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Department of Genetics and Microbiology

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Thesis to obtain the PhD degree in Microbiology from Universitat Autònoma de Barcelona, 2022

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This work was partially funded by an institutional grant from HIPRA.

The printing of this thesis was made possible with the financial aid of the Universitat Autònoma de Barcelona

Cover design and bookmark illustrations by Óscar Blanch Lombarte, 2022

El **Dr. Julià Blanco Arbués**, investigador principal de la Fundació Institut d'investigació Germans Trias i Pujol, (IGTP) i professor de la Universitat de Vic-Universitat Central de Catalunya, i la **Dra. Carmen Aguilar Gurrieri**, investigadora a l'Institut de Recerca de la SIDA, IrsiCaixa,

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Que el treball experimental i la redacció de la memòria de la Tesi doctoral titulada "Engineered enveloped VLPs with high-density antigen coating. Application to Feline leukemia virus" han estat realitzades per la Raquel Ortiz López sota la seva tutoria i considera que és apta per a ser presentada per a optar al grau de Doctor en Microbiologia per la Universitat Autònoma de Barcelona.

I per a que quedi constància, signa aquest document a Barcelona el 29 de setembre de 2022.

Dra. Nuria Rabella Garcia

A mis padres, a mi familia

Antes no había más que dos caminos para la mujer,

ahora se abre un nuevo camino.

Luisa Carnés, *Tea Rooms: Mujeres obreras*

TABLE OF CONTENTS

ABBREVIATIONS

AAFP	American Associaton of Feline Practitioners
ABCD	Advisory Bureau on Cat Diseases
ADCF	Animal-derived component-free
AIDS	Acquired Immunodeficiency Syndrome
ALV	Avian Leukosis Virus
APOBEC3	Apolipoprotein B editing complex
ART	Antiretroviral Therapy
AZT	Zidovudine
BIV	Bovine Immunodeficiency Virus
BLV	Bovine Leukemia Virus
BSA	Bovine Serum Albumin
CA	Capsid protein
CAEV	Caprine Arthritis Encephalitis Virus
cDNA	Complementary DNA
CO2	Carbon Dioxide
ConA	Concanavalin A
Cryo-EM	Cryogenic Electron Microscopy
CTL	Cytotoxic T cell
DNA	Deoxyribonucleic Acid
EIAV	Equine Infectious Anemia Virus
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immuno Absorbent Spot
EMA	European Medicines Agency
Env	Envelope glycoprotein complex
EV	Extracellular vesicles
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
FeLV	Feline Leukemia Virus
FFV	Feline Foamy Virus
FISS	Feline Injection-Site Sarcoma
FIV	Feline Immunodeficiency Virus
FOCMA	Feline oncornavirus cell membrane antigen
GaLV	Gibbon-ape Leukemia Virus

HAART	Highly active antiretroviral therapy
HBV	Hepatitis B Virus
HFV	Human foamy virus
HHV	Human Herpes Virus
HIV-1	Human Immunodeficiency Virus-1
HIV-2	Human Immunodeficiency Virus-2
HRP	Horseradish Peroxidase
HTLV-1	Human T-Lymphotropic Virus 1
HTLV-2	Human T-Lymphotropic Virus 2
IFA	Immunofluorescence assay
IFN	Interferon
IN	Integrase
ISD	Immunosuppressive domain
KoRV	Koala Retrovirus
LPS	Lipopolysaccharide
LTR	Long Terminal Repeats
MA	Matrix
MLV	Murine Leukemia Virus
MMTV	Murine Mammary Tumor Virus
MPER	Membrane - Proximal External Region
MPLA	Monophosphoryl lipid A
mRNA	messenger RNA
MSD	Membrane spanning domain
MW	Molecular weight
NC	Nucleocapsid
OPD	o-Phenylenediamine Dihydrochloride
ORF	Open Reading Frame
P	Cell lysate
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLWH	People Living With HIV
PMN	Polymorphonuclear
PO	Oral Administration
POC	Point-of-care
PR	Protease
R10	RPMI with 10% FBS

RBD	Receptor-Binding Domain
RER	Rough Endoplasmic Reticulum
REV	Reticuloendotheliosis Virus
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute 1640 medium
RSV	Rous Sarcoma Virus
RT	Reverse Transcriptase
Rtemp	Room Temperature
SARS	Severe Acute Respiratory Syndrome
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SC	Subcutaneous
SERINC 3	Serine incorporator protein 3
SERINC 5	Serine incorporator protein 5
SF	Serum-free
SIV	Simian Immunodeficiency Virus
SIV cpz	Chimpanzee's SIV
SIV sm	Sooty Mangabey's SIV
SN	Supernatant
SOSIP	Soluble stabilized gp40 I559P
SP	Signal Peptide
ssRNA	Single-stranded RNA
STLV-1	Simian T-lymphotropic virus 1
SU	Surface protein
TEM	Transmission Electronic Microscopy
Th	T helper
TLR	Toll-Like Receptor
TM	Transmembrane protein
tRNA	Transfer RNA
UC	Ultracentrifugation
UNAIDS	United Nations Program on AIDS
VLP	Virus-Like Particle
VNA	Virus-neutralizing antibodies
WDSV	Walleye Dermal Sarcoma Virus
WHO	World Health Organization
WOAH	World Organization for Animal Health
x g	Relative Centrifugal Force

SUMMARY

Retroviruses are infectious agents that have been identified in many species, causing diseases of major importance. Such is the case of the Human immunodeficiency virus (HIV-1), which is the causative agent of the acquired immunodeficiency syndrome (AIDS). Unfortunately, despite large efforts, the development of a protective vaccine against HIV has proven elusive for more than 40 years.

Not only humans are the target of this family of viruses, but a wide range of animals can also be infected by them. For instance, cats are infected with different retroviruses, one of the most common pathogens being the Feline leukemia virus (FeLV). Near 60 years after the discovery of FeLV, none of the available commercial vaccines provide full protection from infection.

Therefore, retroviruses can dramatically impact human and animal health, but vaccines have the potential to save millions of lives and change history. Efforts to improve current vaccines and design new strategies are one hot topic in medical research. This work particularly focuses on Virus-like particles (VLPs), a vaccine platform which mimics the structure of a virus, lacking the viral genome and thus, being non-infectious. Recently, our group developed a modified VLP platform based on the HIV structural protein Gag. These VLPs show a high density of immunogen on their surface and induce a potent and functional immune response in mice even in absence of adjuvants and at a low VLP dose.

The objective of the present work is to analyze the versatility of our HIV-based VLP platform and its adaptability to another retrovirus. Specifically, this thesis focuses on FeLV. First, we produced and optimized FeLV-Gag based VLPs. Then, we loaded on the surface of FeLV-based VLPs different antigens derived from the viral Envelope glycoprotein. Finally, immunogenicity was tested in two different murine animal models, in which we analyzed different delivery systems (purified VLPs or nucleic acid) and studied the impact of adjuvants in the purified VLPs delivery system.

Altogether, this thesis not only confirms the versatility of the HIV-1 Gag-based VLP vaccine platform but also presents retroviral VLPs as an excellent alternative to more conventional vaccines. Moreover, this work sheds light on FeLV vaccine research, considering that FeLV is not only of interest to veterinary practice but also could be a relevant model to help understand HIV-1 immunology.

RESUMEN

Los retrovirus son agentes infecciosos que han sido identificados en diferentes especies, causando enfermedades de gran importancia. Un ejemplo destacado es el Virus de la inmunodeficiencia humana (VIH-1), el cual es el agente causante del síndrome de inmunodeficiencia adquirida (SIDA). Desafortunadamente, y a pesar de los esfuerzos durante los últimos 40 años, aún no se ha tenido éxito en el desarrollo de una vacuna protectora contra el VIH-1.

Los retrovirus no infectan solamente a humanos, sino que infectan un amplio rango de animales. Los gatos, por ejemplo, pueden ser infectados con distintos retrovirus, siendo el Virus de la leucemia felina (VLFe) uno de los patógenos más comunes. Parecido a lo que ocurre con HIV-1, cerca de 60 años después del descubrimiento del VLFe, no existen todavía vacunas comerciales que ofrezcan una protección completa frente a la infección.

Así, los retrovirus pueden tener un impacto dramático en la vida humana y animal y, en este sentido, las vacunas tienen el potencial de salvar millones de vidas y cambiar la historia. La investigación médica reciente ha dedicado grandes esfuerzos a la mejora de las vacunas existentes y el diseño de nuevas estrategias. En este trabajo nos centramos especialmente en las partículas similares a virus (VLPs, por las siglas en ingles de *Virus-Like Particles*), un tipo de vacuna que simula la estructura del virus, desprovisto sin embargo del genoma viral, siendo de esta forma no infeccioso. Recientemente, nuestro grupo de investigación ha desarrollado una plataforma de VLPs basadas en la proteína estructural del VIH-1, Gag. Estas VLPs muestran una gran densidad de inmunógeno en su superficie, que induce una respuesta inmune potente y funcional, incluso en ausencia de adyuvante y con una dosis baja de inmunógeno administrada.

El objetivo de este trabajo es analizar la versatilidad de nuestra plataforma de VLPs basada en VIH-1 y la posibilidad de que ésta pudiera ser aplicada a otros retrovirus. En particular, esta tesis se centra en el VLFe. En primer lugar, producimos y optimizamos las VLPs basadas en el VLFe. Después, añadimos en la superficie de las VLPs distintos antígenos derivados de la glicoproteína de envuelta del mismo virus. Y finalmente, estudiamos la inmunogenicidad en dos modelos murinos, analizamos distintos sistemas de administración (inmunización con VLPs purificadas o con ácidos nucleicos) y observamos el impacto de adyuvantes en la respuesta inmunológica.

En conclusión, esta tesis no solo confirma la versatilidad de nuestra plataforma de VLPs basadas en Gag de VIH-1, sino que presenta las VLPs basadas en retrovirus como una excelente alternativa a otras vacunas más convencionales. Además, intenta arrojar algo

de luz en la investigación sobre la vacuna del VLFe, considerándola no solo de interés veterinario, sino entendiéndola como un modelo relevante para ayudar a comprender la inmunología del VIH-1.

RESUM

Els retrovirus són agents infecciosos que han estat identificats en diverses espècies, causant malalties de gran importància. Un exemple destacat és el Virus de la immunodeficiència humana (VIH-1), que és l'agent causant de la síndrome d'immunodeficiència adquirida (SIDA). Malauradament i malgrat els esforços dels darrers 40 anys, encara no s'ha aconseguit desenvolupar amb èxit una vacuna protectora contra el VIH-1.

Els retrovirus no afecten únicament als humans, sinó que poden infectar també un ampli ventall d'animals. Els gats, per exemple, poden ser infectats amb diversos retrovirus, sent el Virus de la leucèmia felina (VLFe) un dels patògens més comuns. Similar al que ocorre amb VIH-1, 60 anys després del descobriment del VLFe, no existeixen encara vacunes comercials que ofereixin una protecció completa contra la infecció.

Així, els retrovirus poden afectar dramàticament la vida humana i animal i, en aquest sentit, les vacunes tenen el potencial de salvar milions de vides i canviar la història. La recent recerca mèdica ha dedicat grans esforços a la millora de les vacunes existents i el disseny de noves estratègies. En aquest treball ens centrem especialment en les partícules similivíriques (VLPs, per les sigles en anglès de *Virus-Like Particles*), un tipus de vacuna que simula l'estructura del virus però que no conté el seu material genòmic, convertint-se d'aquesta manera en no infecciós. Recentment, el nostre grup ha desenvolupat una generació de VLPs basades en la proteïna estructural de VIH-1, Gag. Aquestes VLPs mostren una gran densitat d'immunogen en la seva superfície, cosa indueix una resposta immune potent i funcional, fins i tot en absència d'adjuvant i amb una dosi baixa d'immunogen administrada.

L'objectiu d'aquest treball és analitzar la versatilitat de la nostra plataforma de VLPs basada en VIH-1 i la possibilitat d'aplicar-se a d'altres retrovirus. En particular, aquesta tesi es centra en el VLFe. En primer lloc, vam produir i optimitzar les VLPs basades en el VLFe. Després, vam afegir a la superfície de les VLPs diversos antígens derivats de la glicoproteïna de l'envolta. I finalment, vam estudiar la immunogenicitat en dos models murins, vam analitzar diversos sistemes d'administració (immunització amb VLPs purificades o amb àcids nucleics) i vam observar l'impacte dels adjuvants en la resposta immunològica.

En conclusió, aquesta tesi no només confirma la versatilitat de la nostra plataforma de VLPs basades en Gag de VIH-1, sinó que presenta les VLPs basades en retrovirus com una excel·lent alternativa a altres formulacions més convencionals. A més, posa el focus

en la vacuna del VLFe, considerant-la d'interès no estrictament veterinari, sinó entenent-la com un model rellevant per ajudar a comprendre la immunologia del VIH-1.

INTRODUCTION

Vaccines have the power to change society. The recent SARS-CoV-2 pandemic reinforces the importance of vaccination programs. Since the first successful vaccine against smallpox by Edward Jenner in 1796 (1), vaccines have become our principal weapon in the battle against infectious diseases (e.g., polio or hepatitis). Their ability to prevent disease and protect the most vulnerable segments of the population (i.e., infants, and older adults) greatly contributed to decrease mortality and to increase life expectancy worldwide in the past century (2,3).

Despite the great achievements in vaccinology during the 20th century, there are still some devastating epidemics such as malaria, tuberculosis, and AIDS for which attempts at generating effective vaccines remain elusive (4). Vaccines are necessary not only for current but also for future pandemics. More than 60% of human pathogens are zoonotic in origin. This includes a wide variety of bacteria, fungi, protozoa, parasites, and viruses (5). Factors such as climate change, urbanization, animal migration and trade, travel and tourism, and natural factors have influenced the emergence and re-emergence of zoonoses (6). Therefore, we need to consider the complete ecosystem to prevent future epidemics in humans.

In 2008, the World Health Organization (WHO), the World Organization for Animal Health (WOAH), and the Food and Agricultural Organization (FAO) of the United Nations launched an initiative called “One World, One Health,” in which the term “One Health” was a concept highlighting the importance of the interface between public health, veterinary medicine and ecology for improving not only human and animal health, but also environmental health and food safety worldwide (7,8). In 1858, Doctor Rudolf Virchow, known as “the father of modern pathology”, stated: “Between animal and human medicine there are no dividing lines—nor should there be. The object is different, but the experience obtained constitutes the basis of all medicine” (9).

1. Retroviruses

Retroviruses are infectious agents that have been identified in many species, causing diseases of major importance. The first discovered retrovirus was the Equine infectious anemia virus (EIAV) by Doctors Carré and Vallée in 1904 (10,11). Since then,

retroviruses were abundantly identified in many other animals, such as rodents, birds, and primates (12). The first human retrovirus, Human T-lymphotropic virus (HTLV-1), was discovered in 1977 by Doctor Gallo (13,14). And only a few years later, in 1983, Human immunodeficiency virus (HIV) was identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS) by Doctors Barré-Sinoussi and Montagnier (15). Since then, retroviruses have been at the center of biomedical research. They have played a significant role in the advance, not only of modern virology, but also of molecular biology, biotechnology, genetics, and molecular medicine. Some of the major events in the retrovirology field, including the first virus discovered and strategies to fight against them (e.g., therapies, restriction factors), are summarized in Figure 1.

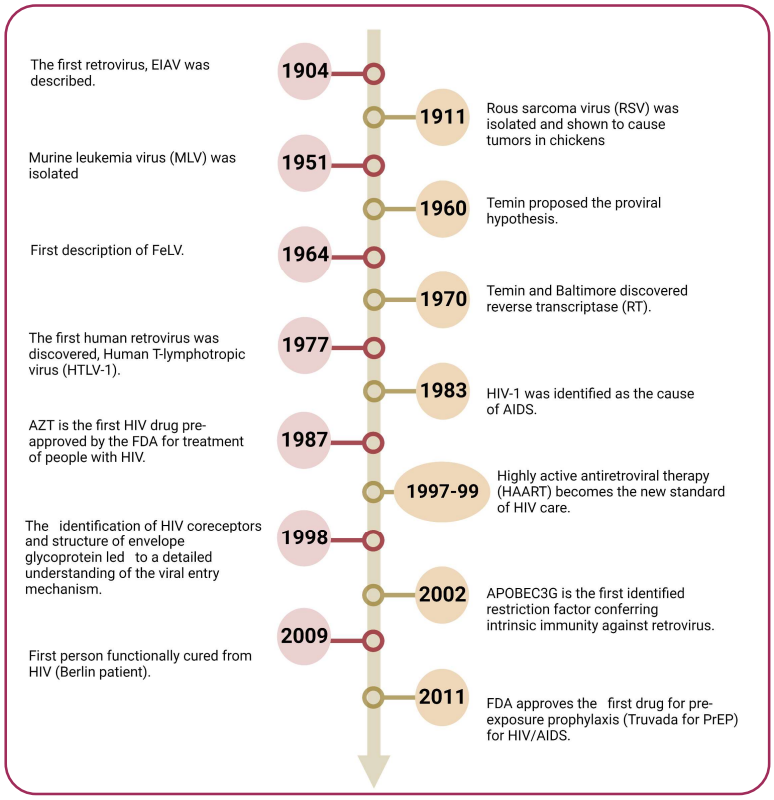


Figure 1. Key events in the history of retroviral research. Adapted from (16).

The main characteristic of retroviruses is their RNA genome; however, they need to produce a DNA copy of it to express their genes in the infected cell and ultimately replicate their RNA (17). This dual genetic system employed by retroviruses allows transmission of their genetic information from cell to cell as packaged RNA while simultaneously leaving a DNA copy integrated into the chromosomes of each infected cell (18). To allow for such a complex replication cycle, the retroviral genome encodes for a particular enzyme called reverse transcriptase (RT), which is responsible for the reverse transcription of genomic RNA to DNA (19). The identification of the RT by Doctors Temin and Baltimore in 1970 transformed the prevailing concepts of the

transmission of genetic information (20). Before that discovery, it was generally assumed that the flow of genetic information from DNA to RNA was irreversible.

1.1 Classification

Retroviruses belong to the *Retroviridae* family and are classified based on their polymerase sequence phylogeny and molecular biology into two subfamilies: orthoretroviruses (*orthoretrovirinae*) and spumaviruses (*spumaretrovirinae*). Each subfamily is subclassified in genera (Figure 2) (21).

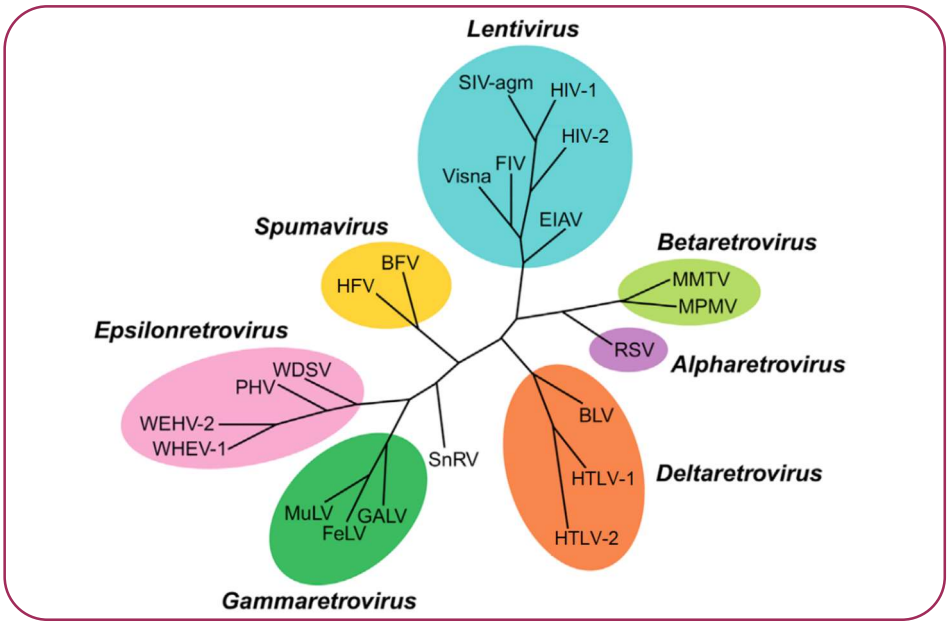


Figure 2. Overview of the phylogeny of the *Retroviridae* family, classification based on conserved regions in the polymerase gene of the virus, including Human immunodeficiency viruses 1 and 2 (HIV-1, HIV-2), Human T-lymphotropic viruses 1 and 2 (HTLV-1, HTLV-2), and Human foamy virus (HFV). Adapted from (20).

Five out of six orthoretroviruses genera (*Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus, and Epsilonretrovirus*) are associated with the development of cancers (22), whereas, *Lentivirus* are associated with the development of immunodeficiency and neurologic symptoms (23). In contrast, the spumaviruses subfamily contains a single genus and is hypothesized not to cause any serious disease (24). Therefore, retroviruses are currently classified into seven genera, and only three of them include human and feline pathogens (Table 1).

Subfamily Orthoretrovirinae			
Genus	**Representative species**	**Host species**	**Diseases**
Alpharetrovirus	Avian leukosis virus (ALV)	Chicken	B-lymphoma
	Rous sarcoma virus (RSV)		Sarcoma
Betaretrovirus	Murine mammary tumor virus (MMTV)	Mouse	Breast cancer
Gammaretrovirus	Murine leukemia virus (MLV)	Mouse	Immunosuppression, lymphomas, leukemia
	Feline leukemia virus (FeLV)	Cat	
	Gibbon-ape leukemia virus (GaLV)	Gibbon	
	Reticuloendotheliosis virus (REV)	Chicken	
	Koala retrovirus (KoRV)	Koala	
Deltaretrovirus	Bovine leukemia virus (BLV)	Cow	B-cell leukemia
	Human T-lymphotropic virus 1 and 2 (HTLV-I and -II)	Human	T-cell leukemia
	Simian T-lymphotropic virus 1 and 2 (STLV-I and -II)	Monkeys	
Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV)	Walleye pike	Dermal sarcoma
Lentivirus	Human immunodeficiency virus 1 and 2 (HIV-1 and -2)	Human	Immunodeficiency, neurologic disease
	Simian immunodeficiency virus (SIV)	Monkeys	Immunodeficiency

	Feline immunodeficiency virus (FIV)	Cat	
	Equine infectious anemia (EAIV)	Horse	Anemia
	Bovine immunodeficiency virus (BIV)	Cow	Immunodeficiency
	Caprine arthritis encephalitis virus (CAEV)	Goat	Arthritis encephalitis
Subfamily Spumaretrovirinae			
Spumavirus	Simian foamy virus	Nonhuman primates	
	Foamy virus of non-primates	Cow, horse, cat	

Table 1. Members of the *Retroviridae* family. Adapted from (23).

1.2 Structural organization of retroviruses

The genome of retroviruses contains three essential genes, reading from the 5′ end: *gag*, *pol,* and *env* genes. Each gene codes for different polypeptides with structural, enzymatic, and tropic functions respectively (Table 2).

Polyprotein	Protein	Abbreviation
Gag	Matrix	MA
	Capsid	CA
	Nucleocapsid	NC
Pol	Protease	PR
	Reverse transcriptase	RT
	Integrase	IN
Env	Surface protein	SU
	Transmembrane protein	TM

Table 2. Retroviral proteins. Adapted from (18,20).

1.2.1 Genome Structure

Retroviruses are enveloped viruses with two single-stranded positive-sense RNAs (ssRNA) as their genetic material (25). The two copies of RNA genomes are attached to each other near the 5' end and are packaged inside the capsid (26). The ORFs of the polyproteins, *gag*, *pol*, and *env*, lie in the middle of the RNA genome (Figure 3). Moreover, the 5′ and 3′ ends of the viral RNA contain sequences identified as R/U5 (5′ end) and U3/R (3′ end). After reverse transcription, the linear double-stranded DNA includes identical long terminal repeats (LTRs) at each end, containing the U3, R, and U5 regions in that order (Figure 3). LTRs play an essential role in transcription, integration, and regulation of expression of the integrated complementary DNA (cDNA) provirus (23).

