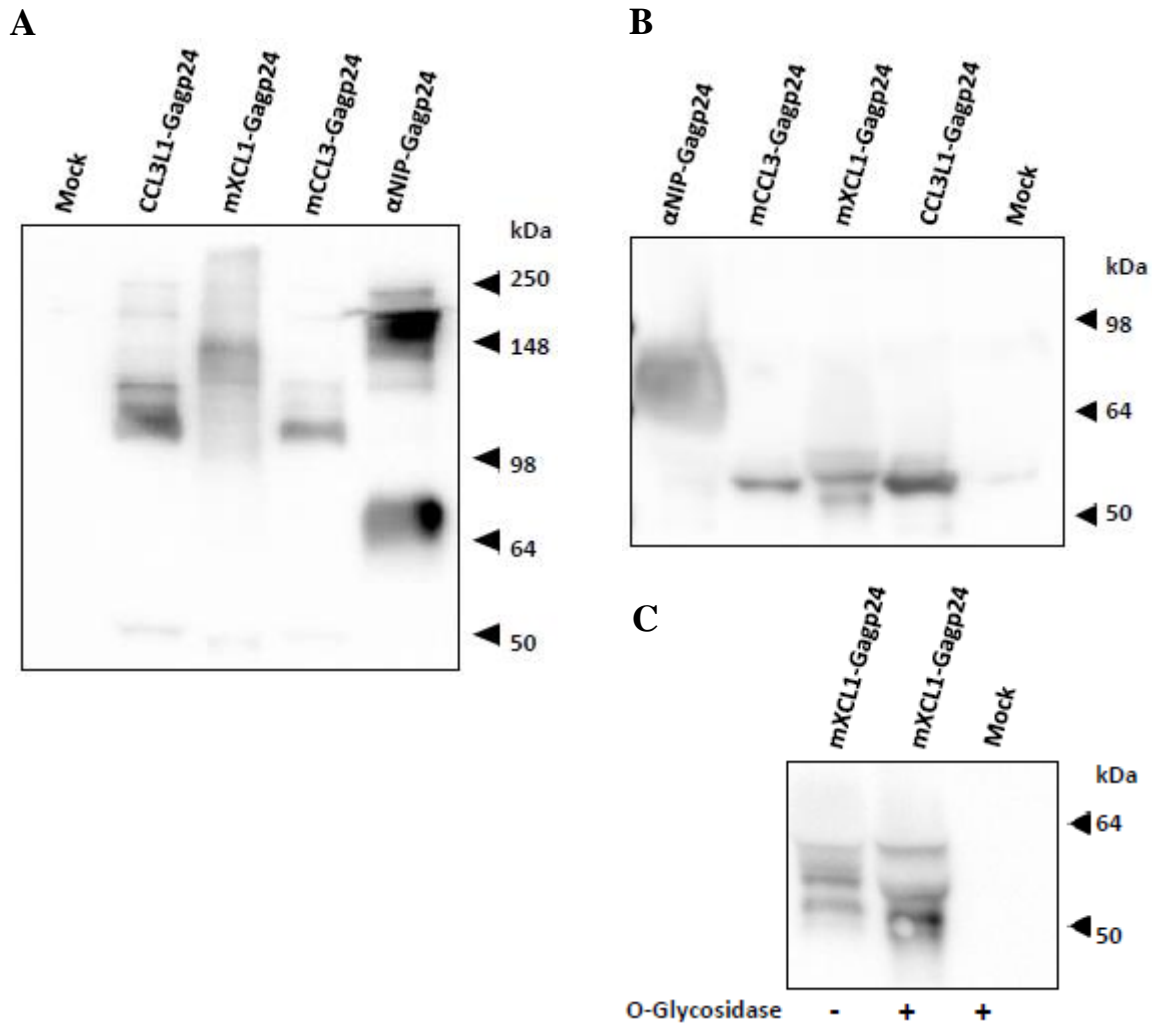


Figure 4.2 Western blotting analysis of vaccibody molecules. SDS-PAGE (10%) and Western blotting of supernatants harvested from vaccibody-transfected HEK293 cell under non-reducing (SDS) or reducing conditions (SDS+DTT) and blotted onto a PVDF membrane (Mock: supernatant from HEK293 cells transfected without plasmid). A) Non-reducing condition, 2 repeats* and B) Reducing condition, 4 repeats*. C) Supernatants from HEK293 cells transfected with DNA encoding mXCL1-Gagp24 were treated with O-glycosidase before adding SDS and DTT and analysis. 2 repeats*. * = The repeats of western blotting gave similar results and representative results from the experiments are presented.