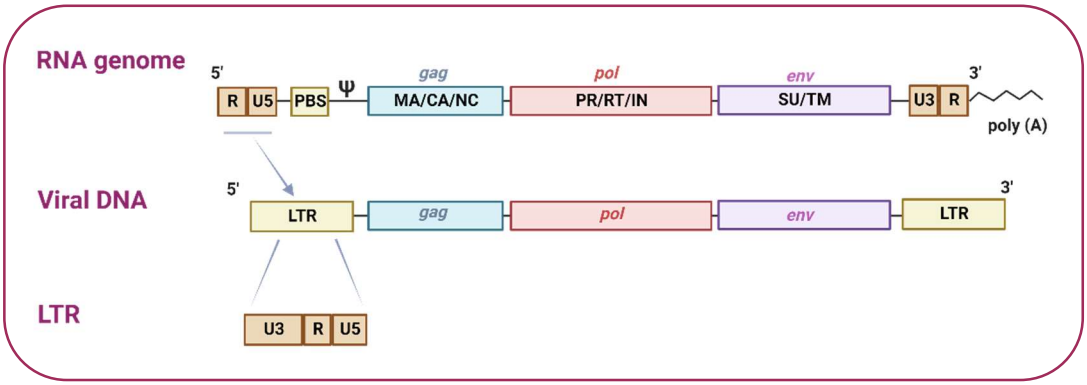


Figure 3. The retroviral genome is a single-strand RNA of 8~10 kb length, containing *gag*, *pol*, and *env* genes. Three elements in the 5' noncoding region (R, U5, and PBS), and two elements in the 3' noncoding region (U3, and R) are denoted. A specific packaging signal (ψ) is indicated. Image created with Biorender.

Additionally, the primer binding site (PBS), which is the binding site for tRNA, is located downstream of the U5 element. In fact, tRNA molecule is utilized as an RNA primer for viral reverse transcription (20). The isoforms of tRNA used as primers are different among retroviruses: a proline-tRNA is used for MLV, whereas a lysine-tRNA is used for HIV. Also, at the 3' end of the RNA genome, a poly (A) tail is located (16).

The *gag* gene encodes a Gag polyprotein which has structural functions and is processed in three viral proteins i) matrix (MA, lying under the envelope, ~10–15 kDa), ii) capsid (CA, the structural protein of the viral capsid particle, ~25–30 kDa), and iii) nucleocapsid protein (NC, which binds and compacts viral RNA, ~10 kDa) (23). The *pol* gene encodes the viral enzymes: protease (PR; ~10–12 kDa), reverse transcriptase (RT, ~80–120 kDa), and integrase (IN, ~30–40 kDa) which are translated as a single polyprotein. Similarly, to Gag, the Pol polyprotein undergoes proteolytic cleavage during

maturation to give the final proteins in the virus particles. (23). The *env* gene encodes the external viral polyprotein, after its cleavage usually carried out by the cellular protease furin during passage through the rough endoplasmic reticulum (RER), two subunits are generated: surface (SU, ~ 70–120 kDa) at the amino terminus, and transmembrane (TM, ~ 15–45 kDa) at the carboxy terminus (23).

According to the complexity of their genome, retroviruses are also classified as simple or complex, e.g., FeLV is a simple retrovirus, on the contrary, HIV-1 is considered a complex retrovirus (27). Complex retroviruses contain a variety of additional regulatory genes coding for accessory proteins involved in the regulation of viral propagation and pathogenesis. For example, HIV-1 encodes six accessory proteins in addition to the three polyproteins: Tat, Rev, Nef, Vif, Vpu, and Vpr (28).

1.2.2 Virion Structure

Retroviruses are generally spherical enveloped particles with an average diameter ranging between 100 to 200 nm (21,29). The viral envelope consists of a membrane bilayer coming from infected cells and containing host-cell proteins and the viral envelope glycoprotein complex (Env). This complex structure encompasses two predominantly N-linked glycosylated proteins: SU and TM (30). SU is exposed on the surface of the viral particle and stays anchored to it by strong interaction (covalent or not) with the membrane-anchored TM protein (Figure 4). SU contains the receptor-binding domain (RBD), which is responsible for the specific attachment of the virus to a susceptible cell at the beginning of the infection cycle (16). TM spans the lipid bilayer, and its external domain (ectodomain) contains sequences involved in the formation of envelope multimers (trimers), as well as sequences that mediate the fusion of viral and cellular membranes during infection (18). The C-terminus of TM contains a short stretch of a polypeptide that is on the interior of the viral envelope (or in the cytoplasm of the infected cell). These residues may interact with viral structural Gag protein to localize Gag to areas of the infected cell membrane where virus particle formation occurs (23).

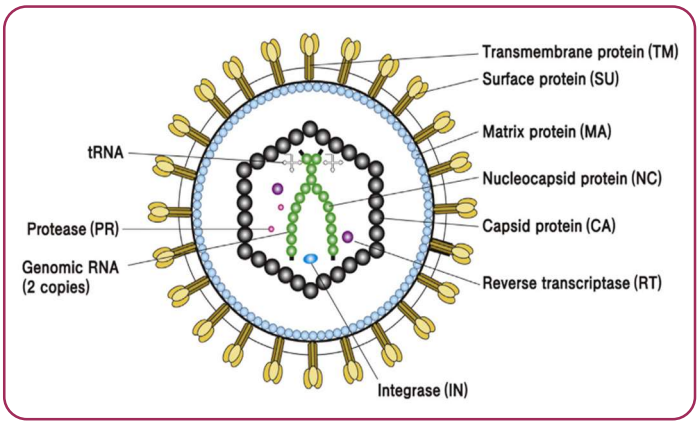


Figure 4. Virion structure of a retrovirus. Two envelope glycoproteins (SU and TM, in yellow) are found in the viral envelope. Capsid (CA, in black) is found inside the viral envelope. Two genomic RNA copies encapsulated by nucleocapsid protein (in green) are found inside the capsid (in black). Two tRNA molecules attached to the viral RNAs are indicated. Three viral enzymes (RT, PR, and IN) are denoted (in pink, purple and blue). Adapted from (18).

The first component found inside the virion is the matrix (Figure 4), which plays a key role in virus assembly by directing the intracellular transport and membrane association of the Gag polyprotein. It is also critical for the incorporation of viral Env proteins into mature virions (31,32). Then, the capsid is found in the mature viral particle and is believed to form a shell surrounding the ribonucleoprotein complex that contains the genomic RNA (Figure 4) (33). Finally, the nucleocapsid promotes the annealing of complementary RNA sequences (34) and stimulates the reverse transcription and the formation of dimeric RNA (33). In a retroviral particle, there are approximately 1000 molecules of NC covering the entire genome (35). Moreover, three enzymes RT, IN, and PR are found in a mature virion (Figure 4).

Each retrovirus has evolved to recognize a specific cell surface molecule in the host cell. This molecule is noted as the viral receptor and defines the tropism of infection to specific cells and/or tissues (36).

1.3 Retroviral replication

The replication cycle of retroviruses comprises four distinct stages: i) Binding and entry, ii) reverse transcription and integration, iii) viral gene expression, and iv) virus assembly, budding, and maturation (Figure 5) (16,37).

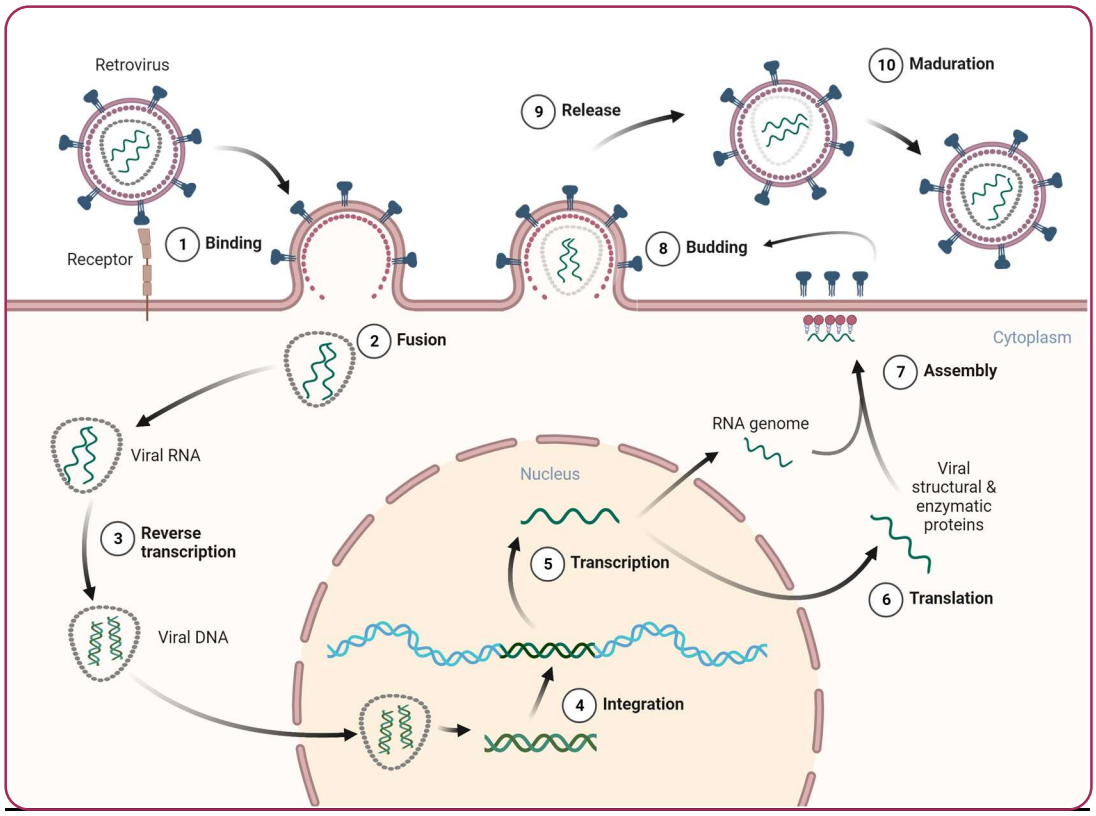


Figure 5. The retrovirus replication cycle. Infection begins at the upper left, with the binding of a virion to its receptor on the host cell surface. It culminates with the release of immature virions. Subsequent maturation involves cleavage of viral polyproteins by viral protease. Image created with Biorender.

Binding and entry

Retroviral entry into the host cells is initiated through the binding of the viral envelope (SU protein) to a specific receptor molecule on the cell's outer membrane (Figure 5). Among different retroviruses, entry occurs either by direct fusion with the plasma membrane or by receptor-mediated endocytosis and subsequent fusion (38). In general, binding to the receptor alters the conformation of the SU domain exposing the TM protein, which carries a hydrophobic fusion peptide sequence (38). The fusion peptide is inserted into the target cell membrane and TM protein mediates the fusion of the cell and viral membranes, permitting the release of the capsid core containing the viral RNA and diverse proteins, including the RT, into the cytoplasm of the infected cell (Figure 5) [23].

Taking HIV-1 as an example, the main viral receptor, CD4, is predominantly expressed on T-helper lymphocytes, macrophages, and dendritic cells; thus, these cells are the primary targets for infection *in vivo* [28]. In addition, in HIV-1 and related simian lentiviruses, successful infection requires interaction with both CD4 and a co-receptor, the chemokine receptors CCR5 or CXCR4 (39).

Reverse Transcription and Integration

Immediately after entry into the cytoplasm of a susceptible cell, the viral RNA is reverse transcribed (Figure 5). Using the genome-associated tRNA as a primer, the reverse transcriptase makes a single-stranded DNA complementary to the infecting RNA and then uses that DNA as template to synthesize a DNA complementary sequences, 'reverse transcribing' the information contained in the viral RNA into a duplex DNA (38). During the reaction, LTRs are created, being the final product a linear duplex DNA with duplicate LTR regions (37). The viral RNA template is degraded during reverse transcription by RNase H enzymatic activity intrinsic to RT (40). Over the past decade, the understanding of capsid function underwent a major paradigm shift. It has been proposed that the capsid provides a close environment for the reverse transcription and protects the cDNA, furthermore, acts as a delivery vehicle towards and through the nuclear pore and even within the nucleus (26).

Once in the nucleus, the cDNA is integrated into the DNA genome (provirus) of the host cell, catalyzed by the viral protein integrase (Figure 5) (37). Insertion usually occurs randomly into actively transcribed regions of the host genome. However, different retroviruses manifest subtle different site preferences, which are mediated by interactions of their IN protein with host chromatin-binding proteins (16).

Viral Gene Expression

Once integrated into the host DNA, the cellular RNA polymerase II transcribes the viral DNA producing new copies of the viral genome and the mRNAs that encode the viral proteins (Figure 5). The 5′ LTR contains the proviral transcription start site and control elements that regulate transcription initiation by cellular RNA polymerase II (41). The 3′ LTR contains a signal that determines the 3′ end of the transcript and its polyadenylation (16). Newly transcribed viral RNA undergoes different levels of splicing in the nucleus before being exported to the cytoplasm (37).

Once in the cytoplasm, the viral mRNAs are translated by host cell ribosomes and translation factors. Simple retroviruses produce only two mRNAs: the full-length viral RNA which acts as the mRNA for gag and gag-pol and the subgenomic mRNA that encodes the env gene (20). In contrast, complex retroviruses transcribe more than two mRNA, differences depending on the virus. Following the HIV-1 example, it has nine genes and produces over 30 different mRNA transcripts by splicing (42).

Virus Assembly and Maturation

The later stages of the life cycle involve the synthesis of the virion proteins and their assembly into viral particles containing two copies of the viral RNA. The virion RNA

contains a specific packaging signal (ψ) located near its 5′ end that is required for the efficient incorporation of RNA into viral particles (16).

Assembly of an infectious virion particle is required for the efficient transfer of the retroviral RNA genome from cell to cell. Particle formation is controlled by the self-assembly of the Gag polyprotein which first assembles into spherical immature particles (43). As the immature virion buds, the viral protease is responsible for post-translational cleavage of the Gag polyprotein to yield the matrix, capsid, and nucleocapsid proteins, and for cleavage of the gag-pol polyprotein to yield the viral enzymes PR, RT and IN (18,20,37,43).

1.4 Retroviruses and Disease

Retroviruses are associated with a wide variety of diseases including an array of malignancies, neurologic disorders, and immunodeficiencies. Syndromes as seemingly diverse as arthritis, osteopetrosis, and anemia can all result from retroviral infection (44). These disorders affect not only humans but also animals and have a relevant impact on global health.

Retrovirus and Cancer

The ability of some retroviruses to induce tumors has been known since 1911 when Doctor Rous discovered an avian virus that induced tumors in chickens, later called Rous sarcoma virus (RSV) (45,46). The list of animals affected by oncogenic retroviruses expanded during the last century to include cats, cows, rats, sheep, goats, koalas, several primates, and some fish (47). Moreover, in 1979 the discovery of HTLV-1 marked that retroviruses could also cause malignant diseases in humans (13,22).

Retroviruses can cause cancer by a wide variety of mechanisms; they are capable to immortalize or destroy the cells. One consequence of progressive cell destruction is immunodeficiencies. A common pathway to cause tumors is the activation of oncogenes by random proviral insertion into regions of actively transcribed chromatin. When a retrovirus integrates near a cellular protooncogene, the viral LTR can act as a dominant control element that stimulates the transcription of the oncogene (16,48).

The proviral DNA can also recombine with cellular genes that are adjacent to its site of integration, giving rise to defective viruses that are capable of transducing fragments of cellular genes (49). Many highly oncogenic viruses are defective viruses that carry oncogenes initially acquired by such nonhomologous recombination events (23).

Neurological diseases

Several retroviruses induce neurologic symptoms such as dementias and neuropathies. HIV-infected individuals frequently develop such symptoms in the central nervous system and in peripheral nerves (50). Some strains of Murine leukemia virus (MLV) also induce neurologic symptoms such as hind limb paralysis (51). Neither HIV nor MLV infects neurons, so the pathogenesis likely results indirectly from the infection of other supporting cells as microglial cells (23).

Immunodeficiencies

Several retroviruses can induce immunodeficiency, most notably the lentiviruses HIV and related Simian immunodeficiency viruses (SIVs), and Feline immunodeficiency virus (FIV).

HIV is classified as a member of the Lentivirus genus, *Retroviridae* family, and, *Orthoretrovirinae* subfamily (52). On basis of the organization of the HIV genome, HIV is classified into type 1 (HIV-1) and type 2 (HIV-2) (53). While HIV-1 is responsible for the worldwide spread of the infection, HIV-2 is restricted to Western and Central Africa (53,54). Phylogenetic data support the hypothesis that zoonotic transmission occurred at the beginning of the 20th century, with simian immunodeficiency viruses transmitted from Central African chimpanzees (*Pan troglodytes*, SIVcpz) and West African sooty mangabeys (*Cercocebus atys,* SIVsm) to humans being the origins of HIV-1 and HIV-2, respectively (53,55–57).

Since the discovery of HIV as the causative agent of AIDS, the United Nations Program on HIV/AIDS (UNAIDS) has reported more than 75 million people infected with HIV and more than 33 million AIDS-related deaths since the beginning of the pandemic (58). In addition, the percentage of people worldwide living with HIV has risen to 38.4 million in 2021. In Spain, about 150,000 people have been estimated by UNAIDS to be living with HIV (58).

A hallmark of HIV infection is the progressive destruction of CD4+ T lymphocytes in peripheral blood and tissues, resulting in immunodeficiency. As a result, opportunistic infection such as pneumonia and viral herpes occurs. In particular, KS herpesvirus (HHV-8) causes Kaposi's sarcoma in AIDS patients (59).

The resulting AIDS epidemic around the world has led to intensive research on the interactions between viruses and cells of the immune system. The last decades have seen the widespread introduction of combined antiviral therapy (ART), contributing to better viral control in people living with HIV, which resulted in less mortality, fewer escape variants, and a reduction in secondary effects (60–62), making HIV-1 infection a chronic

infection but not a deadly one. Furthermore, antiviral therapy substantially reduces sexual transmission, and when given either during pregnancy or to neonates can greatly reduce vertical transmission, thereby offering a practical measure to reduce the future burden of infection (63). Although ART can achieve control of viral load to an undetectable level, it fails to thoroughly clear HIV-1 because ART only acts upon actively replicating viruses rather than the latent reservoirs (64) and it may lose efficacy due to HIV-1 resistance (65). Moreover, limited access to therapy, especially for those who are in high-risk populations, and unknown HIV-positive status are major hurdles to be addressed (66).

Thus far, HIV stands as an infectious agent that has no preventive vaccine and no functional cure. Attempts to develop a vaccine have proved disappointing despite enormous efforts.

2. FeLV and other feline retroviruses

Domestic and non-domestic cats are afflicted with multiple viruses that serve as powerful models for human diseases including cancer, SARS, and HIV/AIDS (67). Feline viruses that cause these diseases have been studied for decades revealing detailed insight concerning transmission, virulence, origins, and pathogenesis.

Cats are susceptible to natural infection by at least three members of the *Retroviridae* family: *Lentivirus*, *Spumavirus*, and *Gammaretrovirus* (68–70).

2.1 Feline lentivirus (*Orthoretrovirinae* subfamily)

Feline immunodeficiency virus (FIV)

Feline immunodeficiency virus (FIV) causes an acquired immunodeficiency syndrome (AIDS) in domestic and non-domestic cats worldwide (71,72). FIV was first reported in 1987 as a cat lentivirus with structural, genomic, and pathogenic parallels to HIV (73–75). Tumors, neurological diseases, and opportunistic infections due to immunosuppression are all recognized presentations of the disease (76). However, while often compared with HIV infection, the degree of immunosuppression in infected cats is typically lower and survival time does not appear to differ between infected (seropositive) and uninfected cats (77). With proper care, FIV-infected cats die at an old age from causes unrelated to their FIV infection (78).

FIV exists in at least five subtypes or clades, A to E, which are defined based on their *env* sequence; there may be up to 30% divergence between members of different clades (79). Similar to HIV-1, each clade is predominant in a specific geographic region in the

world; for example, clade A viruses are common in northern Europe and the western United States, whereas clade B viruses predominate in southern Europe and the eastern United States (80).

FIV, like HIV-1, requires primary and secondary receptors. Its primary receptor is CD134, expressed on feline CD4+ T lymphocytes, B lymphocytes, and activated macrophages (81,82). And its secondary receptor CXCR4, a chemokine receptor, is analogous to that used by HIV; this receptor alone is sufficient for infection with some laboratory isolates of FIV (83).

Even if one vaccine is commercially available (Fel-O-Vax FIV), vaccination against FIV is controversial for several reasons. Vaccination provides only partial protection from infection, interferes with diagnostic tests, and, equally important, increases the risk of sarcoma formation associated with adjuvanted vaccines (84–86). Therefore, FIV vaccine should only be used in high-risk cats.

2.2 Spumavirus (*Spumaretrovirinae* subfamily)

Feline foamy virus (FFV)

Feline foamy virus (FFV), although transmissible, is considered nonpathogenic (87–89). A recent study shows no association between the presence of antibodies to the virus and clinical disease (90). The first isolate of FFV was made in 1969 from a tumor-bearing cat (91). As a unique feature of FFVs, Gag is not sufficient for sub-viral particle release (92,93).

2.3 Feline gammaretrovirus (*Orthoretrovirinae* subfamily)

Feline leukemia virus (FeLV)

FeLV is an enveloped RNA virus that belongs to the genus *Gammaretrovirus* first described in 1964 at the University of Glasglow (94). It was discovered in a household cluster of cats with lymphosarcoma and was first isolated and purified from the plasma of cats in 1967 (95). Following other examples of viral cross-species transmission, it is hypothesized that FeLV probably evolved from a murine or rat leukemia virus and may have crossed species barriers when cats preyed on rodents (96).

In the clinics, the differentiation between the clinical signs of FeLV and FIV infections is rarely possible, as they cause a spectrum of diseases with a certain degree of overlap. Despite these similarities, the nature of infection with each virus and the subsequent immune response and pathogenesis are quite different (83). Also, recovery from FIV infection has never been documented, in contrast, cats exposed to FeLV can clear the

infection. Despite that, FeLV infection can progress more rapidly than FIV infection and is more pathogenic, therefore cats that develop progressive infections ultimately die of FeLV-related disease (97).

Even though FIV and FeLV are known to be common in domestic cats (*Felis catus*) other studies suggest that these viruses can be found in other felines, e.g in kodkod (*Leopardus guigna*), a small south American feline (98), and Iberian lynx (*Lynx pardinus*) (99). FeLV is the main retrovirus studied in this thesis, therefore, a detailed explanation follows in the next section.

Feline sarcoma virus (FeSV)

FeSV was first isolated in 1969 (100). FeSV is a recombinant virus that develops *de novo* in FeLV-A-infected cats by recombination of the FeLV-A genome with cellular oncogenes. Through a process of genetic recombination, FeSV acquires one of several oncogenes, such as *fes, fms,* or *fgr* (101). As a result, FeSV is an acutely transforming (tumor-causing) virus, leading to a polyclonal malignancy with multifocal tumors arising simultaneously after a short incubation period (102). With the decrease in FeLV prevalence, FeSV also has become less common (78). FeSV-induced fibrosarcomas are multicentric and usually occur in young cats (78). Strains of FeSV identified from naturally occurring tumors are defective and unable to replicate without the presence of FeLV-A as a helper virus that supplies proteins (such as those coded by the *env* gene) to FeSV (69).

RD-114

RD-114 virus is a feline endogenous retrovirus (ERV) isolated from human rhabdomyosarcoma in 1971 and classified as endogenous gammaretrovirus in domestic cats (103–105). To date, there is no evidence that RD-114 virus causes any disease in cats, or that RD-114 sequences recombine with exogenous feline retroviruses (30).

To sum up, feline retroviruses are perhaps the most relevant cause of infectious diseases in domestic cats. While FFV is thought to be of minimal clinical significance, both FIV and FeLV result in a variety of immunologic perturbations that impact morbidity and mortality.

2.4 Feline Leukemia Virus (FeLV)

FeLV is a gammaretrovirus of domestic cats, it can be exogenous (foreign, “pathogenic”) or endogenous (inherited, “non-pathogenic”) (106). The endogenous retroviruses (enFeLVs) are integrated into the genome of all domestic cats, in all tissues. EnFeLVs might increase the pathogenicity of exogenous FeLV, for example, by recombining with

exogenous FeLV (FeLV-A) and subsequent development of other more pathogenic FeLV subgroups (107–109).

2.4.1 Virology

Feline leukemia virus has a classical retroviral structure, consisting of an enveloped particle with a diameter of approximately 105-125 nm within which the capsid containing the genetic material (RNA) and viral enzymes are centrally located (110). The envelope glycoprotein spikes, which are composed of transmembrane (p15E) and surface glycoproteins (gp70), are important for the binding of the virus to cell surface receptors, determining cell tropism and represent an important target for the host immune response (111) (Figure 6).