A monoclonal antibody against HIV-1 Gagp24 (clone number: 39/5.4A) was used to detect the antigenic unit of all four vaccibodies. An anti-mouse IgG HRP-linked antibody was applied as secondary antibody in all western blotting analyses. Figure 4.2A and B, show that the vaccibodies could be detected by the anti-HIV1 Gagp24 antibody, meaning that the antigenic units of vaccibodies (Gagp24) were presented when they are combined with all four different targeting units in vaccibody format.

Detection with specific antibody under non-reducing condition revealed two bands (at the size around theoretical Mw and half Mw) for each of vaccibodies in Figure 4.2A. However, the vertical smeared streaking for each of vaccibodies and the presence of several bands around same size seen under non-reducing condition have been observed in Figure 4.2A. For example, two bands are visualized separately at the size around 110-130 kDa and 50 kDa for CCL3L1-Gagp24 in Figure 4.2A. These two observed sizes correlate to the full or half theoretical Mw of CCL3L1-Gagp24 which are 102 kDa and 51 kDa. This result was consistently observed for the rest of vaccibodies on the gel as shown in Figure 4.2A. Those two bands indicate that both homodimers and monomers of vaccibodies are presented. The theoretical protein molecular weight (Mw) of α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24, CLL3L1-Gagp24 are 136, 96, 104, 102 kDa, respectively.

To identify the size of monomers of vaccibodies, treatment with DTT and boiling was performed to reduce disulfide bridges and generates monomers. It resulted in dominant bands around 68, 48, 52, 51 kDa (half amount of 136, 96, 104, 102 kDa) for α NIP-Gagp24, mCCL3-Gagp24, mXCL1-Gagp24, CLL3L1-Gagp2 separately (Figure 4.2B). The results are consistent with predicted molecular weight, and show that the vaccibody molecules could form covalently linked homodimers as expected from their design. In addition, two bands were observed around the same size for mXCL1-Gagp24. This size diversity is also the case for human XCL1, which because it is O-glycosylated *in vivo* [124] and *in vitro* [125] with a lower Mw structure appeared for deglycosylated XCL1.

To investigate whether mXCL was glycosylated when combined with Gagp24 in the vaccibody format, treatment with neuraminidase and O-glycosidase was performed (Figure 4.2C). After the treatment, two bands with lower Mw appeared in the sample treated with O-glycosidase compared to the sample treated without O-glycosidase. Thus, some of the mXCL1-Gagp24 expressed in HEK293 cells is at least partly O-glycosylated when expressed in HEK293 cells.

Taken together, the ELISA and Western blotting results reveal that the targeting, dimerization, and antigenic units of all four vaccibodies are presented and exposed in a way which is detectable by antibodies or ligand when they are combined in vaccibody format without degradation, meaning that the full structure of all four vaccibodies have been produced and secreted *in vitro*. In addition, they have been secreted in right size as mentioned above.

4.3 Functional analysis of the CCL3-targeting unit

As shown above, the vaccibodies were produced and secreted properly regarding to sizes, degradation situation and all units can be detected. However, the chemoattractant ability of targeting units would be preferred to know since the presence of the vaccibodies does not reveal the presence of chemoattractant function. The vaccine format containing mCCL3 is specific for the chemokine receptors CCR1 and 5 [126], [127]. To verify if the targeting units were chemoattractant, a chemotaxis assay employing CCR1⁺CCR5⁺Esb-MP cell line was performed for three repeats (Figure 4.3).

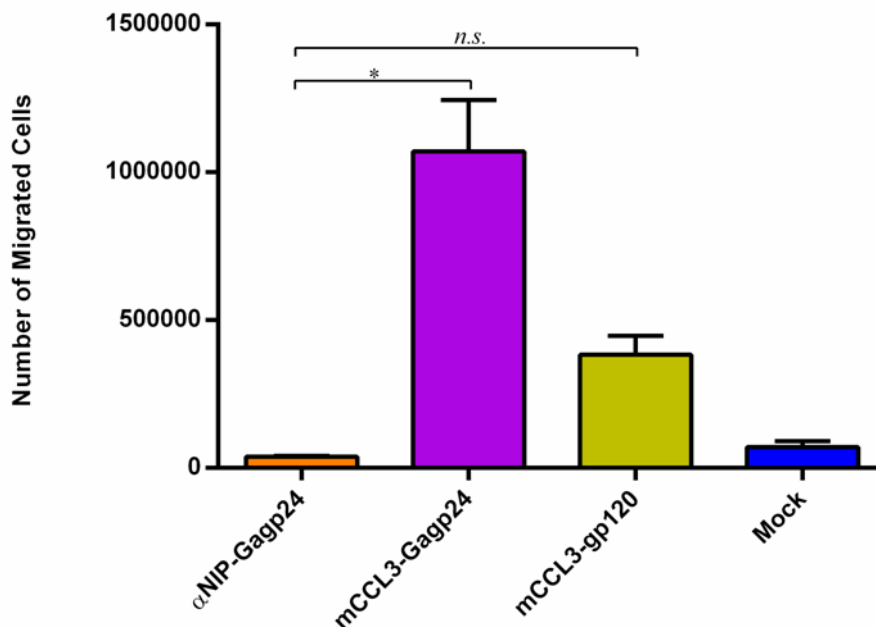


Figure 4.3: Total number of migrated Esb-MP cells in chemotaxis assay. mCCL3 in the vaccine format is chemotactic. Chemotactic activity of vaccibodies on the mouse Esb-MP T cell lymphoma was tested by a Transwell assay. 600 μ l complete RPMI containing vaccine proteins (supernatant from transfected HEK293 with mCCL3-Gagp24 plasmid), positive control (supernatant from transfected HEK293 with mCCL3-gp120 plasmid), non-targeted control (supernatant from transfected HEK293 with α NIP-Gagp24 plasmid) and negative control (supernatant from Lipofecatmine-exposed HEK293) were used to chmoattract 2,000,000 mouse Esb-MP cells respectively. The results are shown as group mean of Number of Migrated Cells \pm SEM. Statistical significance indicated by the use of star symbols (*)

* = p-value < 0.05; n.s. = not significant.

It was previously shown that ESb-MP lymphoma cells migrate *in vitro* toward mCCL3 [128] and towards the gp120-containing vaccibody targeted by CCL3 [91]. Therefore, CCL3-gp120 vaccibody was included as a positive control. The number of cells that migrated towards mCCL3-Gagp24 was significantly higher than the number of cells that migrated towards α NIP-

Gagp24. On the other hand, the number of cells that migrated towards mCCL3-gp120 was not significantly higher but higher than the number of cells that migrated towards α NIP-Gagp24, suggesting mCCL3 did attract Esb-MP cells in our assay but did not work with the same efficiency between the two vaccibodies. Taken together, the functional activity of the targeting unit mCCL3 in the vaccibody structure mCCL3-Gagp24 has been confirmed. The non-targeted α NIP-Gagp24 did not chemoattract Esb-MP cells.

4.4 Investigation of T cell responses *in vivo*

To investigate whether vaccibodies could induce T cell responses, BALB/c mice were immunized by intramuscular injection combined with electroporation by using 50 μ l of saline without or with plasmid DNA encoding vaccibodies. Five weeks after immunization, the spleens were removed and splenocytes were isolated as described in section 3.2.10 for enumeration of T cell responses using IFN- γ ELISpot. As shown in Figure 4.4, the splenocytes were left un-treated or re-stimulated with different peptide pools, or re-stimulated with an HIV1-Gagp24-derived, CD8⁺ T cell-specific epitope (MHC I peptide) to quantify CD8⁺ T cell responses in BALB/c mice. The peptide pools may re-stimulate both CD4⁺ and CD8⁺ T cells.