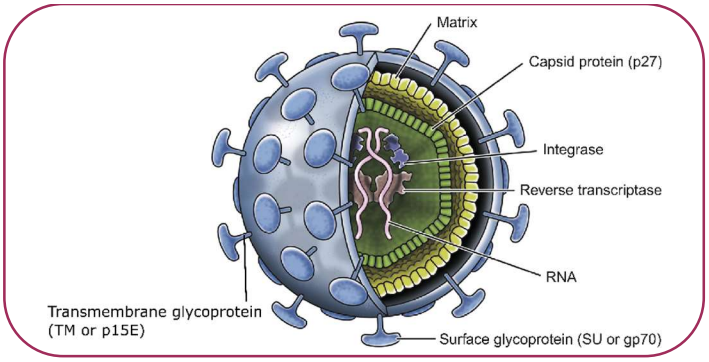


Figure 6. Structure of feline leukemia virus. The virion contains two identical strands of RNA and associated enzymes, which include reverse transcriptase, integrase, and protease, packaged into a capsid composed of p27 protein, with a surrounding matrix, all enclosed by a phospholipid membrane envelope derived from the host cell. The envelope contains a gp70 glycoprotein and the transmembrane protein p15E. Adapted from (112).

The genome of FeLV is organized as a simple retrovirus, where the essential three genes: *gag*, *pol*, and *env* are present in a single viral RNA genome (30). They encode the viral structural proteins, replicative enzymes, and envelope proteins, respectively (Figure 7).

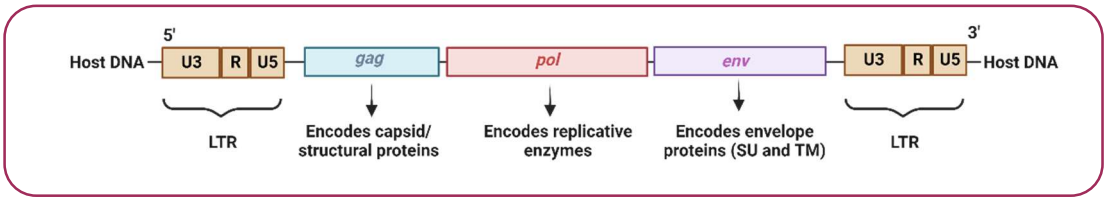


Figure 7. Diagrammatic representation of integrated FeLV proviral DNA. After reverse transcription of viral RNA, the double-stranded DNA provirus is integrated into the host genome. Indicated are the viral genes (*gag*, *pol*, and *env*) and the LTR. Adapted from (113), created with BioRender.

In addition, the FeLV virion carries the reverse transcriptase enzyme, which reverse transcribes the viral RNA genome into a DNA form (30). The genes and proteins of the virus are summarized in Table 3.

Gene	Abbreviation	Function	Protein
env	SU	Surface	gp70
	TM	Transmembrane	p15E
g*ag*	MA	Matrix	p15
	P12	Unknown	p12
	CA	Capsid	p27
	NC	Nucleocapsid	p10
pol	PR	Protease	p14
	IN	Integrase	p46
	RT	Reverse Transcriptase	p80

Table 3. Genes and proteins of the Feline Leukemia Virus.

During the first decades of FeLV research, it was found that FeLV-infected cells produced an antigen on their cell surface called the feline oncornavirus cell membrane antigen (FOCMA) (114,115). This antigen does not appear to be a structural component of the virus (116). Studies suggested that some cats infected with FeLV could produce antibodies against FOCMA which does not neutralize the virus but recognizes transformed cells (117). FOCMA was used as a diagnostic tool but nowadays FOCMA is no longer used as more efficient and effective tools raised.

In addition to the polyprotein precursor of the viral capsid proteins (Pr65gag), gammaretroviruses, including murine, feline, and gibbon-ape leukemia virus, encode an alternate glycosylated form of Gag polyprotein known as glycogag or gPr80gag (118–122).

Glycogag

Glycogag is present in gammaretroviruses, such as MLV (123) and FeLV (119,123). Glycogag is translated from unspliced viral mRNA via an upstream CUG initiation codon in the same reading frame as for Pr65gag (124) (Figure 8A). The N-terminus of gPr80gag contains 88 unique amino acids, including a signal peptide that targets gPr80gag for transport to the rough endoplasmic reticulum (RER), leading to its glycosylation and export to the cell surface (Figure 8B). At the cell surface, mature gPr80gag is cleaved into two proteins of 55 and 40 kDa. The 55-kDa amino-terminal portion is membrane-associated and can be either a type I integral membrane protein, with the N terminus on

the exterior side of the plasma membrane; or a type II protein, with the N terminus on the cytoplasmic side (125,126). The 40 kDa C-terminal protein is secreted to the media. The protein is not thought to be incorporated into virions (118,125).

The glycosylated Gag polyprotein is synthesized and processed independently of Pr65gag, which is the precursor polyprotein of the internal virion proteins (MA, p12, CA, and NC).

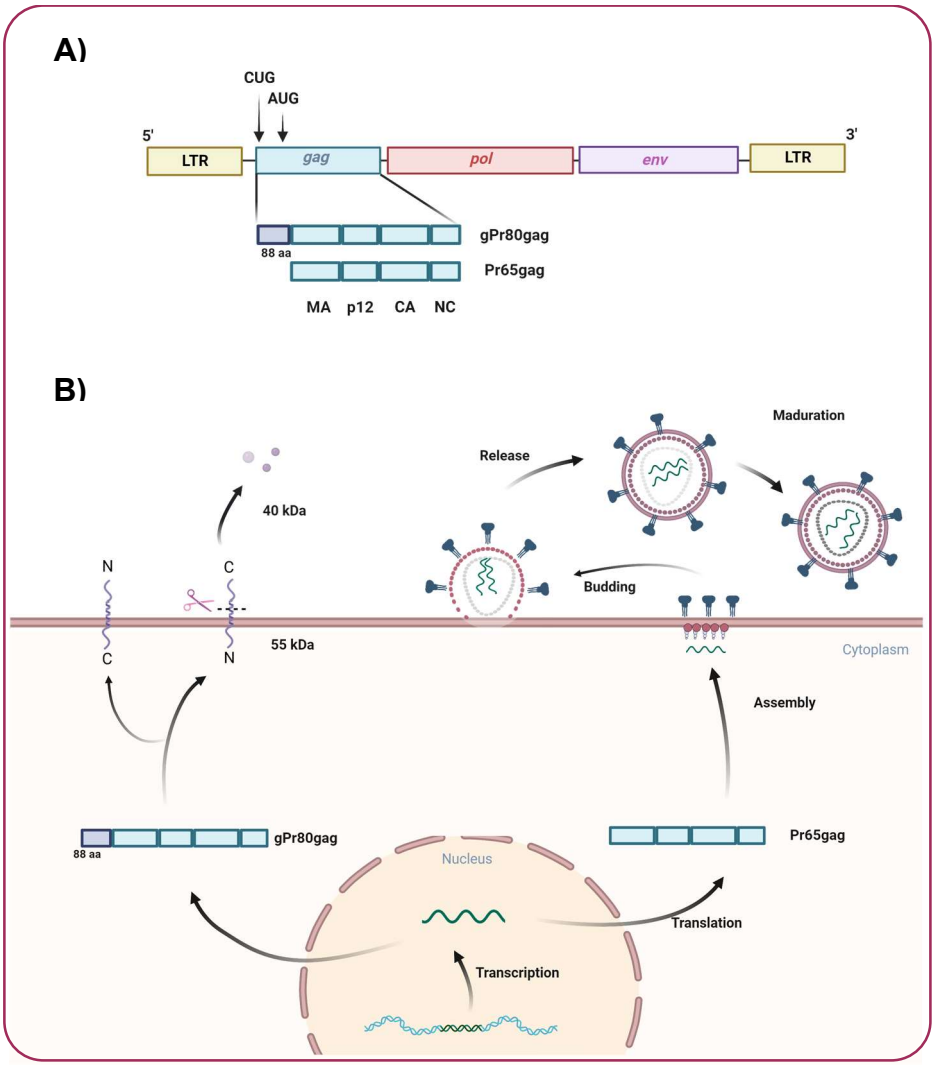


Figure 8. A) gPr80gag is translated from the same reading frame as Pr65gag but from an upstream CUG initiation codon. 88 additional residues at the N-terminus of gPr80gag compared to Pr65gag are denoted. **B)** Schematic figure of synthesis and cleavage of Pr65gag and gPr80gag. Pr65gag is synthesized as a cytosolic protein which is incorporated into virions and processed by the viral protease into the various components of the viral core. gPr80gag is synthesized at the endoplasmic reticulum and exported to the cell surface. Image created with BioRender.

The role of glycogag and its mechanism of action have been unclear for many years. However, research in the last 10 years has provided more information regarding the functionality of glycogag. Its main functions are described below.

- Structural function

Glycogag has been related to an increase in virus release rate, being involved in viral budding or assembly/release. Glycogag negative cells present tube-like structures on the cell surface, reinforcing this hypothesis. In addition, it has been described that glycogag facilitates virion release through cholesterol-rich lipid rafts (124,127,128)

- Pathogenesis

Glycogag has also been described as an accessory protein dispensable for virus replication, however, it is an important virulence determinant (129–131). Also, it remains unclear the correlation between glycogag expression and neurovirulence (132,133).

- Host restriction factor antagonist

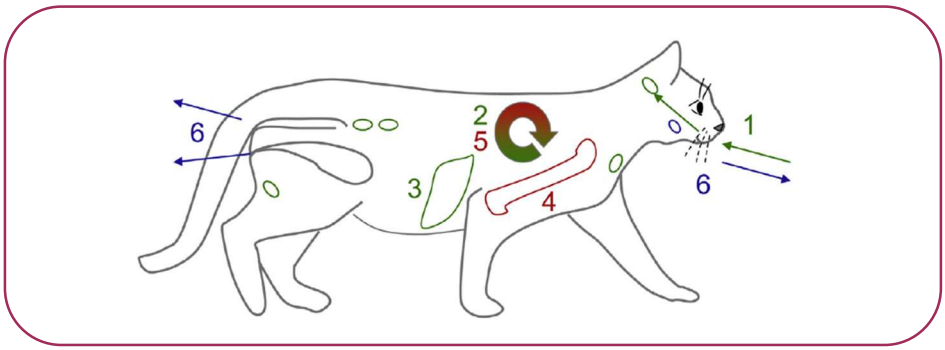
Retroviruses have evolved to evade host restriction factors, which function is to reduce retrovirus infectivity. Some examples of host restriction factors are serine incorporator proteins 3 (SERINC3) and 5 (SERINC5) (134), or apolipoprotein B editing complex (APOBEC3) (135). The last research on glycogag has been shown to counteract different host restriction factors. The first report was published by Doctor Pizzato whose research led to the conclusion that MLV glycogag was able to replace Nef and enhance HIV-1 replication *in vitro* (136–139). Glycogag was also described as an antagonist of SERINC3 and SERINC5 (123,126,140–145). And lastly, different studies suggested that in viruses lacking Vif, such as MLV, glycogag has an analogous function and counteracts APOBEC3 antiviral function (146–149).

2.4.2 Life cycle

FeLV follows the classical life cycle of a *Retroviridae* family member. In brief, once FeLV virion has fused with the host cell, the viral RNA is converted into viral DNA. The viral DNA is imported into the cell nucleus, where it is integrated into the host genomic DNA with the help of the viral integrase. Then, new viral RNA and proteins are produced and assembled at the host cell membrane to build new viral particles that are shed into the blood and saliva (150). In addition to viral particles, also soluble FeLV p27 capsid antigen is shed into the blood and can be detected by point-of-care test (POC) and laboratory ELISA (151).

FeLV does not lyse the cells it infects and in which it replicates (152). The virus buds from the surface of infected cells and is then able to infect other cells. FeLV, however, can transform cells into tumor cells which may continue to divide and produce tumors (152).

Cats typically acquire FeLV via the oronasal route but can also become infected through bite wounds (153). After virus exposure via the oronasal route, FeLV can be found first in the local lymphoid tissues; and then, it spreads via monocytes and lymphocytes (primary viremia) to the periphery. During this primary viremia, the virus can infect the bone marrow (154). After bone marrow infection, a secondary viremia can occur, and the virus spreads readily to the spleen, lymph nodes, intestine, urinary bladder, and salivary glands, where it is shed in high concentrations (154), whereas low virus loads are shed in urine and feces (Figure 9).



1. Oropharynx, local lymphoid tissue	Starting time point up to 2 weeks
2. Primary cell-associated viremia (lymphocytes, monocytes)	
3. Lymphoid tissue throughout the body	
4. Bone marrow (neutrophils, platelet precursors), intestine	1-3 weeks
5. Secondary viremia (neutrophils, platelets, high loads)	2-4 weeks
6. Shedding (mucosal and glandular tissue)	3-8 weeks

Figure 9. Pathogenesis of progressive FeLV infection. Adapted from (151).

2.4.3 FeLV classification

There are three main subtypes of FeLV: FeLV-A, FeLV-B, and FeLV-C. FeLV subtypes are classified based on their *env* sequence (155–157). Recently, a new variant of FeLV associated with severe immunodeficiency has been described and designated as FeLV-T, which has a marked tropism for T lymphocytes [135]. FeLV-T is closely related to FeLV-A, from which it evolves during infection and as a result of multiple mutations throughout the *env* gene [136].

FeLV-A is the predominant subtype in infected cats and is transmitted exogenously between animals (160,161). FeLV-B arises in approximately 50% of cats because of recombination between FeLV-A and endogenous FeLV-related retroviruses in the cat genome (162). Infection with FeLV-B viruses may influence the course of the disease; for example, the infection can accelerate the generation of neoplasia as lymphomas or

increase virus neuropathogenicity (163). FeLV-C viruses arise rarely in cats infected with FeLV-A and is associated with non-regenerative anemia (164,165). The different strains of the virus target different cell types depending on the receptor (Table 4).

FeLV Subtype	Receptor	Tropism	Comments	References
FeLV-A	FeTHTR1 (Thiamine transporter protein)	Kidney, liver, T-cells, small intestines	Present in all cats with FeLV; transmitted exogenously	(166)
FeLV-B	FePit1 or FePit2 (Sodium-dependant inorganic phosphate transporter protein)	Wide range of tissues	Results from recombination between FeLV-A and feline endogenous FeLV-related retrovirus sequences; may accelerate the development of lymphoma or enhance neuropathogenicity	(159,167)
FeLV-C	FLVCR (Heme transporter protein)	Erythroid progenitor cells	Arises from point mutations in FeLV-A env gene; associated with nonregenerative anemia	(168,169)
FeLV-T	FePit1 in combination with co-receptor FeLIX	T-cells	Arises from evolution of FeLV-A	(155,158)

Table 4. FeLV subtypes, each subtype uses different host receptors for cell entry, resulting in different tissue tropisms. Adapted from (170).

2.4.4 Prevalence

FeLV infection exists in domestic and non-domestic cats worldwide. There have been several international studies aiming to determine FeLV prevalence. Despite the difficulties to compare the different studies due to sample size, diagnostic methodology (111), cat habitat, age, or vaccination status; it is commonly accepted that FeLV prevalence varies considerably between geographic areas. Estimations of the last 10 years report a prevalence of progressive FeLV infection of 2.3% to 3.3% in the United States, 0.7% to 15.6% in Europe, 3.0% to 28.4% in South America, and 0.5% to 24.5% in Asia and Australia/New Zealand (171–179).

The prevalence of FeLV infection in cats has decreased worldwide in the last 20 years, mainly due to increased testing and prevention programs, as well as vaccination (180–183). However, recent studies indicated that the decrease in prevalence has now reached a plateau in many countries, and thus, awareness of this important infection and its prevention should not be neglected (153,184). These studies show that although guidelines for the prevention of infection have been available for decades, there remains a need to improve adherence to testing and vaccination recommendations (179).

2.4.5 Transmission

FeLV's transmission occurs mainly horizontally (185), but it can also be transmitted vertically (186). Primarily, transmission results from close contact with salivary secretions, such as through playing, mutual grooming, and shared food and water dishes (187). But, it may also occur via other routes, such as biting, blood transfusion, transplacental, and during lactation (188). Additionally, the infection can also occur via urine and feces, being of much lower probability (150). Fleas may also be a source of transmission (189). Detection of FeLV RNA in secretions has demonstrated that a viremic cat is more likely to transmit the virus than a cat that has recovered from infection (190).

2.4.6 Pathogenesis and Infection outcomes

Whether a cat recovers from infection or develops viremia and ultimately develops disease is governed by several factors. The age of the cat at the time of exposure to infection is essential for the evolution of the cat, being neonatal kittens the most susceptible (191). Infection outcome also depends on the virus strain involved, the challenge dose, the route of inoculation, and factors that influence host immune function, such as genetics, co-infections, stress, and treatment with immunosuppressive drugs (77).

Outcomes of FeLV infection are classified as abortive infection, regressive infection, progressive infection, and focal or atypical infection (192,193) (Figure 10).

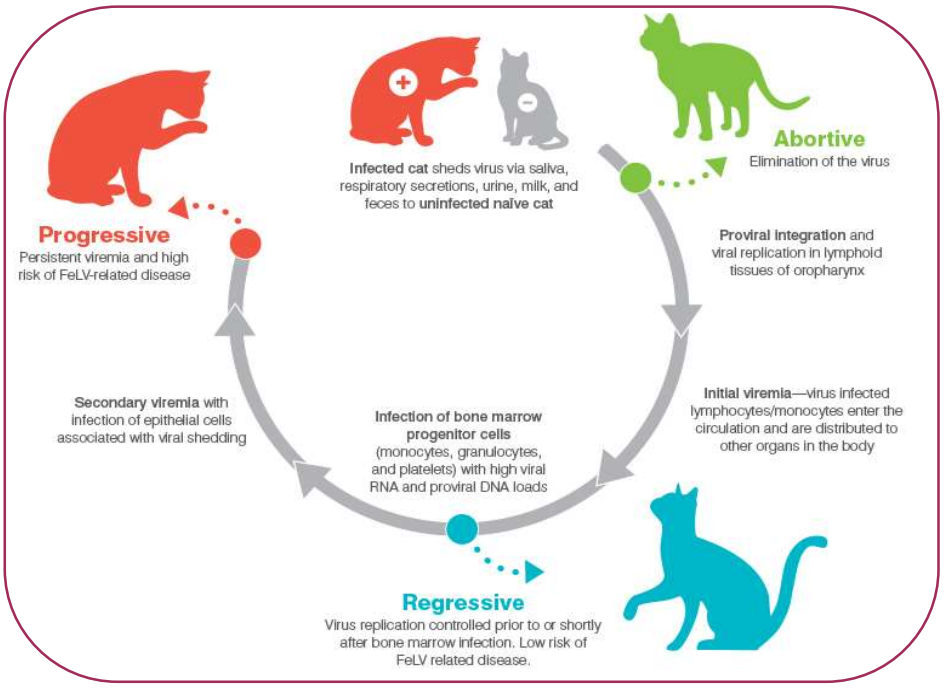


Figure 10. Infection outcomes after FeLV challenge. Adapted from (78).

Abortive infection

After infection, the virus initially replicates in the local lymphoid tissue in the oropharyngeal area (78). This first phase of infection is critical. In immunocompetent cats, viral replication may be terminated by an effective humoral and cell-mediated immune response; these cats never become viremic, this is likely to occur when a cat is exposed to low doses of FeLV (194).

Abortive infection may be the most common outcome following exposure, as approximately 60% of cats exposed to FeLV recover (83,175,182). Animals with abortive FeLV infection present high levels of neutralizing antibodies but neither FeLV antigen nor viral RNA or proviral DNA can be detected in the blood at any time (195). In contrast, when the virus exceeds the ability of the immune response to eliminate infection, the animals develop persistent viremia (112).

Regressive infection

Regressive infection develops following a partially effective immune response that contains but does not eliminate virus replication. After the initial infection, FeLV spreads systemically through infected mononuclear cells. During this stage, cats have positive results on tests that detect free antigen in plasma, shedding viruses, mainly in saliva. About three weeks later, viremia is contained prior to or shortly after bone marrow infection. Even if bone marrow cells become infected, a certain percentage of cats are able to clear viremia. However, they cannot eliminate the virus from their infected cells,

as proviral DNA is integrated into the genome of bone marrow stem cells (193,196,197). Thus, viral replication is controlled, and no infectious viral particles are produced. Therefore, regressively infected cats do not shed FeLV and are not infectious to others. Consequently, cats with regressive infection have negative results in all tests that detect FeLV antigen (196). Sensitive PCR methods can detect provirus in the blood of cats with regressive infection that are antigen-negative (197–199).

Cats with regressive infection demonstrate continuously high titters of virus-neutralizing antibodies (VNA) (200) and are at low risk of developing FeLV-associated diseases (201–203). Despite that, reactivation can occur, particularly if cats are immunosuppressed, so they become viremic and develop FeLV-associated diseases (204,205).

Progressive infection

In cats with progressive infection, FeLV infection is not contained during early infection. Thus, extensive virus replication occurs, first in the lymphoid tissues, followed by the bone marrow, and finally in mucosal and glandular epithelial tissues (206). Mucosal and glandular infections are associated with the excretion of infectious viruses, mainly in saliva but also in other secretions, such as urine and feces (154).

Progressive infection is characterized by persistent viremia/antigenemia and by insufficient FeLV-specific immunity, usually neutralizing antibodies are not detectable. Cats with progressive infection have a shorter survival time than cats with regressive FeLV infection and typically succumb to FeLV-associated diseases within a few years after infection (179,195,197,207).

Regressive and progressive infections can be distinguished by repeated testing for viral antigen in peripheral blood. Regressively infected cats will turn negative at the latest 16 weeks after infection, while progressively infected cats will remain positive. Initially, both, regressive and progressive infections are accompanied by the persistence of FeLV proviral DNA in the blood detected by PCR. However, later on, they are associated with different FeLV loads when measured by quantitative PCR. Regressive infection is associated with a low viral load, while progressive infection is associated with a high viral load [129],[140].

Focal infection

Focal infection or atypical infection has been reported in up to 10% of experimentally infected cats, and may also be observed in natural infections but are probably rare (78,175). Focal infections are characterized by a persistent atypical local viral replication

(e.g., in mammary glands, bladder, and eyes) (195). This replication leads to intermittent or low-grade production of antigen, and therefore, these cats have weakly positive or discordant results in antigen tests, even positive and negative results alternate (209,210).

In summary, different outcomes of FeLV infection exist**.** Testing of cats for FeLV infection is an important task for veterinarians in clinical practice. Interpretation of FeLV tests is not trivial and requires a fundamental knowledge of disease pathogenesis, virus-host interactions, and different FeLV tests and their characteristics (195) (Table 5).

Stages of FeLV infection	p27 antigen in blood	Virus blood culture	Viral RNA in blood	Viral DNA in blood	Viral tissue culture	Viral shedding	FeLV-associated disease
Abortive	-	-	-	-	-	-	Unlikely
Regressive	-	-	-	+	-	-	Unlikely
Progressive	+	+	+	+	+	+	Likely
Focal	Variable	Variable	Variable	Variable	+	Variable	Unlikely

Table 5. The variability of diagnostic results depends on the different stages of FeLV infection.

2.4.7 Clinical manifestations

Progressive FeLV infection can course with neoplastic and non-neoplastic manifestations (152) (Table 6), a large proportion of them are secondary infections attributable to immunosuppression, which are treatable.

Neoplastic diseases	Non-neoplastic diseases
○ Lymphomas	○ Nonregenerative anemia
○ Leukemia lymphoblastic	○ Enteritis
○ Leukemia myeloid	○ Secondary infections attributable to immunosuppression
	○ Thrombocytopenia
	○ Reproductive disorders
	○ Fading kitten syndrome
	○ Glomerulonephritis
	○ Polyarthritis
	○ Osteochondromatosis
	○ Lymphadenopathies

Table 6. FeLV clinical syndromes are classified into neoplastic and non-neoplastic diseases. Adapted from (212).

Therefore, FeLV can cause severe clinical syndromes, and progressive FeLV infection is associated with a decrease in life expectancy (211). When therapy is provided and

with the proper treatment, FeLV-infected cats, especially in indoor-only households, may live for many years with good quality of life (78). Even so, an efficient preventive vaccine would prevent any infection outcomes and their associated syndromes.

2.4.8 Immune responses to FeLV infection

The immune mechanisms that influence the infection outcome after exposure to FeLV have yet to be fully resolved. Virus-neutralizing antibodies (VNAs) have been detected in peripheral blood from FeLV-exposed cats and confer protection in some circumstances (213). Furthermore, the role of cytotoxic T cells (CTLs) in mediating vaccinal protection and recovery is still under research (214,215).

Virus-Neutralizing Antibodies (VNA)

The humoral immune response develops in the first 4 to 8 weeks after infection (200). The antibody response to FeLV infection is directed mainly towards epitopes located on the surface envelope and transmembrane glycoproteins; gp70 and p15E (216–222). Such antibodies block the entry of the virus into the cell. After infection, antibodies against other proteins, mostly p27 (FeLV Gag Capsid), are developed (77). Cats that recover from exposure to the virus develop significant VNA titters (191,218,223), and kittens that passively receive maternal antibodies are transiently protected from infection (213). The presence of a high VNA titter is therefore a good indicator of protective immunity in a naturally exposed cat (83,200).

Cell-Mediated Immune Response

CTLs are recognized to be one of the early host defense mechanisms generated in response to viral infection and have been shown to have a role in clearing viruses and controlling viral replication (224). Also, CTLs are a major factor in eliminating virus-infected cells (223). The majority of cats exposed to FeLV frequently clear the circulating virus and recover. CTLs are induced early after exposure, in the first-second week post-infection, preceding the neutralizing humoral response. Under experimental virus challenge conditions, cats that fail to recover from FeLV infection showed delayed and short-lived virus-specific CTLs (223).

In FeLV DNA-vaccinated protected cats, high levels of FeLV-specific CTLs are present in blood and lymphoid tissues (214,225). In addition, virus-specific CTLs occur at higher levels in vaccinated protected and unvaccinated recovered cats compared with unvaccinated persistently viremic cats (223), suggesting that virus-specific CTLs also play and important role in protective vaccinal immunity.

Immunosuppression

The leading cause of death in FeLV-infected cats is immunosuppression, over and above other FeLV-associated disease (226,227). *In vitro* studies have demonstrated functional suppression of T and B lymphocytes in persistently FeLV-infected kittens, although T lymphocytes were more profoundly affected (228).

The immunosuppressive effect of FeLV does not seem to be restricted to B and T lymphocytes. Persistently viremic cats are highly susceptible to opportunistic bacterial and fungal infections, which might indicate impaired innate immunity. In fact, polymorphonuclear (PMN) cells from FeLV-infected cats were shown to be functionally impaired *in vitro* (229).

Furthermore, evidence suggests that the FeLV transmembrane protein p15E is partly responsible for the immunosuppressive effects of FeLV (230–233).

2.4.9 Diagnostic tests

Diagnosing FeLV infection remains challenging due to the different outcomes of infection. Antigenemia and viral or proviral loads can vary over time depending on the balance between the virus and the host's immune response. Knowledge of FeLV infection pathogenesis and the different detection methods is an important prerequisite for the correct interpretation of any test results and the accurate determination of a cat's FeLV status (195).

Testing for FeLV infection has become more refined over the years and now includes diagnostic assays for different viral and immunological parameters. Diagnostic tests are available to detect FeLV p27 capsid protein, whole virus, or integrated proviral DNA (83). Different assays are available:

- Antigen assays include immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA). IFA detects FeLV cell-associated p27 antigen in circulating leukocytes and platelets (110,234). ELISA detects FeLV p27 not only in serum or plasma but also in the saliva of infected cats (235). More recently, a POC test based on ELISA methodology is commonly used in veterinary to detect FeLV antigen in serum, plasma, whole blood, or saliva (111). Most cats will test positive within 30 days of exposure, although the development of antigenemia can take longer in some cats (179). Noteworthy, when detecting p27 it is not possible to distinguish between protein coming from the virion or soluble p27 forms corresponding to glycogag released from infected cells.
- Virological assays include isolation and culture of the virus (195). This test is currently not used routinely.

- Nucleic acid assays such as PCR, RT-PCR, and qPCR detect proviral DNA or viral RNA. In regressive infection concentrations of antigen and proviral DNA can drop below the threshold of detection of some tests, leading to discordant results that may change over time.
- Serological assays. Detection of antibodies against FeLV is unreliable for the diagnosis of FeLV infection because it is unable to discriminate between viremic cats and immunized cats. FeLV antibody testing is only performed in the research setting to provide a more complete picture of a cat's possible exposure to FeLV (175).

It might not be possible to determine a cat's infection status based on testing at a single time point; therefore, repeated testing using different methods is required to determine the infection status and outcome of the animal (179) (Figure 11).

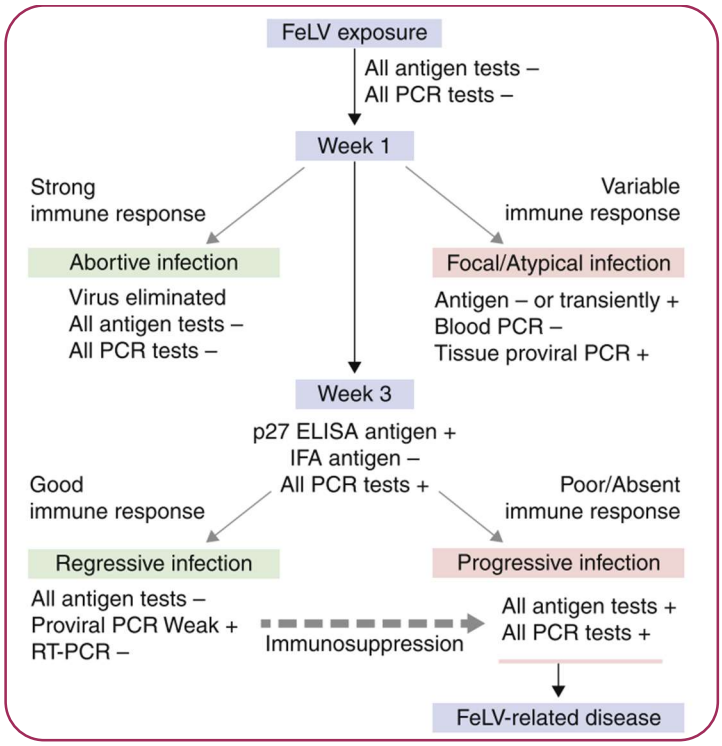


Figure 11. FeLV infection outcomes and diagnostic tests are available for each time point over infection. Adapted from (112).

2.4.10 Treatment

FeLV-infected cats without clinical signs do not require treatment. FeLV infection cannot be eradicated but opportunistic infections and lymphoma can be treated using the same medications and supportive treatments used for FeLV-negative cats with these conditions. Opportunistic infections may require longer periods of treatment or, in some cases, lifelong treatment with antimicrobial drugs.

Antiviral agents and immunomodulators are of limited benefit for the treatment of cats with FeLV infection (112). Most antivirals used in cats are licensed for humans and are specifically intended for HIV infection (236,237). Treatment may include feline recombinant IFN-ω, human recombinant IFN-α (238,239), and antiretroviral Zidovudine (AZT) (Table 7). However, AZT has been reported to be less effective in FeLV-infected cats than in those infected with FIV (238).

Drug	Dose	Route	Interval (hours)
Feline recombinant interferon-omega (IFN-ω)	1 million U/kg	SC	Every 24 h for 15 days
Human recombinant interferon-alfa (IFN-α)	1-50 U/cat	PO	Every 24 h for 7 days
Zidovudine (AZT)	5 mg/kg	PO	Every 12 h, monitoring

Table 7. Suggested medications for the treatment of cats with FeLV. SC: Subcutaneous, PO: Oral administration. Adapted from (112).

2.4.11 Prevention

The most important measure for the control of FeLV infection is the identification and segregation of infected cats. Cats with progressive FeLV infection shed high numbers of FeLV particles and pose a risk to other cats. They should be kept separated from FeLV-naive companions, regardless of the health status of the FeLV-infected cat (240). Thus, the American Association of Feline Practitioners (AAFP) recommends screening all cats for infection at the time they are first acquired, before initial vaccination against FeLV, following potential exposure to infected cats, or if clinical signs of illness are displayed (179). Therefore, cats with progressive infection should always be kept indoors, not only to prevent the spread of infection to other cats but also to decrease the risk of exposing infected cats to opportunistic infections.

Testing and identification of FeLV-infected cats are important to prevent new infections, but vaccination is also an important preventive tool (179). The combined use of testing and vaccination programs is likely the reason for the decrease in FeLV prevalence over the last 20 years (180,241,242).

In 1980, the first experimental FeLV vaccines, based on live tumor cells, although effective, caused neoplasia in some vaccinated animals (243). Despite these early setbacks, further research efforts led to licensing of the first commercial FeLV vaccine in 1985 (244). Since that time, several improved vaccines have been developed.

Current available FeLV vaccines include: i) classical inactivated virus vaccines (245,246); ii) subunit vaccines based on FeLV recombinant antigens (247); and iii) infectious recombinant canarypox virus engineered to express FeLV genes (248,249). The two first vaccines, contain adjuvant (248,250–252). Vaccines positively impact disease progression, protecting cats against progressive infection and FeLV-associated diseases. However, vaccination not always prevents proviral DNA integration after FeLV exposure (253). Comparative studies show that none of the currently available vaccines provides full protection against FeLV infection (254–256).

The AAFP highly recommends that all FeLV antigen-negative kittens should be vaccinated against this pathogen. Also, vaccination is indicated for all at-risk cats, such as those with outdoor exposure or those that reside in households with other FeLV antigen-positive cats. (257). AAFP recommended vaccination scheme is two doses given, 3 to 4 weeks apart from 8 to 9 weeks of age, followed by a booster at 1 year and then every 1 to 3 years thereafter. The European Advisory Bureau on Cat Diseases (ABCD) also recommends a booster every 2 to 3 years for cats older than 3 to 4 years of age. Acutely ill cats should not be vaccinated, but it is acceptable to vaccinate cats with chronic diseases, such as chronic kidney disease (112). More information is required on the duration of immunity for FeLV-vaccinated animals, as data on longitudinal studies is currently very limited.

Unfortunately, a significant correlation between vaccination with FeLV vaccines and the development of feline injection-site sarcomas (FISS) has been documented (258). FISS has been recognized since the early 1990s, contemporaneous with the implementation of stricter vaccination recommendations and the development of adjuvanted FeLV vaccines (258). Pathologists recognized an increase in the development of sarcomas at vaccination sites (259,260). Over the past 2 decades, this problem has been recognized worldwide (261).

To summarize, testing and identification of FeLV-infected cats are equally important as vaccination programs to prevent new infections. Actual commercial vaccines provide substantial but not complete protection and may cause FISS, therefore, there is room for improvement and new vaccine designs are needed.

3. Vaccines

Vaccines have transformed public health, particularly since national programs for immunization first became properly established and coordinated in the 1960s. In countries with high vaccine program coverage, many of the diseases that were

previously responsible for the majority of childhood deaths have essentially disappeared (e.g., smallpox, polio, measles) (262). The World Health Organization (WHO) estimates that 2–3 million lives are saved each year by current immunization programs (263). The widespread use of vaccines substantially contributed to public health and animal welfare. Consequently, reducing the transmission of zoonotic diseases and securing the food supply for humans.

Advances in virology, molecular biology, and immunology have created many alternatives to traditional vaccines. Modern vaccines include nucleic acid-based vaccines (mRNA, DNA), viral vectored vaccines, virus-like particles (VLPs), and recombinant protein (subunit) vaccines (Table 8), each having advantages and limitations (264). Choosing a particular vaccine can depend on several factors such as the level of protection or the expected mode of action (265). There are still some diseases that have yet to be successfully treated, therefore, vaccine research must continue and adapt pre-existent technologies or create new platforms.

In the present work, we have focused on two types of vaccines: i) nucleic acid vaccines and ii) multivalent platforms, specifically VLPs.

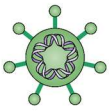
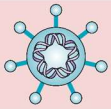
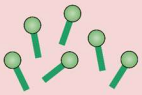
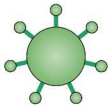
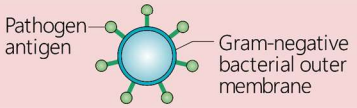
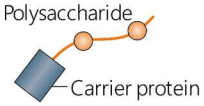
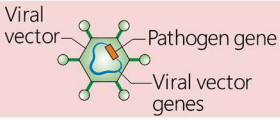
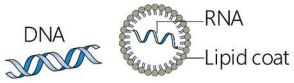
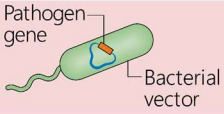
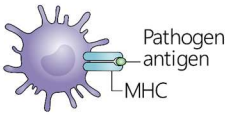
Type of vaccine		Licensed vaccines using this technology	First introduced
Live attenuated (weakened or inactivated)		Measles, mumps, rubella, yellow fever, influenza, oral polio, typhoid, Japanese encephalitis, rotavirus, BCG, varicella zoster	1798 (smallpox)
Killed whole organism		Whole-cell pertussis, polio, influenza, Japanese encephalitis, hepatitis A, rabies	1896 (typhoid)
Toxoid		Diphtheria, tetanus	1923 (diphtheria)
Subunit (purified protein, recombinant protein, polysaccharide, peptide)		Pertussis, influenza, hepatitis B, meningococcal, pneumococcal, typhoid, hepatitis A	1970 (anthrax)
Virus-like particle		Human papillomavirus	1986 (hepatitis B)
Outer membrane vesicle		Group B meningococcal	1987 (group B meningococcal)
Protein–polysaccharide conjugate		*Haemophilus influenzae* type B, pneumococcal, meningococcal, typhoid	1987 (*H. influenzae* type b)
Viral vectored		Ebola	2019 (Ebola)
Nucleic acid vaccine		SARS-CoV-2	2020 (SARS-CoV-2)
Bacterial vectored		Experimental	–
Antigen-presenting cell		Experimental	–

Table 8. Schematic representation of different types of vaccines against pathogens. Adapted from (2,265) with Inkscape.

On one hand, in the last years, nucleic acid vaccines have become of significant relevance since the SARS-CoV-2 pandemic accelerated the need for readily accessible vaccines (266). The main advantage of this type of vaccines is the easiness of producing and adapting the platform to express different immunogens since the modifications are performed on the DNA level. This strategy facilitates vaccine production, purification, and quality control since all the downstream processes will be similar independently of the

antigenic content (267). Nucleic acid vaccines can be categorized into three major groups: viral vectored vaccines, messenger RNA (mRNA) vaccines, and DNA vaccines.

DNA vaccines are often administrated in the context of a plasmid DNA vector that encases the gene of interest. These plasmid vectors may also contain an antibiotic resistance gene for selection in prokaryotes during production. For example, the pVAX1 vector is a reduced version of the eukaryotic expression plasmid pcDNA3.1 that contains a CMV promoter and a BGH polyadenylation site while having a kanamycin resistance gene for selection. pVAX1 follows the FDA recommendations for DNA vaccines (268).

On the other hand, one strategy to increase the immunogenicity of the antigens is their administration formulated or conjugated with a multivalent platform (269). These platforms rely on nanotechnology-based strategies like polymeric carriers and liposomal formulations but also include biological approaches that aim at mimicking the virus morphology, such as VLPs.

3.1 Virus-Like Particles (VLPs)

In the 1980s, subviral particles were found in the blood of patients infected with the Hepatitis B Virus (HBV). Plasma-derived subviral particles from these patients were administered to healthy individuals, providing protection against HBV infection and giving rise to the first VLP-based vaccine (270). The success of this vaccine motivated further research on VLP vaccines over the last twenty years (271).

VLPs are self-assembled particles that mimic the virus structure. However, they do not contain the viral genome and are thus non-infectious (272). Due to their repetitive organized structure and particulate nature, they are very efficiently uptaken by antigen-presenting cells giving rise to a potent immune response with stimulation of both arms of the immune system: humoral and cellular (273). These unique properties of VLPs make them appealing in many aspects as an alternative to the existing vaccines and are therefore the subject of intensive research and represent an advanced vaccine technology platform (274,275).

3.1.1 Types of VLPs

VLPs are diverse and functionally versatile. They are classified as non-enveloped or enveloped, according to the absence or presence of a lipid bilayer. Non-enveloped VLPs are further categorized as simple- or multiple-capsid protein VLPs (276). A structurally simple VLP is a non-enveloped single capsid VLP, such as the licensed human papillomavirus (HPV) VLP vaccine. These simple VLPs, composed of a single capsid protein, can be produced in both prokaryotic and eukaryotic expression systems (277).

In contrast, non-enveloped multiple-capsid protein VLPs are more complex and challenging to produce. These complex VLPs are usually produced in higher eukaryotic hosts such as yeast (278,279), insect cells (280,281), and plants (282). Some examples of non-enveloped multi-capsid proteins VLPs in experimental development are bluetongue virus (BTV) (283), Enterovirus 71 (284,285), infectious bursal disease virus (286), poliovirus (287), and rotavirus (279,288).

Enveloped VLPs acquire a lipid membrane during their assembly and budding in eukaryotic cells. One or more types of glycoprotein spikes can be embedded in the lipid bilayer and these glycoproteins are the antigenic target for generating neutralizing antibodies. Influenza VLPs are possibly the most studied enveloped VLPs, commonly consisting of matrix M1 proteins that provide the inner structure to the VLP and glycoprotein hemagglutinin (HA) and/or neuraminidase (NA) proteins embedded in the lipid bilayer and exposed on the surface (289). Human immunodeficiency virus (HIV) VLPs (290), Ebola virus VLPs (291), and Chikungunya virus VLPs (277, 278) have also been explored as enveloped VLP platforms.

3.1.2 HIV-1 Gag-based VLPs

HIV-1 Gag-based VLPs are enveloped particles that mimic the structure of immature HIV-1 virions but are non-infectious and non-replicative, hence they are a good vaccine platform to induce potent immune responses by vaccination (274). HIV-1 Gag-VLPs have been used as immunogens to elicit potent cellular responses against Gag or Gag-Pol proteins by vaccination but also as multivalent immunogen carriers since they can accommodate immunogens at the surface of their lipid membrane (274). The benefit of using Gag-VLPs as immunogen carriers is that balanced humoral and cellular responses against the immunogen and Gag, respectively, can be elicited. Furthermore, immunogen presentation at VLPs' surface faithfully mimics viral proteins at the surface of a virus and presents the immunogens in the right orientation, and hence it induces responses that will better engage the virus (294).

HIV-1 VLPs are produced by the expression of Gag, the main HIV-1 structural protein. Upon synthesis, Gag migrates to the host cell membrane, where it buds producing enveloped VLPs and incorporates around 2,500 Gag monomers/VLP (295).

However, one of the main disadvantages faced by HIV-1 VLPs is that, since VLPs mimic the HIV-1 structure, immunogens are incorporated at a low density on the surface of VLPs (296). That is why many efforts have been invested in increasing antigen density at their surface (297–299). Some groups have tried to incorporate mutations or to substitute the cytoplasmic tail of Env to achieve higher densities (300,301). In this sense,

our group has been working to overcome the limitation of poor incorporation of immunogens. To achieve higher densities of antigen at the VLP surface, first, a gp41-derived miniprotein, including the MPER and HR2 domains, named Min was selected as the immunogen for the study (302). When co-transfecting Min and Gag, low-density VLPs were produced; but high-density VLPs were generated when the immunogen was fused to HIV-1 Gag through a transmembrane domain and a linker, increasing the density of the immunogen of interest at the surface of the particles (Figure 12). Interestingly, our design induced a potent and functional immune response against Min, even with no adjuvants, and administered at a low VLP dose (303).

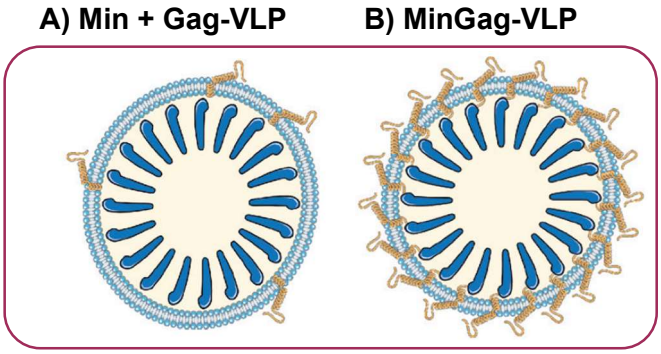


Figure 12. Schematic HIV-1 Gag-based VLPs. A). Low-density VLPs are produced by co-transfection of Min and gag. **B)** High-density VLPs were produced by transfection of a fusion protein MinGag construct. Figure provided by Dr. Tarrés-Freixas.

3.2 Adjuvants

Vaccine formulation development has traditionally focused on ensuring that the marketed therapeutic products are efficacious, safe to administer and remain stable during shipping and storage (304). Adjuvants and excipients are commonly used to achieve such goals. But from a more immunologic view, vaccine adjuvants are an essential tool to enhance immunogenicity. The adjuvant concept is more than 80 years old with the first adjuvant present in human vaccines, an aluminum salt (aluminum potassium sulfate), appearing in the 1920s (305). The development of new alternative adjuvants to aluminum salt was developed after 70 years, in the 1990s (306). The efficacy of a vaccine depends not only on the antigen components but also on adjuvants that are often used in order to stimulate the immune system in a more effective way (307).

Therefore, adjuvants are defined as constituents added to vaccines to improve immune responses toward an antigen (264). In addition, adjuvants have several benefits, such as i) reducing the amount of antigen or the number of immunizations needed for protective immunity; ii) improving the efficacy of vaccines in newborns, older adults or

immuno-compromised persons; and/or iii) increasing the stability of the antigen component (308).

Adjuvants can be grouped according to different criteria, such as their physicochemical properties, origins, and mechanism of action (309). Based on their mechanism of action, adjuvants are divided into two main categories: delivery systems (particulate) and immune potentiators (Table 9) (310). A further class of adjuvants is mucosal adjuvants, a group of compounds that shares some features with the previous ones (307).

Adjuvant Groups	Types of Adjuvants
Delivery systems	
Mineral salts	Aluminium salts
Emulsions	Freund's adjuvants
	MF59
	AS03
Microparticles	Virus-like particles
	Virosomes
	PLA/PLGA
Immune Potentiators	
TLR1/2 agonists	L-pampo, MALP-2, Pam2CSK4 and Pam3CSK4
TLR3 agonists	Poly(I:C) (polyinosinic:polycytidylic acid)
	Poly-ICLC
TLR4 agonists	Monophosphoryl lipid A (MPL)
TLR5 agonists	Flagellin
TLR7/8 agonist	Imiquimod (R837; (R837; 1-(2-methylpro- pyl)-1H-imidazo[4,5-c]quinolin-4-amine)
	Resiquimod (R848, 4-amino-2-(etoximetil)-a,a-dimethyl-1H-imidazo [4, 5-c]quinoline-1-ethanol)
TLR9 Agonists.	CpG ODNs
Combined adjuvants	AS01 and AS02
	AS04
Mucosal adjuvants	Cholera toxin (CT)
	Heat-labile enterotoxin (LTK3 and LTR72)
	Chitosan

Table 9. Classification of adjuvants according to their main mechanism of action. Adapted from (307,308)

The different classes of adjuvants have different capabilities to induce antigen-specific antibodies and/or T cells. For example, mineral salts (e.g., aluminum phosphate, aluminum hydroxide, calcium phosphate) or emulsions (e.g., oil-in-water, water-in-oil, or multiphasic water-in-oil-in-water) are generally more proficient in stimulating an increase

in the antigen-specific antibody response and accompanying T-helper (Th) type 2 response. Whereas toll-like receptor (TLR) agonists (e.g., MPL, ODN) are more capable of inducing a Th1 and cytotoxic CD8+ T-cell response (305,311). Combinations in adjuvant formulations, where adjuvants of different classes are combined (e.g., aluminum salts with MPL), have also been employed to generate a more balanced Th1/Th2 response (312).

Currently, several licensed adjuvants are in use for human vaccines. Most vaccines licensed by EMA and FDA for human use include aluminum salts as an adjuvant, (307,312) (Figure 13). However, classic aluminum salts are not always capable of eliciting the desired immune response and a more complex adjuvant may be required. One of the promising approaches to improving the efficacy of newly developed prophylactic and therapeutic vaccines is the use of innovative adjuvants or combinations of them. As a result, in addition to aluminum, TLR agonists, MPL and CpG 1018 are approved and used as vaccine adjuvants (264). In addition, several other adjuvants are in current use including virosomes, MF59, ISA51, and a line of adjuvant systems (313).