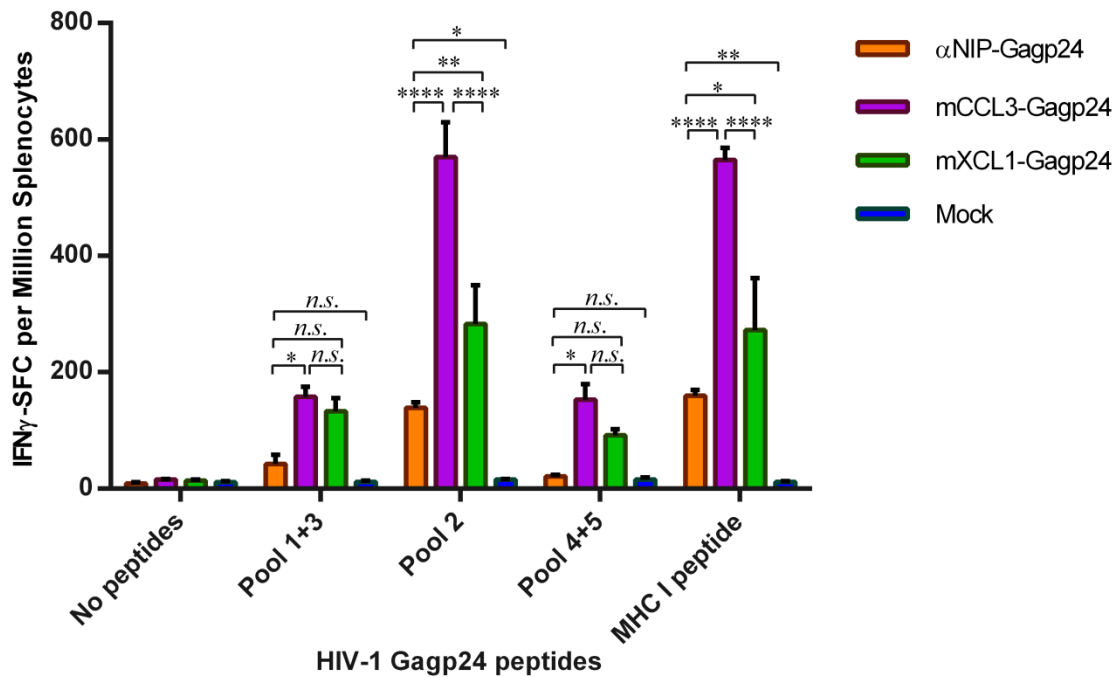


Figure 4.4: Investigation of T cell response *in vivo*. BALB/c mice were immunized by intramuscular injection combined with electroporation by using 50 μ l of saline without (Mock) or with plasmid DNA encoding homodimer vaccibodies α NIP-Gagp24, mCCL3-Gagp24, or mXCL1-Gagp24. Five weeks after immunization, the splenocytes were harvested and assigned to IFN- γ ELISpot for enumeration of antigen-specific T cell responses. The splenocytes were re-stimulated with different peptide pools (Pool 1+3, Pool 2, Pool 4+5) or without (No peptide), or alternatively, an HIV1-Gagp24-derived CD8⁺ T cell-specific epitope (MHC I peptide). The results are shown as group mean IFN- γ spot forming cells (SFC) \pm SEM. Statistical significance is indicated by the use of star symbols (*) and the number of stars indicates the significance level.

‘*’ = p-value < 0.05; ‘**’ = p-value < 0.01; ‘****’ = p-value < 0.0001; ‘n.s.’ = no significant.

All vaccibodies induced IFN γ -positive T cell responses towards HIV1 Gagp24 compared to the mock control. In addition, the CD8⁺ T cell-specific HIV-1 Gagp24 (199-207) peptide (MHC I peptide) was used to quantify CD8⁺ T cell responses. T cell response was significantly stronger compared to non-targeted control (α NIP-Gagp24) upon the peptide pools re-stimulation while there was no difference between vaccibodies group and negative control in the absence of peptide pools re-stimulation (Figure 4.4).

Targeting by mCCL3 resulted in a significantly higher number of T cells compared to the non-targeted control (α NIP-Gagp24) in vaccinated mice. This was the case for T cells towards peptides of pool 1+3, pool 2, pool 4+5 and MHC I peptide, these are peptides derived from whole HIV1 Gagp24 protein. In comparison to the non-targeted control, targeting by use of mXCL1 showed significantly higher T cell responses towards the Pool 2 and MHC I peptide. There was no significant difference in T cell numbers in vaccinated mice towards Pool 1+3,

Pool 4+5 by using mXCL1-Gagp24 compared to non-targeted control. Next, when comparing the number of IFN γ -positive T cells elicited by the mCCL3- and mXCL1-targeted vaccibodies, targeting by mCCL3 induced significantly more T cells towards the peptides in pool 2 and MHC I peptide, whereas there was no difference in the number of IFN γ -positive T cells towards pool 1+3 or pool 4+5 for mCCL3-Gagp24 and mXCL1-Gagp24. In short, mCCL3-Gagp24 vaccibody not only result a broader immune response towards peptides of pool 1+3, pool 2, pool 4+5 and MHC I peptide but also show stronger immune response towards peptides of pool 2 and MHC I peptide.

4.5 Humoral immune responses

To examine the ability of vaccibodies to induce humoral immune responses *in vivo*, blood samples were harvested after 5 weeks from vaccinated mice as described in section 3.2.10. The amount of IgG HIV-1 Gagp24-reactive antibodies was measured by sandwich ELISA combined with endpoint titer method (Figure 4.5).

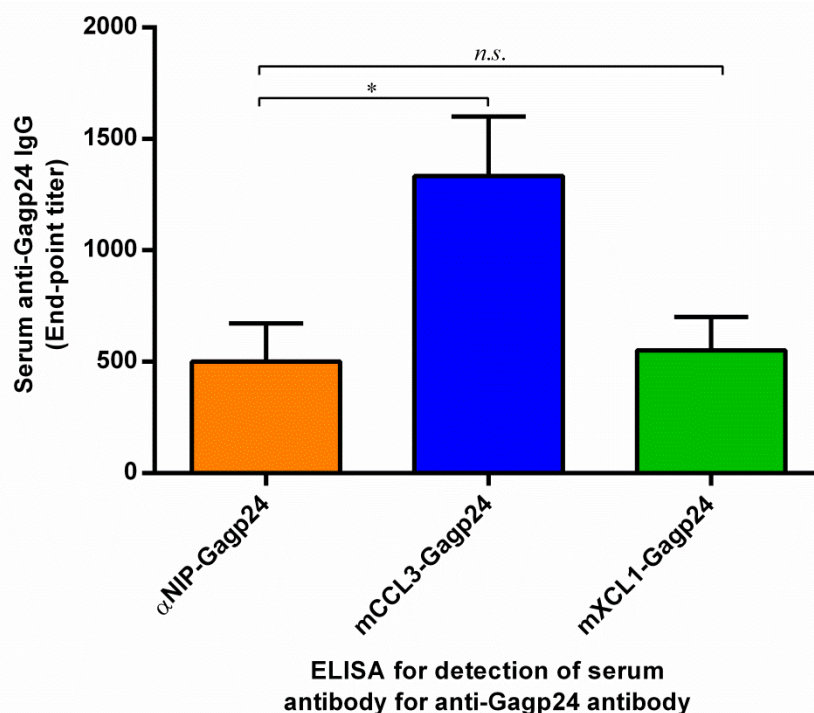


Figure 4.5: Comparison of HIV1 Gagp24-specific antibody level in serum from vaccinated mice. Antibodies were measured in ELISA by using HIV-1 Gp24 antigen and Anti-Mouse IgG, Horseradish Peroxidase-Linked Antibody as coating and detection reagent, respectively. The results are shown as group mean of Serum IgG amount \pm SEM. Statistical significance indicated by the use of star symbols (*)

‘*’ = p-value < 0.05; ‘n.s.’ = no significant.

End-point titer was determined as the serum dilution that gave OD at 450 nm at least two times higher than the averaged OD value measured from serum samples of the NaCl group. The level of antibody was significantly higher for the mCCL3-Gagp24 group compared to the non-targeted control. On the other hand, the level of antibody was not significantly different for the mXCL1-Gagp24 group compared to the non-targeted control. These results suggest that targeting by CCL3 to APCs is more beneficial for stimulation of humoral immune response compared to targeting of mXCL1 at early time points after immunization (5 weeks).