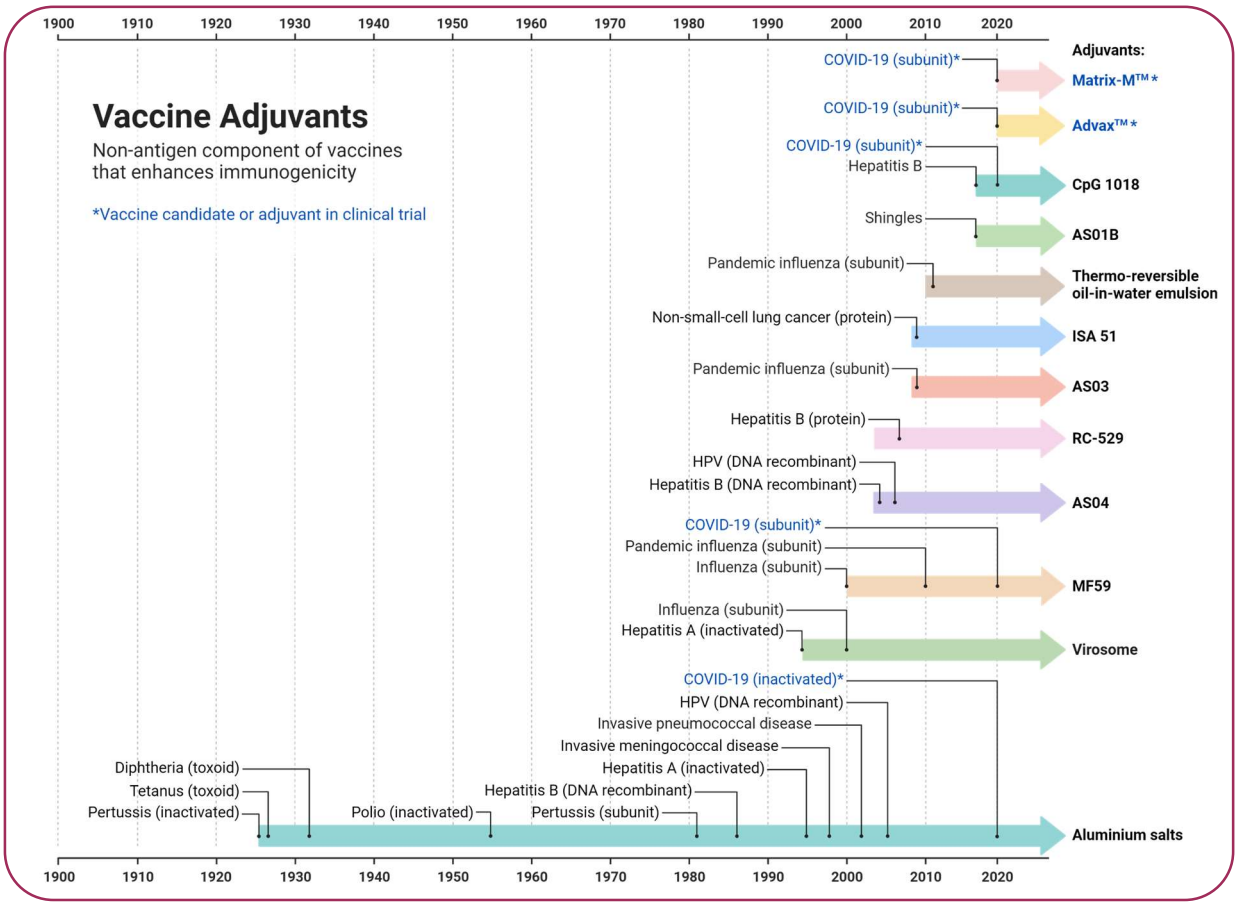


Figure 13. Timeline of adjuvant used in human vaccines. Created with BioRender

HYPOTHESIS AND OBJECTIVES

Hypothesis

Even though FeLV prevalence has decreased in the past 20 years, FeLV continues to be one of the most common and important pathogens in domestic and probably in non-domestic cats worldwide. Considering that in some countries the prevalence of FeLV has plateaued and that none of the available commercial vaccines provide full protection, improvements in testing and vaccination are still necessary.

Recently, our group has developed a novel HIV-1 Gag-based VLP platform, in which the antigen of interest is expressed at a high density on the surface of the VLP by fusing an HIV-1 immunogen to Gag through a transmembrane domain and a short linker. These VLPs induced a potent and functional immune response even in absence of adjuvants and at a low VLP dose (303).

Therefore, we hypothesized that our HIV-based VLP platform could be a good candidate to develop a vaccine against FeLV. Considering that FeLV and HIV belong to the same viral family, we replaced extracellular HIV-1 antigens and HIV-1 Gag by their FeLV equivalents and evaluated the generation of FeLV Gag-based VLPs.

As for HIV-1 Gag-based VLPs, we hypothesized that FeLV Gag-based VLPs will generate relevant humoral and cellular immune responses and that these responses could be enhanced by the addition of adjuvants.

Objectives

The main aim of this project is to generate FeLV Gag-based VLPs as a novel vaccine strategy against this retrovirus.

Hence, the specific objectives to fulfill this aim are:

Objective I: To adapt the HIV-1 Gag-based VLP platform to a FeLV Gag-based VLP.

Objective II. To optimize the production of FeLV Gag-based VLPs.

Objective III. To test the immunogenicity (humoral and cellular responses) of FeLV Gag-based VLPs in murine models.

Objective IV. To maximize humoral and cellular immune responses by identifying optimal adjuvants and antigens.

RESULTS

SECTION 1: Exploring FeLV Gag-based VLPs as a new vaccine platform. Analysis of production and immunogenicity.

FeLV is a common gammaretrovirus infecting domestic and non-domestic cats. In the last two decades, testing, isolation, and vaccination programs helped to decrease the prevalence of FeLV in cats. Although, it has also been described in some countries that this tendency has reached a plateau. Among other measures, it is indispensable a good vaccination program and vaccination at an early age for those cats that are at risk to prevent a fatal outcome of the disease.

As the available commercial vaccines do not provide full protection, there is still room for improvement. In this chapter, we present a novel design of FeLV Gag-based-VLPs. This strategy is based on an analogy to the group's previous work on HIV-1 Gag-based VLPs. First, we adapted the HIV-1 VLP platform to FeLV. We selected a fragment of FeLV envelope glycoprotein, p15E as the antigen to be exposed on the surface of the VLPs. Then, characterization *in vitro* of different FeLV Gag-based VLPs lead us to select the best candidates and analyze their immunogenicity in murine animal models.

Different variables such as the delivery system of the vaccine (DNA or VLP), animal models (C57BL/6 and BALB/c mice), and adjuvants (presence or absence) were also considered.

This chapter corresponds to the manuscript:

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Exploring FeLV-Gag based VLPs as a new vaccine platform. Analysis of production and immunogenicity.

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Keywords: FeLV vaccination, Virus-like Particle, humoral immunity, glycogag, veterinary science

Abstract

Feline leukemia virus (FeLV) is one of the most prevalent infectious diseases in domestic cats. Although different commercial vaccines are available, none of them provides full protection. Thus, efforts to design a more efficient vaccine are needed. Our group has successfully engineered HIV-1 Gag-based VLPs that induce a potent and functional immune response against the HIV-1 transmembrane protein gp41. Here, we propose to use this concept to generate FeLV Gag-based VLPs as a novel vaccine strategy against this retrovirus. By analogy to our HIV-1 platform, a fragment of the FeLV transmembrane p15E protein was exposed on FeLV Gag-based VLPs. After optimization of Gag sequences, the immunogenicity of the selected candidates was evaluated in C57BL/6 and BALB/c mice, showing strong cellular and humoral responses to Gag, but failing to generate neutralizing anti-p15E antibodies. Altogether, this study not only tests the versatility of the enveloped VLP-based vaccine platform but also sheds light on FeLV vaccine research.

Introduction

Feline leukemia virus (FeLV), an enveloped RNA virus belonging to the genus *Gammaretrovirus* of the *Retroviridae* family (30,83), was first identified in the early 1960s by Jarret et al (94,241). Last decade's prevalence of FeLV infection in cats, ranged between 1% and 5,5% depending on the location (USA, Canada, or Southern Europe) (153,171,178,179,314). Even though the prevalence has decreased in the past 20 years due to testing, isolation, and vaccination programs, FeLV is still one of the most common and important pathogen in domestic cats worldwide (183,315). FeLV infection can present with different clinical symptoms: neoplasia, such as lymphomas and leukemia; and non-neoplastic diseases, such as anemia, enteritis, and secondary infections attributable to immunosuppression (111,112,151). Humoral and cellular immune responses have been associated with protection from FeLV infection and disease; however, the mechanisms that determine the infection outcome have yet to be fully resolved (83).

FeLV is considered a simple retrovirus with three genes: *gag*, *pol*, and *env*. The *env* gene encodes the Env viral envelope glycoproteins which is responsible for the specific attachment of the virus to host target cells. It consists of 2 proteolytically cleaved subunits: gp70 (surface, SU, 70 kDa) and p15E (transmembrane, TM, 15 kDa). Both subunits are targeted by the humoral response to generate protective neutralizing antibodies (200,218,219,316). However, following infection and proviral integration into the host's cell genome, the magnitude of viral replication and clinical progression seems to be defined primarily by the cellular response (214,223).

Since FeLV discovery, there have been many efforts to design a vaccine able to induce both humoral and cellular responses (214,223,317). Currently, three vaccine formulations against FeLV are commercially available: i) classical inactivated virus vaccines (245,246); ii) subunit vaccines based on FeLV recombinant antigens (247); and iii) infectious recombinant canarypox virus engineered to express FeLV genes (248,249). However, they all fail to provide full protection against FeLV infection, most likely due to their incapacity to induce a potent neutralizing immune response (243,255,256,318,319). Therefore, new efforts are needed to design more efficient vaccines protecting against infection.

Virus-Like Particles (VLPs) represent an emerging vaccine platform that may enhance both humoral and cellular immune responses, making them a good vaccine candidate against FeLV. VLPs consist in self-assembled complex protein or lipoprotein particles that mimic the structure of native viruses, but do not contain the viral genome and thus

are non-replicative. VLPs can carry antigens in a particulate form and with repetitive surface patterns, making them efficient immunogens (320–323). In particular, VLPs based on Human immunodeficiency virus (HIV) are nanoparticles wrapped by a lipid bilayer and can be generated by the sole expression of the HIV structural Gag protein (274,324). Our group developed a novel approach by directly fusing an antigen of interest to the N-terminal region of HIV Gag. This strategy allowed the generation of VLPs with a high density of immunogen on its surface which could induce a potent and functional immune response even in absence of adjuvants and at a low VLP dose (302,303). Retroviral VLPs could represent an excellent alternative to more conventional vaccine platforms.

Here, we propose to generate FeLV-based VLPs as a novel vaccine strategy against this retrovirus. By analogy to our HIV strategy, and considering that p15E is one of the targets of neutralizing antibodies (218–220,222), a fragment of this protein was exposed on the surface of HIV-based VLPs. As an alternative, we also designed FeLV-Gag based VLPs loaded with the same immunogen, which may elicit not only humoral responses against p15E but also against FeLV Gag (214). After FeLV Gag-based VLP optimization, immunogenicity of the selected candidates was evaluated in two different animal models, C57BL/6 and BALB/c mice, showing a humoral and cellular response against FeLV Gag, but no response against the TM antigen.

Material and Methods

Plasmids

All DNA sequences were synthetized at GeneArt (ThermoFisher Scientific) and cloned into pcDNA3.4-TOPO vector (ThermoFisher Scientific). In addition, they were subcloned into pVAX1 vector (ThermoFisher Scientific) using FastDigest *KpnI* and *XhoI* restriction enzymes (ThermoFisher Scientific). All plasmids were transformed in One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) for plasmid DNA amplification. Plasmids were purified in endotoxin-free conditions using the ZymoPure II Plasmid Maxiprep Kit (Zymo Research) and sterile filtered at 0.22 µm (Millipore). Nucleic acid concentration was measured using NanoDrop One/One (ThermoFisher Scientific), based on the absorbance at 260 nm.

Cell line, culture conditions, and transfection

The Expi293F cell line (ThermoFisher Scientific) was used for protein and VLP production. Cells were cultured in Expi293 Expression Medium (Gibco) at 37ºC, 8% CO_2, and under agitation at 125 rpm. All transfections were performed using ExpiFectamine

transfection kit (Gibco) following the manufacturer's recommendation. Cells and supernatants were harvested 48 h after transfection.

Cell disruption, VLP extraction, and purification

A method to recover intracellular VLPs was adapted from Titchener-Hooker, N et al (325,326). Cell pellet was resuspended in 1 pellet volume (PV) of lysis buffer at 4°C (20 mM Phosphate buffer pH 7.4 (Merck), 2 mM EDTA (ThermoFisher Scientific), 2 mM EGTA (Merck), and Protease Inhibitor (cOmplete™ ULTRA Tablets EDTA-free, Merck).

Cell disruption was carried out by manual homogenization with a tissue grinder (CS1, KIMBLE) for 1 min on ice. After cell disruption, 2 PV of lysis buffer supplemented with 0.2% Triton X-100 were mixed for 4 h at 4°C to ensure VLP release. Centrifugation at 3,000 x g for 15 min was carried out to remove cellular material and contaminating solids. Supernatant was incubated with Amberlite XAD-4 beads (Merck) for 2 h at 4°C, for removal of Triton X-100. Triton-free supernatant was recovered after centrifugation at 800 x g for 5 min.

Supernatant from cell disruption and intracellular VLP extraction was filtered through 0.22 µm pore size (Millipore) for sterility. Samples were further purified by ultracentrifugation in a 70% and 30% double sucrose cushion at 40,000 x g for 2,5 h. Sucrose was removed from the sample by dialysis with Spectra-Por Float-A-Lyzer G2 (Merck) following the manufacturer's recommendation against 1x PBS. Final sucrose concentration in VLP vaccine preparation was expected to be lower than 5%.

Analysis of VLP and protein production

Western Blotting

Samples (15 µg of total protein) were boiled for 5 min at 95°C and subjected to electrophoresis in NuPAGE Bis-Tris 4% to 12% (Thermo Fisher Scientific). Proteins were transferred onto a PVDF membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 h at room temperature with blocking buffer (5% (w/v) non-fat skim milk powder in 1x PBS and 0.05% Tween20). Membranes were incubated overnight at 4°C with the primary antibody anti-FeLV p27 monoclonal antibody [PF12J-10A] (1:2000, Abcam), anti-Flag monoclonal antibody [FG4R] (1:1000, ThermoFisher Scientific) or anti-HIV p24 monoclonal antibody [39/5.4A] (1:2000, Abcam). After washing, incubation with the secondary antibody, HRP-conjugated AffiniPure Donkey anti-mouse IgG (H+L) (1:10000, Jackson ImmunoResearch) was done for 1 h. Membranes were developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) or SuperSignal West Femto

Maximum Sensitivity Substrate (ThermoFisher Scientific), depending on band's signal and according to the manufacturer' s protocol.

Flow Cytometry analysis of VLP expression

Transiently transfected Expi293F cells were analyzed by flow cytometry. VLP-producing cells were stained with APC-labeled anti-DYKDDDDK Tag Antibody (1:500, BioLegend), anti-HIV p24 Gag KC57-FITC monoclonal antibody (1:200, Beckman Coulter), or FITC anti-FeLV p27 polyclonal antibody (1:100, ThermoFisher Scientific). For cell surface protein expression, cells were stained with anti-Tag antibody, then fixed and permeabilized with FIX&PERM (Invitrogen) and stained with the specific anti-Gag antibody. For intracellular staining, cells were fixed and permeabilized before incubation with antibodies. Cells were acquired using a BD FACS LSRII Flow Cytometer with DIVA Software. The flow cytometry results were analyzed using FlowJo™ v10.6.1 Software (BD).

Enzyme-Linked Immunosorbent Assay (ELISA) for VLP quantification

FeLV-based VLP quantification was evaluated indirectly assessing the concentration of p27 by an in-house sandwich-ELISA. Nunc Maxisorp ELISA plates (ThermoFisher Scientific) were coated with 100 µl of anti-FeLV p27 biotinylated polyclonal antibody (1µg/mL) (Abcam) overnight at 4ºC in a wet chamber. Then, plates were blocked using 1x PBS, 1% of bovine serum albumin (BSA, Miltenyi biotech), and 0.05% Tween20 (Merck) for 2 h at room temperature. Recombinant feline leukemia virus p27 (ProSpec) was used as standard. Samples were treated with 0.1x RIPA Buffer (Cell Signaling Technology) for 1 h and added (100 µl) to the plate. Overnight incubation was performed at 4ºC in a wet chamber. After that, incubation with anti-FeLV p27 antibody (PF12J-10A) (1:1000, Abcam) was used as primary antibody for 2 h at room temperature. HRP-conjugated AffiniPure Goat polyclonal anti-mouse IgG (1:10000, Jackson ImmunoResearch) was used as detection antibody. Plates were revealed with o-Phenylenediamine dihydrochloride (OPD) (Sigma Aldrich) and stopped using 2N of H_2SO_4. The signal was analyzed as the optical density (OD) at 492 nm with noise correction at 620 nm. For the quantification of HIV p24 in HIV-based VLPs, INNOTEST HIV Antigen mAb (Fujirebio) assay was performed according to the manufacturer's protocol.

Quantification of Gag content was performed in cellular pellets and culture supernatants. The fraction of Gag in both preparations was calculated.

Transmission (TEM) and Cryo-transmission electron microscopy (Cryo-EM)

Cells producing VLPs were analyzed by transmission electron microscopy (TEM). Briefly, transiently transfected Expi293F cells were fixed with 2.5% glutaraldehyde in PBS 0.1 M for 2 h at 4ºC, post-fixed with 1% osmium tetroxide with 0.8% potassium ferrocyanide for 2 h and dehydrated in increasing concentrations of ethanol. Then, pellets were embedded in epon resin and polymerized at 60ºC for 48 h. Sections of 70 nm in thickness were obtained with a Leica EM UC6 microtome (Wetzlar), stained with 2% uranyl acetate and Reynold's solution (0.2% sodium citrate and 0.2% lead nitrate), and analyzed using a JEM-1400 transmission electron microscope (Jeol Ltd.). All images were taken at 120 kV.

Purified VLP preparations were analyzed by Cryo-EM. VLPs were deposited on a carbon-coated copper grid and prepared using a Leica EM GP workstation (Leica). VLPs were observed with a Jeol JEM-2011 (Jeol Ltd.), equipped with a CCD 895 USC4000 camera (Gatan).

Nanoparticle tracking analysis (NTA)

Quantification of vesicles in each vaccine vial was performed by NTA to assess both particle size and concentration (327) using a Nanosight NS300 instrument (Malvern Panalytical). Vesicles were resuspended in 1x PBS and diluted to the working range of the system (10^6–10^9 particle/ml). Videos were captured using a sCMOS camera and analyzed with the Nanosight NS300 software (version 3.4).

Mice immunization and immunogenicity analyses

All experimental procedures were done under the Spanish and European laws and the Institutional Animal Care and Ethics Committee of the center for comparative medicine and bioimage (CMCiB, Badalona, Spain). They were performed by trained researchers and approved by the regional authorities (Generalitat de Catalunya, Authorization ID: 10583 and ID: 11617). All experimental protocols were performed following the principles of the 3Rs, prioritizing the welfare of the animals used in the research.

DNA immunization was performed in groups of ten six-week-old C57Bl/6JOlaHsd mice (Envigo). Male and female were equally represented in each group. Two doses, at weeks 0 and 3, of sterile endotoxin-free DNA were electroporated intramuscularly at the hind leg (20 µg DNA in physiological saline). Electroporation protocol consisted of 8 pulses of 20 ms with a 1 s interval at 60 V. Prior to each immunization, blood was sampled via facial vein puncture. Serum was recovered from whole blood after coagulation by centrifugation for 10 min at 4000 x g and heat-inactivated for 30 min at 56°C. Euthanasia

of all animals was performed at week 6 when blood samples and spleens of each animal were taken for *ex vivo* immune analysis.

Immunizations with purified VLPs (ranging from $1.27x10^8$ to $1.7x\ 10^8$ particles/dose) or TM peptide (20 µg/dose) were performed in groups of ten six-week-old BALB/cOlaHsd mice (Envigo). Male and female were equally represented in each group. Two doses, at weeks 0 and 3, of purified VLPs were injected at the hock. Different adjuvants were tested: MPLA + $Al(OH)_3$, QuilA + cholesterol + DDABr, MF59, and QuilA + $Al(OH)_3$. Adjuvants were mixed at a ratio 1:1 with VLP preparations. Sample collection and processing were performed as described for the DNA electroporation experiment.

Evaluation of humoral response by ELISA

The levels of antibodies against HIV Gag, FeLV p27, and FeLV p15E in mouse serum samples were determined by an in-house sandwich-ELISA.

For anti-HIV Gag and anti-FeLV p27 antibody quantification, Nunc MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 100 ng/well of recombinant Gag p55(303) or FeLV p27 (ProSpec), and incubated overnight at 4°C in a wet chamber. Then, coated plates were blocked with PBS, 1% of bovine serum albumin (BSA, Miltenyi biotech), and 0.05% Tween20 (Merck) for 2 h at room temperature. Mouse anti-HIV p24 monoclonal antibody (clone 39/5.4A, Abcam) or mouse anti-p27 monoclonal antibody (clone PF12J-10A, Abcam) were used as standards for HIV Gag and FeLV p27 determination, respectively. Blocking buffer-diluted serum samples (1:100 and 1:1000) were added and incubated overnight at 4°C in a wet chamber. Total bound IgG was determined with a secondary HRP-conjugated AffiniPure Goat polyclonal anti-mouse IgG (1:10000, Jackson ImmunoResearch). Plates were developed with o-Phenylenediamine dihydrochloride (OPD, Sigma Aldrich) and stopped using 2N of H_2SO_4 (Sigma Aldrich). The signal was analyzed as the optical density (OD) at 492 nm with noise correction at 620 nm.

For anti-p15E antibody quantification, a recombinant human Fc-fusion protein containing the p15 peptide in the N-terminal region (TM-huIgG) was used as antigen. Nunc MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 100 ng/well of Pure goat anti-human IgG Fc (Jackson ImmunoResearch) and incubated overnight at 4°C in a wet chamber. Then, plates were blocked using PBS, 1% BSA, and 0.05% Tween20 for 2 h at room temperature. After that, plates were incubated with TM-huIgG for 2 h at room temperature. Mouse anti-human IgG_1 Fc (Merck) was used as standard. The rest of the method followed the same scheme as for anti-Gag HIV Gag and anti-FeLV p27.

Design and production of TM-huIgG

TM-huIgG used for p15E quantification is a fusion protein designed in-house. We inserted the p15E fragment (residues 541 to 582 of Env FeLV) into a huCD4-huIgG(328). It was cloned using FastDigest *KpnI* and *NheI* restriction enzymes. Supernatant of transiently transfected Expi293F cells was used directly for the in-house ELISA. The fusion protein was characterized by Western Blot using an HRP-AffiniPure Goat polyclonal anti-human IgG (Jackson ImmunoResearch) and a rabbit polyclonal anti-TM antibodies and quantified by ELISA (30 µg/mL) as described elsewhere (328) (Figure 14).

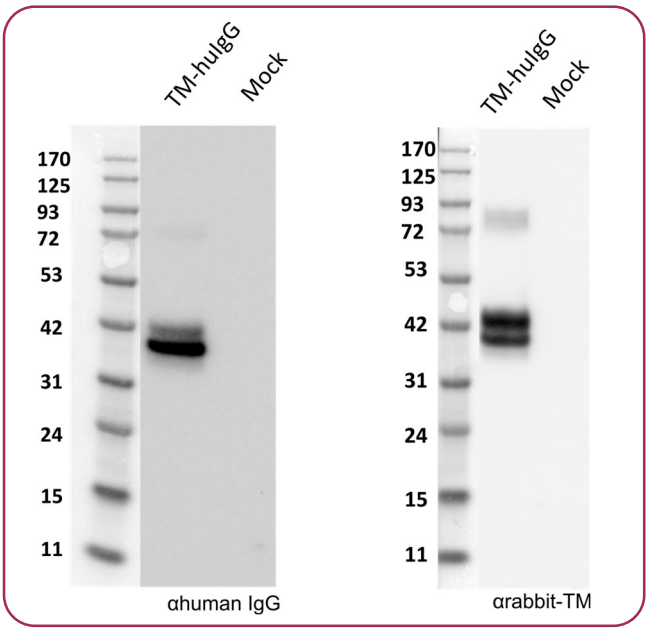

Figure 14. Western Blot of TM-huIgG. Western blot developed with anti-human IgG or anti-TM antibodies to analyze the expression of TM-huIgG fusion protein transiently transfected in Expi293F cells.

Evaluation of cellular immune responses by ELISpot

Splenocytes from vaccinated animals were seeded at $0.4x10^6$ cells/well in ELISpot white PVDF plates (Merck) precoated with 0.2 µg/well of anti-mouse IFN-γ antibodies (AN18; Biolegend) and blocked with 10% FBS-supplemented RPMI (R10). Cells were stimulated overnight at 37°C and 5% CO_2 with either recombinant p27 protein at 14 µg/mL (ProSpec), p15E peptide at 14 µg/mL (residues 541 to 582 of Env FeLV), or a pool of 10 overlapping Gag 15-mer peptides (covering residues 314 - 412) at a concentration of 14 µg/mL per peptide. Concanavalin A (ConA; 7µg/mL; Merck) and R10 alone were used as positive and negative controls, respectively. IFN-γ secretion was detected with an anti-mIFN-γ biotinylated-mAb (clone R4-6A2, 1:2000, Biolegend) and streptavidin-AP (1:2000, Mabtech) and developed with AP Conjugate Substrate Kit (Bio-Rad) following manufacturer's protocol.