5. Discussion

It was previously reported that vaccibody utilizing CCL3 as targeting unit induced a higher number of HIV-1 gp120-specific T cell responses compared to non-targeted control [91]. In this study, the vaccibodies have been examined by using different *in vitro* and *in vivo* methods in order to investigate the effect of replacing the antigenic unit HIV-1 gp120 with HIV-1 Gagp24 regarding cellular and humoral immune responses.

5.1 Expression of vaccibody proteins *in vitro*

Before performing *in vivo*-studies it was confirmed that vaccibody proteins were expressed, secreted in expected size and the targeting unit maintained its chemoattractant ability. Analyses by use of different antibodies against targeting or dimerization units of all four vaccibodies in ELISAs, revealed that all vaccibodies were expressed, and they were secreted in different amounts. The ELISA results show that all targeting units and dimerization units of the vaccibodies remain their binding specificity *in vitro* (Figure 4.1) and the difference of targeting units maybe the cause for the differences regarding the amount of secreted vaccibodies.

The secretion levels of vaccibodies were compared by using two antibodies against the dimerization unit in ELISA. The different secretion levels varied as described in Section 4.1 and could be due to different targeting units-caused influence such as structural misfolding and degeneration, post-transcriptional modification, post-translational modification, etc. Previous study shown the glycosylation could occur when human chemokine XCL expressed in insect S2 cells [125].

The western blotting was used to examine the size and whether the homodimers were formed. This was examined in supernatants three days after transfection of HEK293 cells. Two bands in each lane of the gel were observed under non-reducing conditions in all samples and the positions of two bands corresponded to theoretical sizes of homodimer and monomer of each vaccibody. This result indicates that monomers are secreted in much lower magnitude than homodimers

except for α NIP-Gagp24. There are two possible reasons for the existing two forms. The first one is that both homodimers and monomers are secreted. The other possibility is degradation of secreted homodimers occurred in the medium during three days after transfection. Since the stability of homodimeric vaccibody is uncertain, there is no evidence to predict the main possibility for this result. Previous studies with vaccibody has shown that the dimeric fusion formats were more potent than the monomers in chemotactic abilities, induction of antibody responses and T-cell proliferation in mice [108]. However, α NIP-Gagp24 was desired as non-chemotactic, non-targeted control while Mock (saline) is desired as negative control at *in vitro* and *in vivo* experiments. Based on this purpose, the degradation of α NIP-Gagp24 is not crucial for itself as control for evaluation of novel vaccibody, mCCL3-Gagp24.

Based on the comparison of bands in each lane on the gel (section 4.2a), the monomers' amount is low and remain in a small fraction compared to the dimer amount for each vaccibody except α NIP-Gagp24 which remains a big fraction of monomers. Reasons for this could be that the various targeting units differently affect the enzymes which catalyze the formation of disulfide bonds, alternatively, overexpression of α NIP-Gagp24 overload the intracellular enzymes. Finally, it cannot be excluded that the various vaccibodies show different stability after secretion.

Under reducing conditions in western blotting, mXCL1-Gagp24 appeared in two distinct bands. Post-translational modifications of cytokines affect their biochemical and biologic properties [129]. The glycosylation of XCL1 has been reported to significantly increase its biologic activity *in vitro* [125]. Given the result that treatment (Figure 4.2C) with O-glycosidase resulted in bands of lower molecular weight (kDa), it can be concluded that XCL1 in the vaccibody format was at least partly glycosylated.

5.2 Effect of vaccibody on T cell and B cell immune responses in mice

To evaluate the T cell response, the splenocytes were stimulated with a peptide that binds to MHC class I or several pools of 15-mer peptides covering Gagp24 partly with overlapping sequences to each other. Because of the peptide length and loading capacity of MHC peptide-binding groove, the peptides of the pools are expected to bind MHC class II molecules and be indicative of CD4⁺ T cell responses, but CD8⁺ T cell responses cannot be excluded considering cross-presentation. In the present study, both of the vaccines which utilize CCL3 as targeting

unit induced a higher number of Gagp24-reactive T cells against all three pools of peptides and a higher amount of antibodies towards HIV-1 Gagp24 than the non-targeted control. In the meantime, the vaccine utilizing XCL1 shows significant result only against pool 2 peptides and the MHC class I-restricted peptide, suggesting CCL3 can be used as a better immune adjuvant to enhance the potency of DNA vaccine than XCL1. This could be due to several reasons.