Evaluation of anti-Expi293 humoral responses by flow cytometry

The presence of antibodies targeting proteins at the surface of Expi293F cells in immunized mice sera was determined by flow cytometry with BD FACS LSRII Flow Cytometer. In short, Expi293F cells were incubated with mouse sera samples (1:1000) and stained with AlexaFluor647 Goat anti-mouse IgG Fc (1:400, Jackson Immunoresearch). Flow cytometry results were analyzed using FlowJo™ v10.6.1 Software (BD).

Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc.) and R v4.1.1. Comparisons in immunogenicity were tested including undetectable data (under LOD) using the Peto-Peto rank test for cross-sectional comparisons and Petro-Pentrice generalized Wilcoxon test for longitudinal ones. Multiple comparisons were adjusted using Benjamini & Hochberg method (FDR). For all analyses, a P-value of less than or equal to 0.05 was considered significant.

Results

Generation of HIV and FeLV-based Virus-Like Particles

Our group has developed an HIV-based VLP vaccine platform in which a small fragment of HIV envelope glycoprotein gp41 subunit was fused via its transmembrane domain to the N-terminal part of HIV Gag protein (303). This strategy allows for a high-density display of the antigen on the surface of VLPs (a 1:1 ratio with the Gag protein, about 2500 copies of HIV Gag are estimated per VLP particle (295)). To study the versatility of our platform and adapt it as a FeLV-based VLP vaccine, we selected p15E of FeLV envelope glycoprotein as the antigen to be presented on the surface of the VLPs.

To characterize FeLV-based VLPs and compare them to HIV-based VLPs, two different recombinant proteins based on each retrovirus were designed. A fragment of the p15E protein (residues 542 to 610) of FeLV Envelope glycoprotein, including the membrane spanning domain (MSD), was fused to either HIV Gag (TM-HIV) or FeLV Gag (TM-FeLV) (Figure 15A). Both fusion proteins contained a signal peptide (SP) and a Flag Tag at the N-terminus (Figure 15A). Additionally, two control fusion proteins containing the SP, the Flag Tag, the MSD, and the respective Gag but lacking the extracellular part of the p15E fragment, were designed as controls for immunization experiments (Figure 15A).

Expression of recombinant fusion proteins in transiently transfected Expi293F cells was analyzed by western blot using antibodies targeting the TM antigen (Flag) and Gag (HIV p24 or FeLV p27). The results showed a band of the expected molecular weight

(asterisks in Figure 15B, P lanes), indicating that the full protein was properly expressed (TM-HIV = 67.1 kDa; TM-FeLV = 69.2 kDa). Remarkably, the recombinant proteins were hardly detected in the supernatant, except for FeLV Gag containing proteins (Figure 15B, SN lanes) indicating that the fusion proteins might be retained inside the cells and not secreted to the extracellular media. In addition to the main band corresponding to the whole fusion protein, several low weight molecular bands were detected, probably due to protein processing or degradation. Low molecular weight fragments were differentially identified in both constructs suggesting that Gag from HIV and FeLV were not equally processed (Fig. 15B). In this sense, a 35KDa fragment was detected in the cell lysate lane of TM-FeLV using the anti-Flag antibody that was not observed in TM-HIV (Figure 15B). In line with that, when TM-FeLV was analyzed with an anti-p27 antibody a clear band of 42 kDa was present in the supernatant of the cell culture (Figure 15B), probably corresponding to the other part of the cleaved protein observed with the anti-Flag antibody. This observation was confirmed when the two control fusion proteins lacking the p15E protein were also transiently transfected in Expi293F cells. Both proteins were expressed at their expected molecular weight (Control-HIV = 62.1 kDa; Control-FeLV = 64.3 kDa, Figure 15C). Similar to Figure 15B, when developed with an anti-p27 antibody, a prominent 42 kDa band was observed in the supernatant of Control-FeLV fusion protein, reinforcing the idea of the proteolytic cleavage of Gag (Figure 15C).

In order to evaluate the localization of the expressed proteins, transiently transfected Expi293F cells were stained at the cellular surface with an anti-Flag antibody and intracellularly with anti-HIV p24 or anti-FeLV p27 antibodies, showing that the extracellular fragment of the fusion protein, including the TM immunogen, was barely exposed on the surface of Gag positive cells (Figure 15D). Instead, a much stronger signal was detected when staining for p27 was performed intracellularly, further supporting the intracellular retention of all fusion proteins (Figure 15D). In addition, quantification of HIV p24 and FeLV p27 by ELISA of the cell lysates and supernatants of the transiently transfected cells showed that more material was present in the cell lysate, 83% in TM-HIV and 74% in TM-FeLV (Figure 15E).

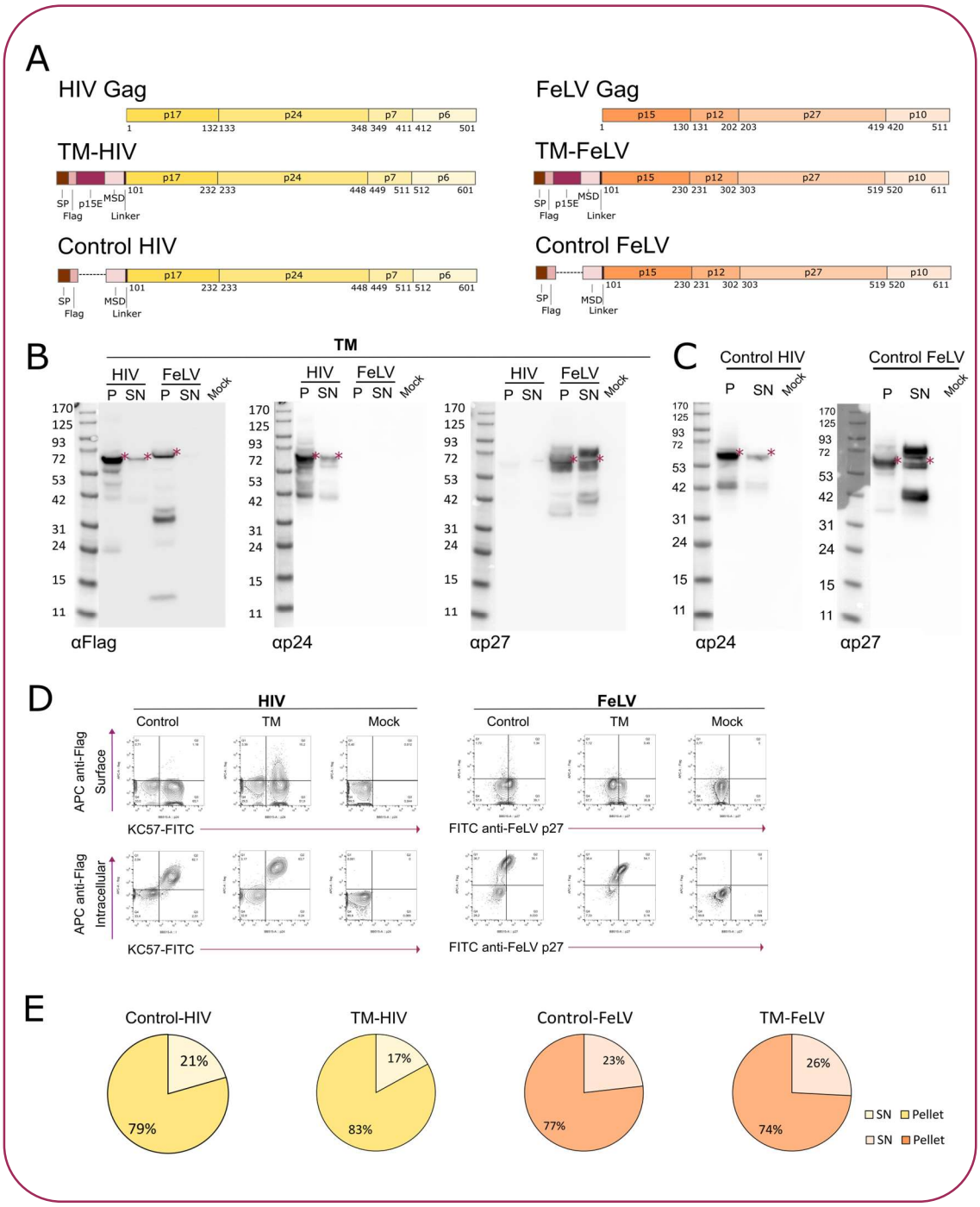

Figure 15. Design and characterization of HIV and FeLV fusion proteins. (A) Schematic representation of the fusion proteins TM-based VLPs with HIV Gag or FeLV Gag. A control fusion protein for each Gag is also represented. SP: Signal Peptide; MSD: membrane spanning domain. **(B)** Western blot analysis of the expression of TM-based fusion proteins transiently transfected in Expi293F cells. Asterisk indicates the expected molecular weight. P: Cell lysate; SN: Supernatant **(C)** Western blot analysis of the expression of Control fusion proteins transiently transfected in Expi293F cells. Asterisk indicates the expected molecular weight. **(D)** Representative flow cytometry panels for extracellular and intracellular expression of the fusion proteins detected with an anti-Flag antibody and anti-p24 or anti-p27 antibody. **(E)** Schematic representation of p24 and p27 quantification by ELISA of the cell lysates and supernatants of the transiently transfected Expi293F cells.

In order to confirm the intracellular retention of all fusion proteins, we evaluated whether VLP assembling, and budding were occurring intracellularly or not. For that, transiently transfected Expi293F cell pellets were fixed and analyzed via TEM. Both FeLV- and HIV-based VLPs were found to accumulate in the perinuclear area at the rough endoplasmic reticulum (RER) of producing cells, showing a similar morphology and diameter (Figure 16). The confirmation of the presence of intracellular VLPs led us to adapt a VLP extraction protocol from Titchener-Hooker, N et al (325,326). The extracted material was visualized by cryo-EM, showing again spherical VLPs with a similar morphology among all of them, and a slightly higher diameter for HIV-based (120nm) than for FeLV-based VLPs (100nm, Figure 16).

Taken together, these data confirmed that both TM-HIV and TM-FeLV VLPs are produced but retained inside the cells. In addition, a large proportion of FeLV-Gag proteins undergo truncation. As explained in the following section, this truncation could be related to the presence of proteolytic cleavage sites associated with glycogag formation during FeLV life cycle (119).

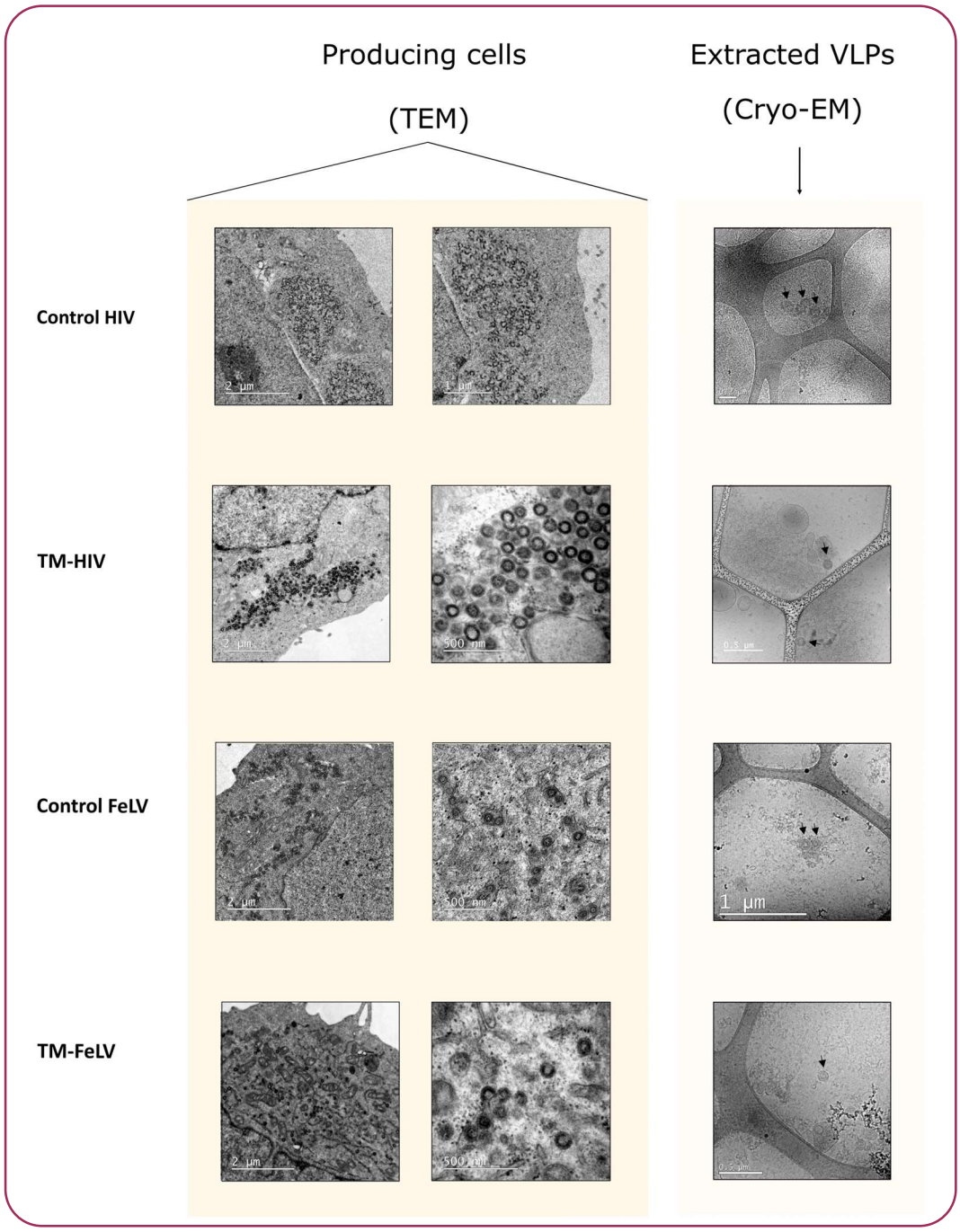

Figure 16. TEM and Cryo-EM of control and TM-Gag fusion proteins. TEM images were obtained from transiently transfected Expi293 cells expressing the indicated VLPs. Cryo-EM images were obtained using purified VLPs.

Sequential deletion of Gag to reduce proteolytic cleavage.

With the aim to reduce TM-FeLV processing we tested two different signal peptides: human GMCSF (MWLQSLLLLGTVACSIA) and the immunoglobulin H5 SP (MDWTWRFLFVVAAATGVQS). No differences in VLP production, cellular localization and VLP morphology were noticed (Figure 17), indicating that the signal peptide had no impact in protein processing.

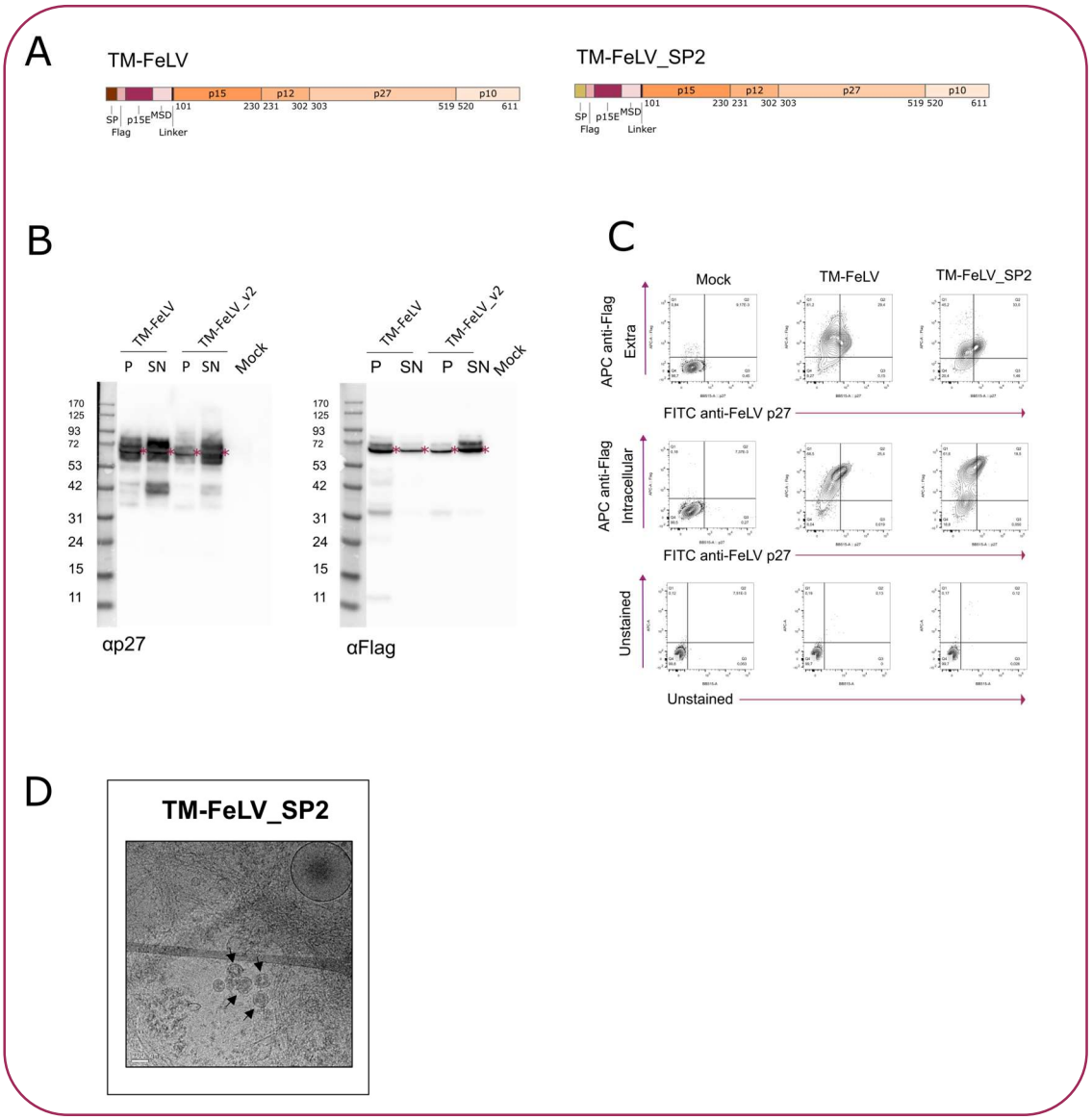

Figure 17. Comparison between two different signal peptides. (A) Schematic representation of the fusion proteins TM-based VLPs with two different signal peptides (SP). TM-FeLV present GMCSF SP and TM-FeLV_SP2 fusion protein present the immunoglobulin H5 SP. **(B)** Western blot was developed with an anti-Flag or anti-p27 antibodies to analyze the expression of TM-based fusion proteins transiently transfected in Expi293F cells. Asterisk indicates the expected molecular weight. **(C)** Representative flow cytometry panels for extracellular and intracellular detection of the fusion proteins using anti-Flag and anti-p27 antibodies. **(D)** Cryo-EM image of the extracted and purified VLPs from cell pellet are shown.

FeLV, as well as other gammaretroviruses, such as MuLV and KoRV (129,149), generates an alternative form of the Gag polyprotein by an alternative translation of the initiation codon (129), resulting in a secreted and glycosylated form of the Gag polyprotein (glycogag) (329). Upon translation, FeLV glycogag is translocated to the plasma membrane of the infected cell, where it is cleaved and the C-terminal product of 40 kDa is released (126). To reduce the possible proteolytic cleavage of FeLV Gag and considering that matrix (MA or p15) is dispensable for retroviral particle formation(330–

332), four new fusion proteins were designed including three N-terminus Gag deletions (TM-ΔMA, TM-Δ153, and TM-ΔMA-Δp12) and one rearrangement, in which the p12 subunit was shifted to the C-terminus (TM-ΔMA-p12CT), mimicking the HIV Gag organization domains: HIV p6 is located in the C-terminus and has a similar function to p12 of FeLV(333,334) (Figure 18A).

Initial characterization by western blotting of cell lysates and culture supernatants of transiently transfected Expi293F cells indicated that all four proteins were expressed at their expected molecular weight (TM-ΔMA = 54.6 kDa; TM-Δ153 = 51.9 kDa; TM-ΔMA-p12CT = 54.6 kDa; TM-ΔMA-Δp12 = 47.3 kDa) (Figure 18B). The corresponding bands were identified in both culture fractions when developed with anti-Flag and anti-p27 (FeLV) antibodies (Figure 18B). A faint band at 20 and 15 kDa were identified at the cell lysate fraction of TM-ΔMA and TM-Δ153, respectively, when the western blot membrane was developed with an anti-Flag antibody (Figure 18B). The same fusion proteins showed a 42 kDa band at the supernatant of the cell culture when western blot membrane was developed with an anti-p27 (FeLV) antibody (Figure 18B). Both bands of each fusion protein suggest that the deletion of the first 153 amino acids on FeLV Gag is not sufficient to avoid glycogag formation and the subsequent protein cleavage. In contrast, when analyzing the cell lysate of the fusion proteins TM-ΔMA-p12CT and TM-ΔMA-Δp12, no cleavage bands were observed when developed with anti-Flag antibody, nor the 42 kDa band on the cell culture supernatant when developed with an anti-p27 antibody (Figure 18B).

We also evaluated the possible retention of the fusion proteins inside the cells by flow cytometry. Transiently transfected Expi293F cells were surface or intracellularly stained with an anti-Flag antibody and intracellularly stained with an anti-p27 antibody (Figure 18C). Flag was mainly detected when intracellular staining was applied, suggesting that all the fusion proteins were retained inside the cells and were not reaching the plasma membrane, similarly to TM-FeLV (Figure 18C). When quantifying p27 by ELISA, the different fusion proteins were mainly accumulated inside the cells (>70% in all cases) (Figure 18D), reinforcing our previous observation that the fusion proteins are retained inside the cells.

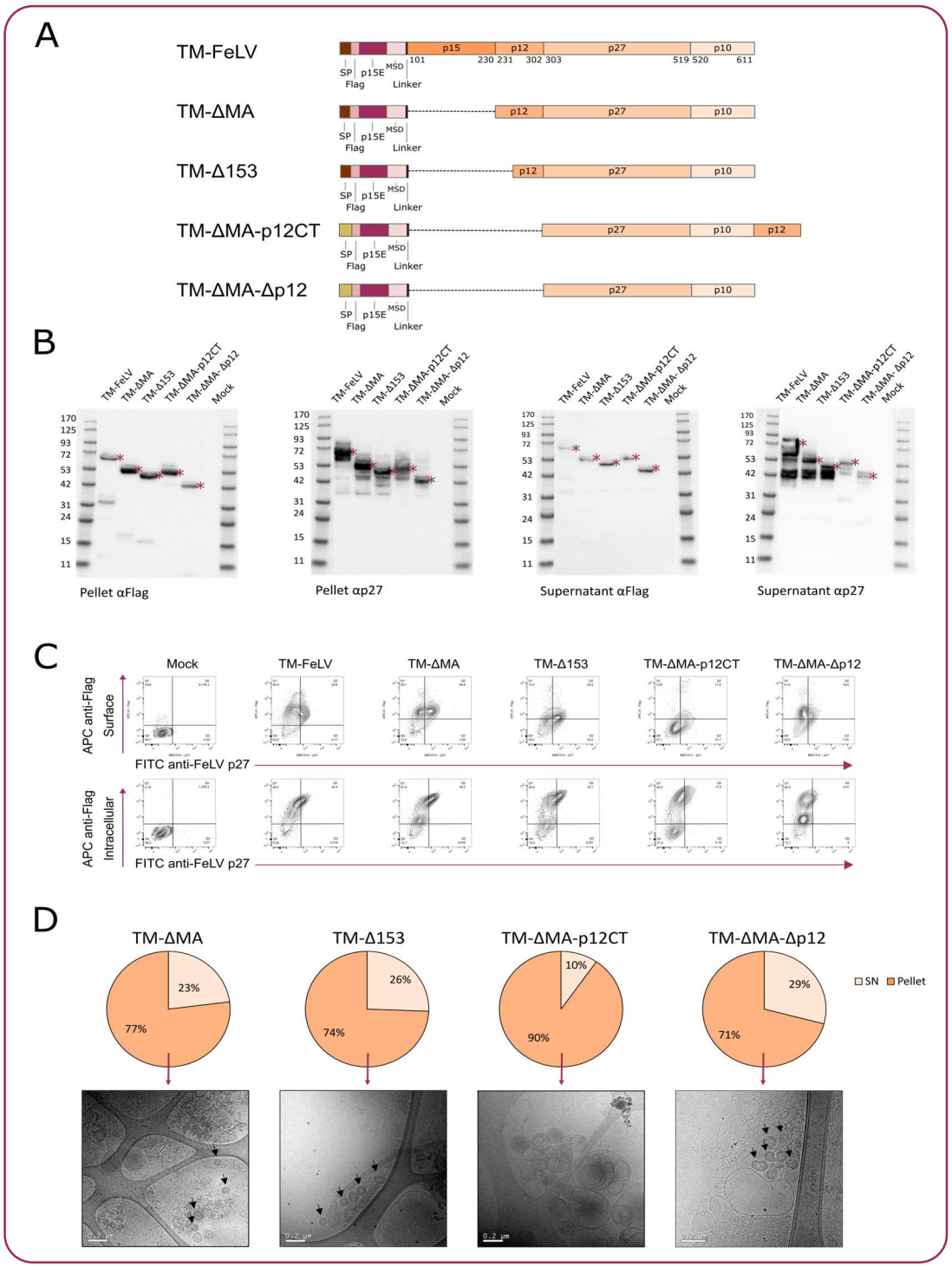

Figure 18. Design and characterization of TM-Gag fusion proteins lacking gag subdomains. (A) Schematic representation of the modifications for each fusion protein. The screening was performed using two sets of signal peptides (as indicated). SP: Signal Peptide; MSD: membrane spanning domain. **(B)** Western blot was developed with an anti-Flag or anti-p27 antibodies to analyze the expression of TM-based fusion proteins transiently transfected in Expi293F cells. Asterisk indicates the expected molecular weight. P: Cell lysate; SN: Supernatant **(C)** Representative flow cytometry panels for extracellular and intracellular detection of the fusion proteins using

anti-Flag and anti-p27 antibodies. **(D)** p27 quantification of the cell lysates and supernatants of the transiently transfected Expi293F cells. Cryo-EM images of the extracted and purified VLPs from cell pellet are shown.