First of all, a previous study has shown that the T cell response is improved by codelivering antigen and adjuvant because of antigen processing and presentation are improved by colocalization of antigen and stimulus in the same phagosome [130]. In our approach, one of the reasons that increased T and B cell responses could also be a benefit from our codelivering antigen and adjuvant system which allows simultaneous endocytosis of both immune adjuvant and antigen.

Secondly, the induction of adaptive immune responses is controlled by the innate pattern recognition receptors (PRRs) which sense pathogen-associated molecular patterns (PAMPs) [131]. APCs expressing the relevant PRRs can be directly activated by PAMPs for the induction of adaptive immunity. Alternatively, APCs can be indirectly activated by proinflammatory factors secreted by cells which are sensing the same PAMPs [132], [133]. However, there is increasing evidence that indirectly activated APCs differ qualitatively compared to directly activated APCs and they are able to drive CD4⁺ T cell expansion but result in lacking helper function of T_H1 and T_H2 cells in priming of naïve CD4⁺ cells [134]. Moreover, indirectly activated APCs can promote proliferation of naïve CD8⁺ T cells but fail to support their survival and cytotoxic T cell differentiation compared to directly activated APCs. CD8⁺ T cells primed by indirectly activated APCs also fail to reject tumors [135]. Therefore, the second reason for increased T and B cell immune responses could be the codelivering method in our approach which ensures activated APCs have seen the antigen directly and this direct activation is crucial for efficient CD4⁺ and CD8⁺ T cell priming for induction of adaptive immunity.

Finally, injection of DNA encoding CCL3 into mice muscle was shown to recruit APCs to the injection site [136] and 75% of cellular infiltrate consisted of DCs [137] which are crucial for antigen transportation and T cell response. Previous reports suggested that XCL1 might attract T cells [111], [138], B cells [138], [139], NK cells [140], [141], and neutrophils [139], [142] whereas some reports claim there is no XCL1-mediated attraction of T cells [124], [143], B cells [144], NK cells [145] or neutrophils [124]. In recent studies, it has been indicated that XCL1

specifically attracts CD8⁺ DCs in mouse and CD141⁺ DCs in human but not CD8⁻ DCs, T cells, B cells in mouse or T cells, B cells, NK cells, granulocytes, monocytes, plasmacytoid DCs in human [146]–[148]. Collectively, the CCL3 and XCL1 as targeting unit in our approach could be the third reason that both vaccibodies works efficiently to significantly increased T cell responses against pool 2 peptides.

As a result of binding between CCL3 and its receptor CCR5, a major signal occurs for induction of IL-12 synthesis by the CD8⁺ subset of DC [149]. This pathway is important for production of IFN- γ dependent T cells which is essential for development of Th1 immunity whereas XCL1 do not has such function [149], [150]. And this could be the reason why CCL3 works more efficiently compared to non-targeted control or XCL1 considering we are aiming to increase CD8⁺ T cell responses. The high antibody titers induced by the immunization could be beneficial on rapid formation of a neutralizing response which would contribute to the vaccine-mediated protection. The functional study based on antibody is needed.

5.3 Concluding remarks and future perspectives

The *in vitro* experiments and analysis by ELISA showed different secretion levels of the vaccibodies, therefore, the differences in T and B cell immune responses could be caused by either the targeting units or dose effect. Based on the discussion before, the application of chemokine CCL3 and XCL1 as targeting unit in vaccine construct successfully stimulate immune responses compared to non-targeted control. In our study, the magnitudes of T cells were determined by IFN- γ -measurement, indicating Th1 and CD8⁺ T cell responses. The potency of immune protection due to CD8⁺ T cell-mediated cytotoxicity remain unknown and need to be further analyzed.

The human version of CCL3-Gagp24 is CCL3L1-Gagp24, has been examined in my study for its expression and secretion. For this vaccine construct, further studies are needed to prove its efficacy, for example, investigation for the chemoattractant ability, its efficiency in human cell lines as well as animal studies. Furthermore, given the positive effect of CCL3, it could be interesting to look at the mechanism and effects of CCL3 - how it promotes an immune responses investigated here.

6. References

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