Further characterization by Cryo-EM of extracted VLPs from transiently transfected Expi293F cells showed that TM-ΔMA and TM-Δ153 generated VLPs with similar morphology to TM-FeLV (Figure 16-18D). However, no VLPs were detected for TM-ΔMA-p12CT fusion protein, and heterogenous VLPs were observed for TM-ΔMA-Δp12 (Figure 18D). Taken together, these data indicate deletions removing the cleavage site in Gag impact on VLP formation.

Improving FeLV-based VLP production

Previous results suggest that FeLV-Gag cleavage site is located in MA or p12 sequences. In order to identify the protease responsible for the cleavage of FeLV-Gag, as well as its putative cleavage sites, we submitted the protein sequence to the MEROPS database (335). Different cleavage sites possibilities were given and ADAMDEC1 protease was referenced as the possible protease responsible for the cleavage at positions 198-201 of our recombinant protein (336,337). Furthermore, we identified a late domain in this region corresponding to residues 126-130, sequence LYPVL that could be the target of the viral protease (338,339). Therefore, new fusion proteins were designed: 1) TM-VA, with two mutations L199V and R200A in FeLV-Gag ; 2) TM-ΔProR, deletion of the late domain region (FeLV-Gag residues 128 to 153); 3) TM-ΔProR-VA, deletion of the late domain site plus the double mutation; 4) TM-ΔMA-VA, deletion of the full matrix plus the double mutation; and 5) TM-Δ153-VA, deletion of the region containing first 153 aa of FeLV-Gag plus the double mutation (Figure 19A).

Expression of recombinant fusion proteins in transiently transfected Expi293F cells was analyzed by western blot, showing specific bands at the expected molecular weight (TM-VA = 69.2 kDa; TM-ΔProR = 66.5 kDa; TM-ΔProR-VA = 66.5 kDa; TM-ΔMA-VA = 54.6 kDa; TM-Δ153-VA = 51.9 kDa). All recombinant proteins were identified in both fractions of cell cultures, cell lysates and supernatants (Figure 19B). Even though all FeLV-Gag modified fusion proteins showed a 42 kDa band at the supernatant when revealed with anti-p27 (FeLV), only TM-VA and TM-ΔProR-VA did not show a clear band of around 30 kDa (TM-FeLV and TM-ΔProR) or around 20 kDa (TM-ΔMA-VA and TM-Δ153-VA) when the cell lysate was revealed with anti-Flag antibody (Figure 19B). This suggests that TM-VA and TM-ΔProR-VA reduce the cleavage of FeLV-Gag.

All fusion proteins examined were retained inside the cells, as suggested by Figure 19C in which intracellular staining with anti-p27 and anti-Flag antibodies of transiently transfected Expi293F cells, showed similar levels of expression (around 25% of double

positive cells detected). In addition, all cell cultures presented more than 60% of p27 in the cell lysate when quantified by in-house ELISA (Figure 19D), while a slightly lower percentage was observed for TM-ΔProR (53%). To confirm the formation and presence of FeLV-based VLPs in cell extracts, cryo-EM was performed on all extracted samples (Figure 19D) and used as a decision criterion to evaluate VLP presence and morphology. Even though FeLV-based VLPs were observed for all fusion proteins, TM-ΔProR-VA and TM-VA showed a more homogeneous morphology.

Taken together, these data suggest that TM-ΔProR-VA and TM-VA fusion proteins promote the expression of uncleaved, morphologically correct intracellular FeLV-based VLPs. Therefore, we retained both as vaccine candidates for subsequent analyses.

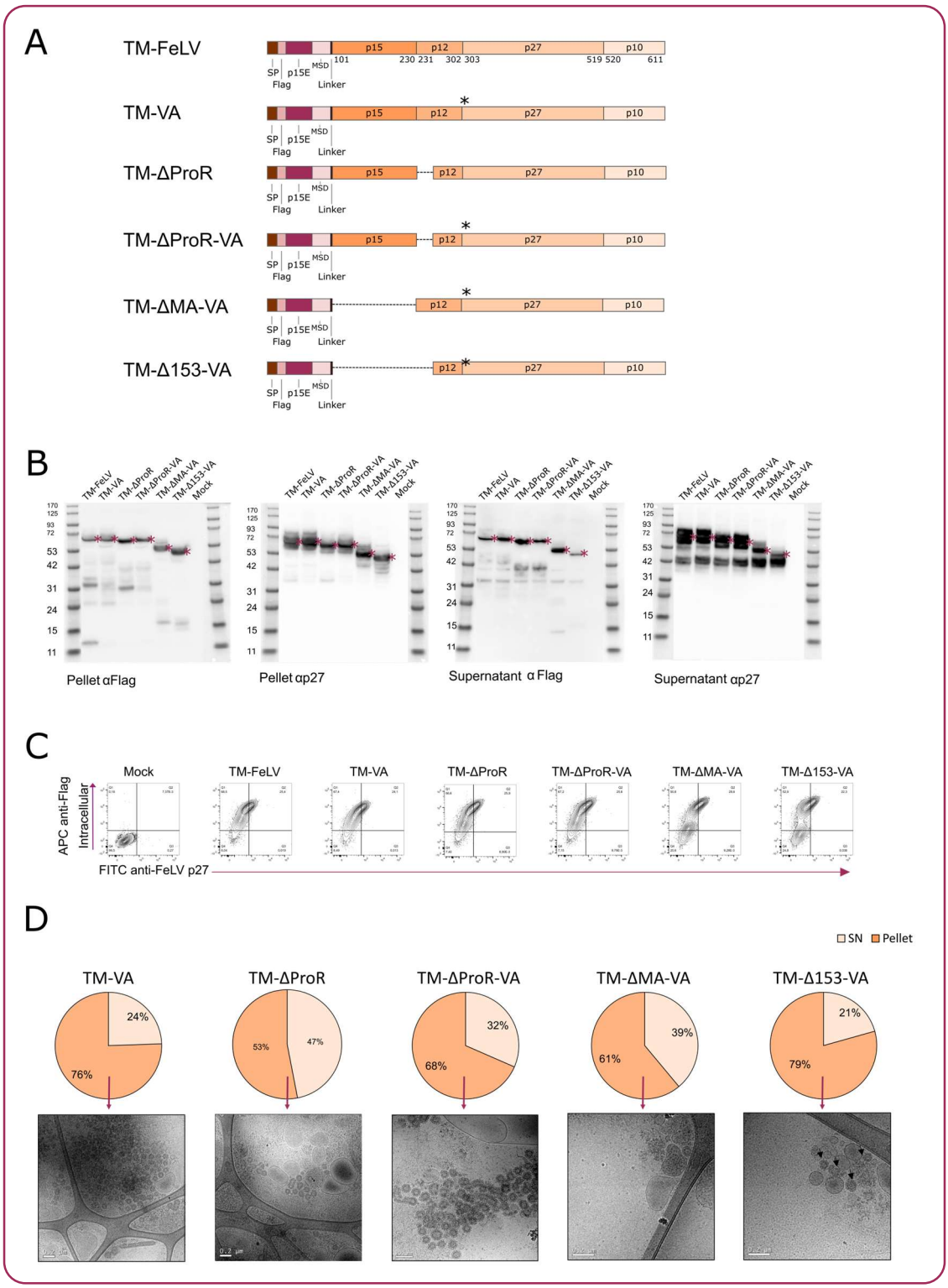

Figure 19. Design and characterization of TM-Gag fusion proteins with altered Gag processing. **(A)** Schematic representation of the modifications for each fusion protein. Asterisk indicates two mutations of L>V and R>A. SP: Signal Peptide; MSD: membrane spanning domain. **(B)** Western blot developed with an anti-Flag or anti-p27 antibodies to analyze the expression of TM-based fusion proteins transiently transfected in Expi293F cells. Asterisk indicates the expected molecular weight. P: Cell lysate; SN: Supernatant **(C)** Representative flow cytometry panels for intracellular detection of the fusion proteins

using anti-Flag and anti-p27 antibodies. **(D)** p27 quantification of cell lysates and supernatants of the transiently transfected Expi293F cells. Cryo-EM images of the extracted and purified VLPs from cell pellet are shown

Immunogenicity of TM-FeLV VLPs in C57BL/6 mice

In order to evaluate the immunogenicity of FeLV-based VLPs, C57BL/6J mice were electroporated intramuscularly with plasmid DNA encoding the fusion proteins TM-HIV, TM-FeLV, TM-VA, and TM-ΔProR-VA. We also included the two controls Control-HIV and Control-FeLV. Prior to immunization, all fusion proteins were transiently transfected in Expi293F cells and cellular pellets were imaged by TEM to ensure VLP formation (Figure 20). Animals were vaccinated with two doses of plasmid DNA (20 µg/dose), at weeks 0 and 3, and end point of the experiment was set at week 6 post-first immunization (Figure 21).

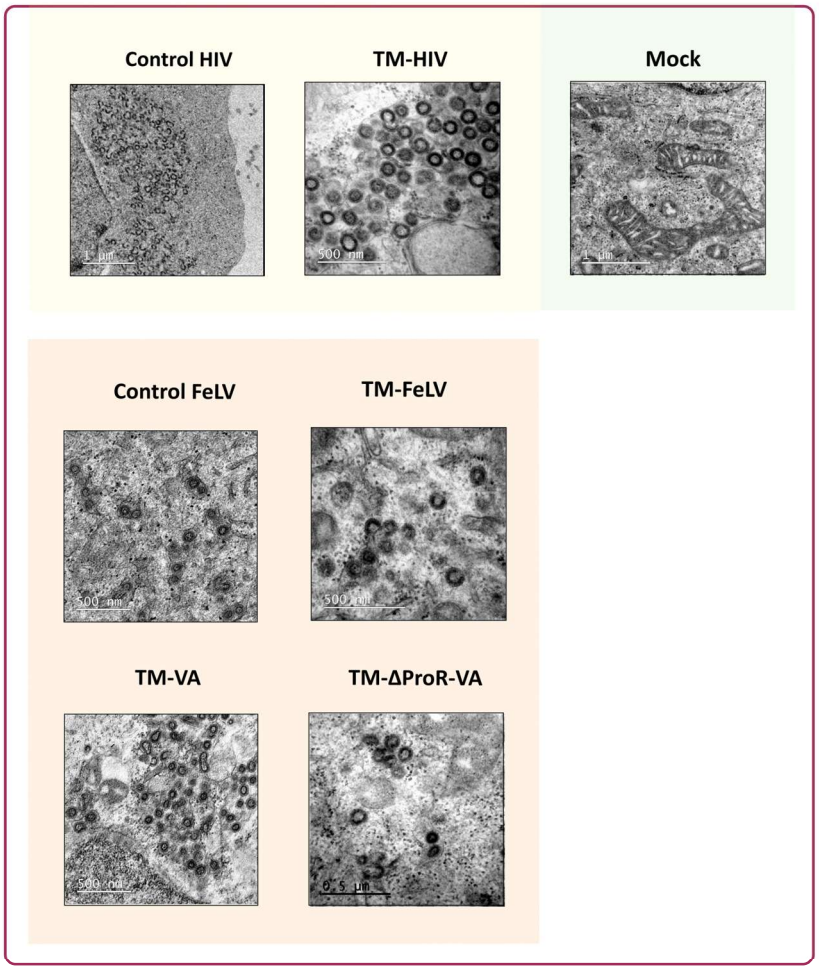

Figure 20. TEM of Expi293 cells expressing HIV or FeLV-Gag fusion proteins. Intracellular VLP formation was evaluated in transient transfected Expi293 cells by TEM. Cells transfected with an empty pcDNA3.4 plasmid were used as control (Mock).

First, we evaluated the humoral response against the TM antigen using an in-house ELISA. Neither HIV- nor FeLV-based VLP vaccinated animals generated detectable antibodies against TM (Figure 21B). To understand these results, we tested the possible immunodominant role of the Flag tag in the fusion protein; however, no humoral response against this epitope was detected in any group. Control-HIV and TM-HIV were included in the analysis as negative controls, and as expected, no humoral response was generated in these groups (Figure 21C). Next, we analyzed the humoral response against Gag proteins. Analysis of the response against FeLV p27 showed a homogeneous signal in all FeLV-based VLPs vaccinated groups. All groups reached a plateau after a single dose of electroporated DNA, which was maintained three weeks after the second immunization (Figure 21D). No statistically significant differences were observed among FeLV-based VLPs vaccinated groups. Similarly, the humoral response against HIV p24 was analyzed by an in-house ELISA assay, and it followed a similar kinetics, reaching comparable levels in TM-HIV and Control-HIV groups (Figure 21E). Very low or no responses against FeLV-Gag were observed in HIV-based VLPs vaccinated animals and vice versa, confirming the specificity of the detected elicited responses (Figure 21D-E).

Finally, the elicitation of cellular responses against TM, p27 (FeLV), and p24 (HIV) was evaluated by IFNɣ ELISpot assay using cryopreserved splenocytes from all vaccinated groups. Consistent with the lack of humoral responses against TM, none of the VLPs, neither FeLV nor HIV, were able to generate a detectable T-cell response against this protein, but both were able to generate a T-cell response against FeLV-Gag (p27 protein) or HIV-Gag (p24 protein), respectively (Figure 21F).

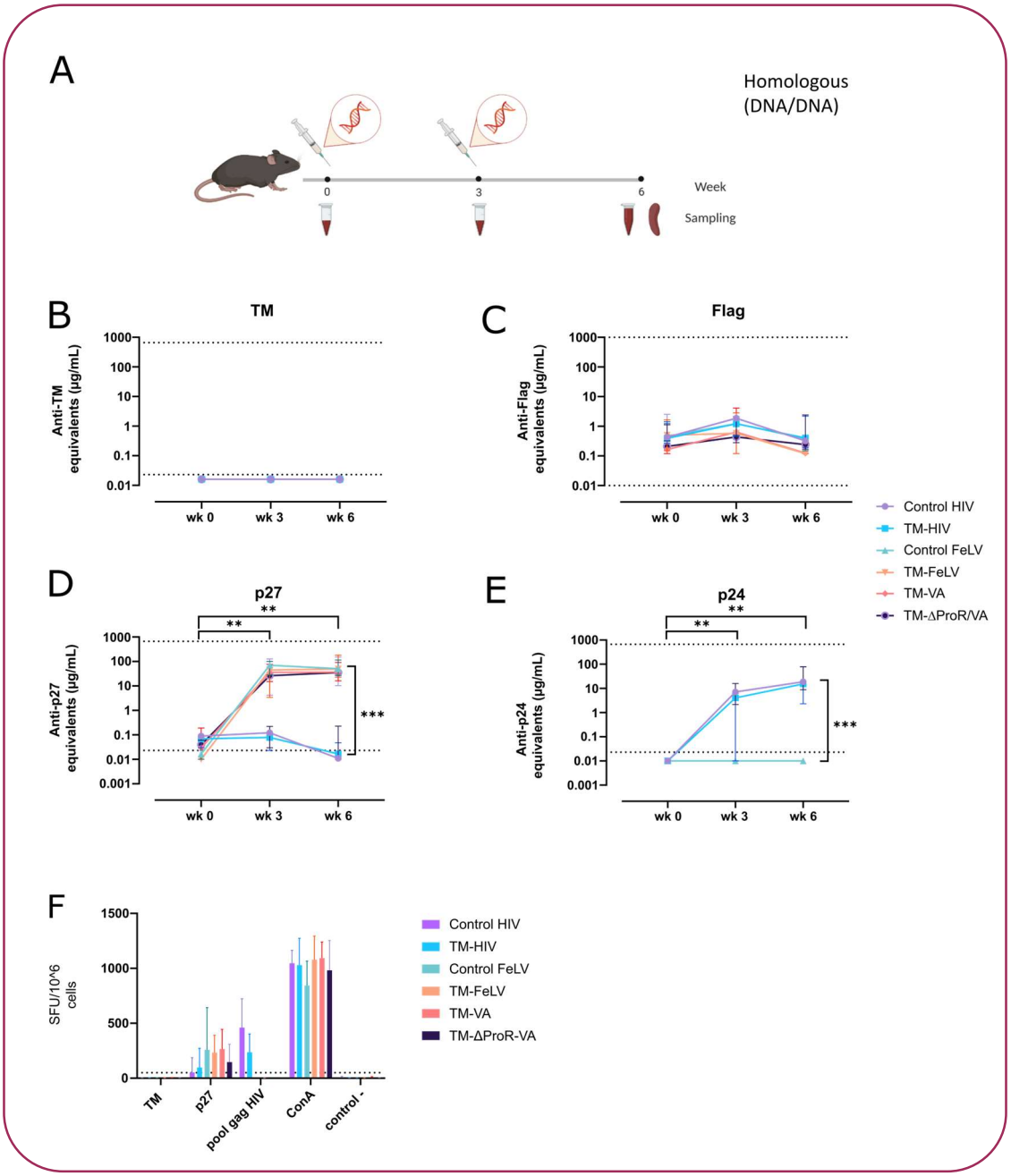

Figure 21. Immunogenicity of TM-FeLV VLPs in C57Bl/6. (A) Graphical scheme of the experimental procedure. C57Bl/6 mice were electroporated twice with 20ug of DNA. Antibodies titters against TM **(B)**, Flag **(C),** p27 **(D)**, and p24 **(E),** are shown. Data is presented as Median with 95% CI of antibody concentration in mouse sera. Statistical differences were found using the Peto-Peto rank test for wk6 and Paired Prentice-Wilcoxon test for longitudinal comparison (*p<0.05; **p<0.01; ***p<0.001). (F) T-cell responses against TM, p27, and p24 were evaluated by IFNɣ ELISpot. Data is presented as Mean with SD.

Altogether, these data indicate that both HIV-based and FeLV-based VLPs generated humoral and cellular responses against their respective Gag protein. In contrast, no humoral nor cellular responses were observed against the TM antigen. Such poor

responses might be due to the restrictive nature of C57BL/6 mice model (340,341), along with the immunization regime (DNA) chosen for this experiment.

Immunogenicity of TM-FeLV VLPs in BALB/c mice

In order to evaluate whether the lack of generation of immune responses against TM in C57BL/6 was strain specific, we also tested BALB/c mice, a model that tends to produce a stronger humoral response than the C57Bl/6 strain (341). BALB/c mice were immunized in a VLP/VLP regime allowing us to add adjuvants to the FeLV-based VLP vaccine preparations in order to enhance immune responses (Figure 23A).

FeLV-based VLPs were extracted from transiently transfected Expi293F cells and purified by double cushion sucrose ultracentrifugation (Figure 22A). Purified material was then analyzed by NTA, which allowed for quantification (ranging from to $1,7x10^{10}$ particles/mL) and calculation of particle size (ranging from 155nm to 180nm in diameter) (Figure 22B). Western blot evaluation of purified fractions confirmed the presence of all fusion proteins at their expected molecular weight (Figure 22C).

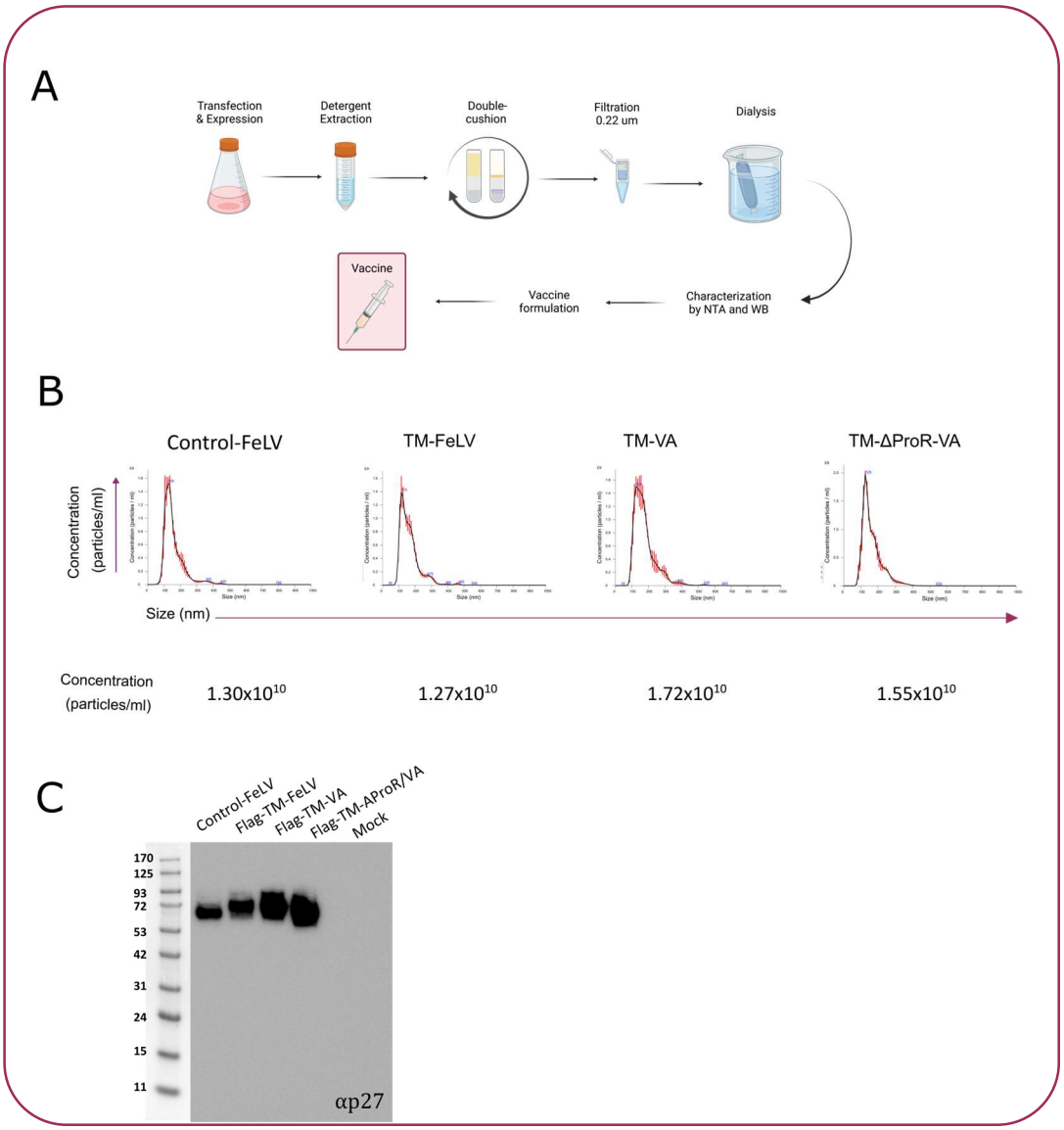

Figure 22. Quantification and analysis of VLPs by NTA, and vaccine preparation. (A) Graphical scheme of the experimental procedure for the preparation of VLPs vaccines: production, extraction, purification, quantification, and vaccine formulation. **(B)** Estimation of size and concentration of the final preparation performed by NTA. **(C)** Western blot developed with anti-p27 antibody to analyze the expression of the vaccine preparation before formulation.

In a first set of experiments, the TM-ΔProR-VA vaccine candidate was formulated with different adjuvants: i) MPLA + $Al(OH)_3$, ii) QuilA + cholesterol +DDABr, iii) MF59, and iv) QuilA + $Al(OH)_3$. A synthetic TM peptide (residues 541 to 582 of Env FeLV) formulated with MPLA + $Al(OH)_3$ and a non-adjuvanted TM-ΔProR-VA VLP preparation were also included as controls. BALB/c mice were immunized subcutaneously at the hock following a homologous VLP regimen of two doses, at weeks 0 and 3. Experimental endpoint was set at week 6 post-first immunization (Figure 23A).

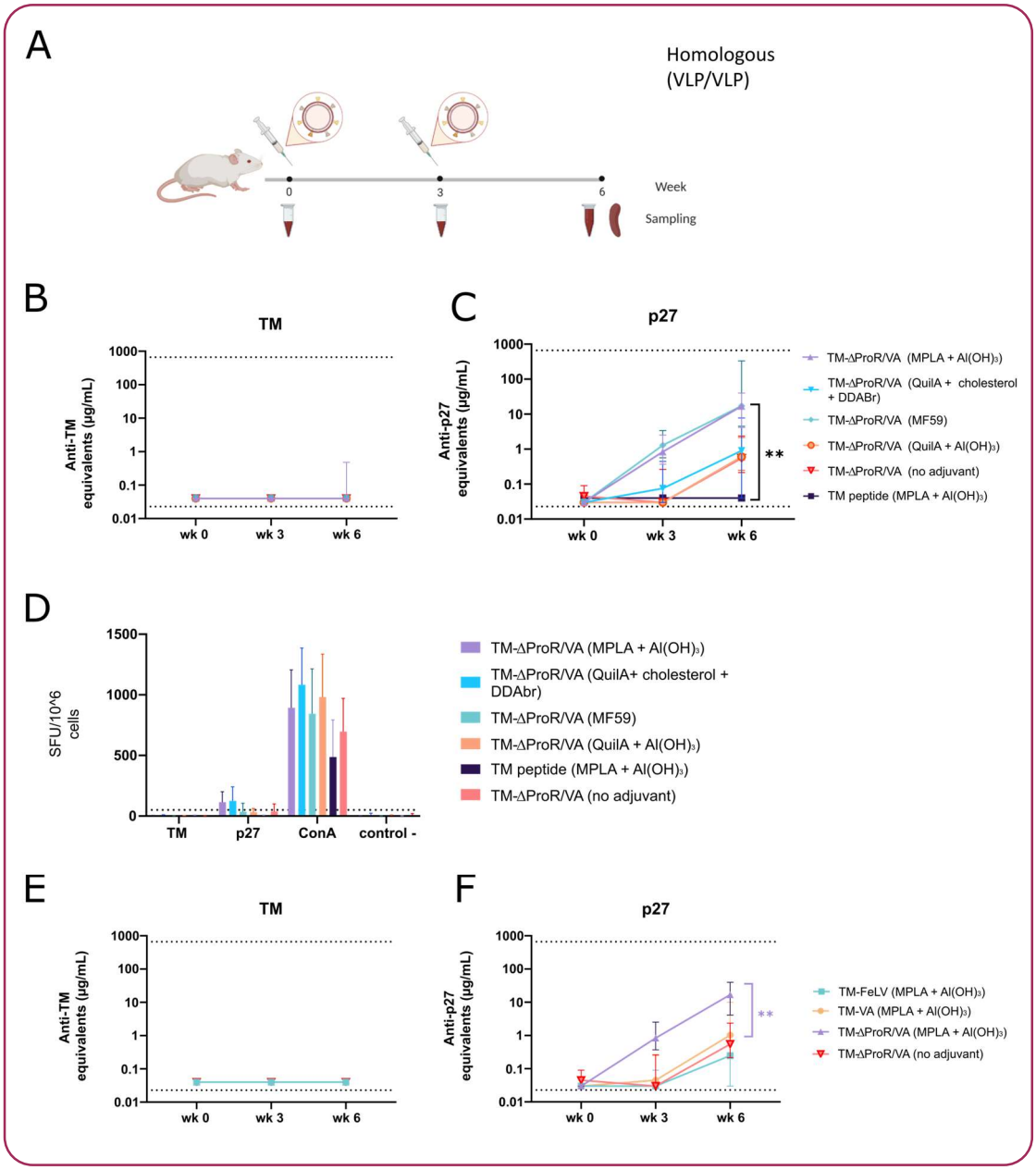

Figure 23. Immunogenicity of TM-FeLV VLPs in BALB/c mice. **(A)** Graphical scheme of the experimental procedure. BALB/c mice were immunized twice with purified and adjuvanted VLPs. Antibodies titters against TM **(B)** and p27 **(C)** are shown. Data is presented as Median with 95% CI of antibody concentration in mouse sera. Statistical differences were found using Peto-Peto rank test for wk6 (*p<0.05; **p<0.01; ***p<0.001). **(D)** T-cell responses against TM and p27 were evaluated by IFNɣ ELISpot. Data is presented as Mean with SD. Humoral responses against TM **(E)** and p27 **(F)** when immunizing with different VLPs adjuvanted with MPLA + Al(OH)3 are shown. Data is presented as Median with 95% CI of antibody concentration in mouse sera. Statistical differences were found using Peto-Peto rank test for wk6 (*p<0.05; **p<0.01; ***p<0.001).

We evaluated the humoral response against TM antigen showing that no humoral immune response against the TM antigen was induced in any of the tested conditions (Figure 23B). Even though all vaccine formulations increased the antibody titter against

p27 after the second immunization, TM-ΔProR-VA vaccine candidate formulated with MF59 and MPLA + $Al(OH)_3$ induced a statistically significant difference compared to the non-adjuvanted group (Figure 23C). Further, T cell responses against TM and p27 were evaluated by IFNɣ ELISpot using cryopreserved splenocytes from all vaccinated groups (Figure 23D). Unfortunately, none of the tested adjuvants was able to enhance a T cell response against TM. However, a T-cell immune response was generated against p27 in MPLA + $Al(OH)_3$ and QuilA + cholesterol +DDABr groups. Taken together and regardless of the immune response against TM antigen, the highest humoral and cellular immune responses against p27 were generated by the animals vaccinated with TM-ΔProR-VA formulated with MPLA + $Al(OH)_3$. Consequently, we have kept MPLA + $Al(OH)_3$ as the adjuvant combination to test in further experiments.

As FeLV-based VLPs are produced in Expi293F cells, a human cell line, we expected that Expi293F proteins (of human origin) were incorporated into the VLPs and could be immunogenic in mice. The analysis of the humoral response against Expi293F cell proteins was performed for all experimental groups by Flow Cytometry (Figure 24A). The data suggest that the adjuvants inducing a higher humoral response against p27, are also the ones that induce higher titers of antibodies against the Expi293F cells, specifically the MF59 adjuvant. Animals immunized with the synthetic TM peptide served as negative control.

Once the optimal adjuvant was selected, we screened the different VLP candidates. In a second experiment, BALB/c mice were immunized subcutaneously following the previous protocol (Figure 23A), with three FeLV-based VLPs: TM-FeLV, TM-VA, and TM- ΔProR-VA (Figure 20) adjuvanted with MPLA + $Al(OH)_3$; a non-adjuvanted TM-ΔProR-VA was included as a control. Again, humoral response against TM was negative for all fusion proteins (Figure 23E). However, antibody titers against p27 were detectable in all vaccinated groups (Figure 23F). Statistically significant differences were observed between TM-ΔProR-VA adjuvanted with MPLA + $Al(OH)_3$ and the other experimental groups (Figure 23F), confirming that this is the most immunogenic preparation. Analysis of the humoral response against Expi293F cell proteins was performed by Flow Cytometry (Figure 24B), showing a similar response between the three adjuvanted VLP preparations. In contrast, non-adjuvanted VLPs elicited a weak response against the Expi293F cells.

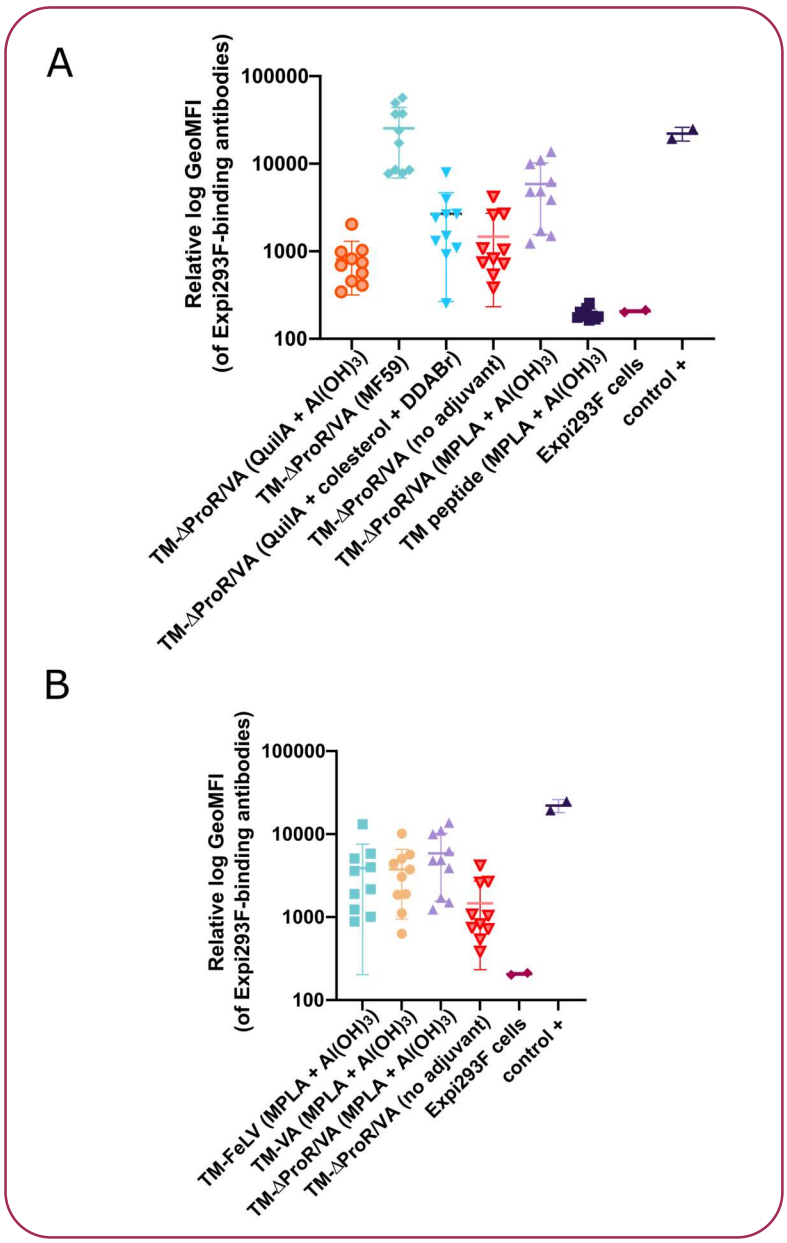

Figure 24. Humoral immune response against Expi293F host cell proteins. Levels of IgG against Expi293F host cell proteins analyzed by flow cytometry. Data presented as geometrical Mean Fluorescence Intensity (Geo-MFI) ± SD. **(A)** Response of immunization with TM-ΔProR-VA plus different adjuvants. **(B)** Response of immunization with the selected fusion proteins adjuvanted with MPLA + Al(OH)3.

To sum up, homologous TM-ΔProR-VA VLPs immunization regimen in BALB/c mice successfully induced humoral responses against p27, especially when MPLA + $Al(OH)_3$ or MF59 are used as adjuvants; however, this formulation fails to induce immune responses against the FeLV TM immunogen, confirming the poor immunogenicity of this Env fragment.

Discussion

Several vaccines are available for prevention of FeLV infection, but none provides full protection (112). Therefore, new vaccine platforms are needed for the development of a successful FeLV vaccine. Enveloped Gag-based VLPs mimic the virus morphology and therefore may display viral immunogens in a more natural manner and in a higher density. This potentiate immune responses against weak immunogens (270,342). Several VLP-based vaccines for humans are commercially available including vaccines against Human Papilloma Virus (HPV) (343) and Hepatitis B Virus (HBV) (344). Several other VLP-based vaccines are currently undergoing preclinical and clinical development (272). Here we propose an innovative design for a FeLV-based VLP vaccine. This is to our knowledge the first study of FeLV Gag-based VLP vaccine.

Recently, we have developed a high-density platform of HIV-based VLPs, where the antigen of interest is exposed on the surface of the VLPs by fusing an HIV-1 immunogen to Gag through a transmembrane domain and a linker. Considering these results (303), we hypothesized that our VLP platform could be versatile enough and could be adapted as a vaccine for other retroviruses. Specifically, we focused on FeLV retrovirus, not only because there is room for improvement regarding the commercial vaccines available, but also because of previous descriptions of neutralizing FeLV antibodies directed against the TM subunit of Env glycoprotein being similar and comparable to that described for HIV (345). In that way, FeLV is not only of interest to veterinary practice but could also be a relevant model to help understand HIV immunology.

First, we decided to use p15E of FeLV Env as the immunogen presented on the VLP surface considering that the literature indicates that virus-neutralizing antibodies (VNAs) have been found to target epitopes located in that region (218–220,222). A fragment of the p15E protein was fused to HIV-Gag or to FeLV-Gag. After characterization of these fusion proteins by western blot, flow cytometry, and ELISA, we concluded that the designed fusion proteins were successfully produced, even though VLPs were retained inside the cells not reaching the extracellular compartment. Moreover, we noticed that a large proportion of FeLV Gag-based fusion proteins underwent truncation as a band of 42 kDa was observed in the supernatant of transfected cells. We hypothesized that this instability may be originated from the existence of a glycosylated form of Gag of FeLV, a remarkable difference between FeLV and HIV Gag proteins. It has been described that FeLV has two starting codons in Gag. One of them encodes for the polyprotein precursor of the internal capsid protein and the other encodes for an alternative type II transmembrane glycoprotein form, known as glycogag, which has an expected

molecular weight of 80 kDa and a soluble cleavage product of 40 kDa. (118–122). Glycogag is dispensable for virus replication but appears to be an important virulence determinant (129). It is involved in a late step of viral budding or assembly (124,127). Our fusion proteins are structurally similar but probably dissimilar to glycogag. Therefore, the potential expression of our immunogen as type II transmembrane proteins (suggested by the presence of large amounts of a 42 kDa Gag fragment in cell culture supernatant), could have a detrimental impact on of FeLV Gag-based VLPs production. To avoid this potential effect, we explored different strategies to stabilize FeLV-Gag, firstly by sequential deletions of several Gag domains and then by analyzing *in silico* the proteolytic cleavage site. We performed several N-terminus modifications such as deletion of specific regions of MA or p12, and we introduced mutations to avoid the proteolytic cleavage site. TEM confirmed that our fusion proteins were capable of producing VLPs, but they were retained into the cell. Nevertheless, FeLV Gag-based VLPs could be successfully extracted from transiently transfected Expi293F cells. Microscopy images were used as a selection criteria to identify the best vaccine candidates and test them in *in vivo* models.

Immunogenicity studies were performed in two different animal models C57BL/6 and BALB/c mice, with two different regimens: two doses of DNA or two doses of purified FeLV-based VLPs. Moreover, different adjuvants were added to enhance immune responses induced by FeLV-based VLPs. Comparison between VLP regimen and DNA regimen showed similar profiles of humoral responses. Although high antibody titers against FeLV-Gag were elicited, no antibodies against TM were detected. Absence of humoral and cellular response against TM could be due to the small size of the protein, unproper epitope expression on the surface of the VLPs, or to the immunosuppressive function of p15E, although the reported immunosuppressive domain was excluded of our immunogen. (346–349).

In vivo experiments demonstrate that FeLV Gag-based VLPs are immunogenic when administered as DNA vaccine or as purified VLPs. From all fusion proteins designed and tested we conclude that the best fusion protein producing VLPs and inducing a good humoral and cellular response is TM-ΔProR-VA. Even if high titers of antibodies are elicited without adjuvant when animals are immunized with DNA, we tested different adjuvants to analyze if they could induce a higher cellular response. Two adjuvant formulation, MPLA + $Al(OH)_3$ and MF59 were eliciting higher titers of antibodies against p27 FeLV Gag. However, MF59 is a squalene-based oil-in-water and we do not know how this could affect VLP's morphology; moreover, based on cellular immune response, we can observe that the best formulation includes MPLA + $Al(OH)_3$ in the final vaccine.

Taken together, our results confirm the versatility of our VLP platform, being able to adapt it to other retroviruses such as FeLV. Even though no response against the surface antigen loaded on the FeLV-based VLPs was generated, biologically relevant humoral and cellular responses against p27 (FeLV Gag capsid) are elicited. For this reason, it is necessary to explore other FeLV antigens maintaining the FeLV Gag-based VLPs as a vaccine platform.

As there is no response against the antigen on the surface, future perspectives could include trying to generate new FeLV Gag-based VLPs presenting more complex immunogens, such as larger antigens, which are characterized by their ability to induce better immune responses against other neutralizing epitopes. However, cellular responses against Gag are reported (214,223), reinforcing that of our FeLV-VLPs are good vaccines candidates as we elicited cellular response against FeLV Gag.

Conflict of Interest

ABarreiro, AG, MC and LF are employees of HIPRA. IrsiCaixa received an institutional grant from HIPRA to conduct the work. Outside this work, BC, JB and JC are founders and shareholders of AlbaJuna Therapeutics, S. L.

Author Contributions

RO wrote the paper and performed research; ABarajas, AP-G, BT, FT-F, CR, performed the research, VU performed statistical analysis; ABarreiro, AG, MC, LF, BC, JC, CA-G and JB designed the research and laboratory experiments, supervised experimental design, analysis and interpretation of data. All authors read and approved the final manuscript.

Funding

This work has been funded by HIPRA (Spain). This work has also been funded by Secretaria d'Universitats i Recerca of Generalitat de Catalunya and the European Social Fund through the project 2022 FI_B 00698.

Acknowledgments

The quantification of the particle concentration and size on the final vaccine preparations by NTA has been performed by the ICTS "NANBIOSIS", more specifically by the Unit 6. Unit of the CIBER in Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) at the Barcelona Materials Science Institute. We are grateful to CMCiB staff for their excellent

technical help with animal care. We would also like to thank the staff from the Servei de Microscòpia of Universitat Autònoma de Barcelona.

SECTION 2: Production and immunogenicity of FeLV-based VLPs exposing a stabilized envelope glycoprotein.

In the last section result, we demonstrated the versatility of our HIV-1 Gag-based VLP platform. Not only we were capable to produce intracellular FeLV Gag-based VLPs but optimized the production of FeLV VLPs by stabilizing FeLV Gag with several N-terminus modifications and different mutations. After different *in vitro* techniques, we were able to identify the best vaccine candidates and test them in *in vivo* models.

By analogy to our previous work on HIV-1 Gag-based VLPs, we selected a fragment of p15E FeLV envelope glycoprotein Even though we tested different vaccine regimens (DNA/DNA or VLP/VLP), different mice animal models, and in the presence or absence of adjuvants, unfortunately, we couldn't generate a humoral response against p15E. However, the selected FeLV VLPs candidates showed a strong cellular and humoral response against p27 (FeLV Gag capsid). Therefore, our hypothesis now is whether different antigen on the VLP's surface will induce antibodies against the immunogen presented on the VLP.

As VNAs have been found against the surface unit (SU) envelope glycoprotein gp70 (247), we design a FeLV Gag-based VLP presenting SU on the surface. Unfortunately, when produced *in vitro*, a fragmentation of SU was observed (data not shown). Therefore, in this section, we present a novel strategy displaying a more complex antigen, such as a modified FeLV Env, on the surface of VLPs to induce a better neutralizing immune response.

This chapter corresponds to the manuscript:

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Production and immunogenicity of FeLV-based VLPs exposing a stabilized envelope glycoprotein

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Keywords: Env, vaccine, VLP, FeLV, SOSIP, veterinary science.

Abstract

The Envelope glycoprotein complex (Env) of retroviruses, such as the Feline leukemia virus (FeLV), is the main target of the host humoral immune response, and therefore a major vaccine antigen candidate. Presenting FeLV Env, along with other viral proteins, to elicit a full humoral and cellular protective response may mimic natural immune responses, which may fully control infection and disease progression.

Based on HIV-1 research, and the strategy to develop an immunogen in a particular soluble native Env trimer, here we present a novel Stabilized FeLV Env (SFE) immunogen. Characterization of SFE soluble protein and its presentation on the surface of FeLV Gag-based VLPs was performed. Immunization assays in C57BL/6 mice showed a poor humoral and cellular response against FeLV Env, but potent responses against FeLV Gag were detected.

Introduction

A major challenge in the development of vaccines against retroviruses is the induction of neutralizing antibodies. Such antibodies bind to the viral envelope glycoprotein complex (Env) and prevent viral entry into target cells and the subsequent integration of the provirus into the cellular genome, where it may persist (350). Retroviral Env is composed of two highly N-linked glycosylated proteins, known as surface (SU) and transmembrane (TM) subunits (18). These proteins orchestrate the fusion of the viral and the host cell membranes, SU acts as the receptor recognizing subunit and TM harbors the membrane fusion machinery (111). Therefore, envelope glycoproteins represent a major target for the host immune response.

Feline leukemia virus (FeLV) is a simple retrovirus infecting cats, among other felines (112). Its Env consists of two subunits, gp70 (SU) and p15E (TM) forming trimers (351). FeLV is one of the few examples of a retroviral infection from which some infected cats can completely recover (151). Furthermore, FeLV is one of the few retroviruses for which commercial vaccines exist (83,243,249,254,319). However, available vaccines do not provide full protection and can cause adverse reactions, such as Feline injection site sarcomas (FISS) (258). Therefore, efforts to develop more efficient and safe vaccines continue.

The Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus, whose Env is a trimeric protein complex composed of gp120 (SU) and gp41 (TM) subunits that are held together by weak non-covalent interactions (352,353). Unfortunately, after 40 years of the discovery of HIV-1, there is no available vaccine yet. Different platforms and strategies, like soluble proteins, multivalent platforms, and nucleic acid vaccines encoding for the pathogen-derived antigen, have been in the spotlight of research (354). Immunization with unmodified Env proteins has proven to be insufficient to generate a protective response, therefore, more sophisticated immunogens have been engineered to present key epitopes targeted by broadly neutralizing antibodies (bNAb) (355,356). The initial hypothesis was that stable trimeric proteins displaying Env's native conformation would be a better immunogen than individual subunits (357–359). Specifically, soluble-stabilized gp140 with I559P (SOSIP) is one of the prototypes which presents Env in a stable native conformation by the introduction of a disulfide bridge between subunits and the addition of one proline residue (357,360,361). Unfortunately, thus far, the induction of bNAbs after immunization with these variants in combination with adjuvants remains challenging (362,363).

An alternative approach to favor the elicitation of bNAbs is to optimize the antigen delivery (269). Among different strategies, the multimeric presentation of antigens on the surface of nanoparticles to induce an immune response comparable to natural infections is an attractive option (364–366). Specifically, virus-like particles (VLPs) have been used to express and display HIV-1 Env (367,368). Our previous work developed a novel approach to optimize antigen delivery by directly fusing an antigen of interest to the N-terminal region of HIV-1 Gag. This strategy allowed the generation of VLPs with a high density of immunogen on its surface with the potential to induce a potent immune response even in absence of adjuvants and at a low VLP dose (302,303).

By analogy to the HIV-1 Gag-based VLP platform, we have recently generated FeLV Gag-based VLPs as a novel vaccination strategy against this retrovirus (369). Considering that p15E is one of the targets of neutralizing antibodies (218–220,222,350), a fragment of this protein was exposed on the surface of the FeLV Gag-based VLPs. After optimization, the immunogenicity of the selected FeLV Gag-based VLPs candidates was evaluated in two different animal models, C57BL/6 and BALB/c mice, showing a strong humoral and cellular response against p27 (FeLV Gag capsid), but no response against the p15E antigen (369).

Here, we hypothesized that presenting a more complex immunogen, such as a Stabilized FeLV Env (SFE) on the surface of VLPs would induce a better neutralizing immune response. By analogy to HIV-1 research on engineered Env, we applied the concept of SFE soluble protein to FeLV Env, as a novel approach to present this antigen on the surface of the FeLV Gag-based VLPs. Immunogenicity of SFE VLPs was evaluated in C57BL/6 mice.

Material and Methods

Plasmids

Fusion proteins were designed by concatenating (from N' terminus to C' terminus) a Signal Peptide (SP), the antigen of interest, the FeLV Env membrane spanning domain (MSD), a short GS linker, and the selected FeLV Gag sequence. All DNA sequences were synthesized at GeneArt (ThermoFisher Scientific) and cloned into pcDNA3.4-TOPO vector (ThermoFisher Scientific). They were also subcloned into pVAX1 vector (ThermoFisher Scientific) using FastDigest *KpnI* and *XhoI* restriction enzymes (ThermoFisher Scientific). All plasmids were transformed in One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) for plasmid DNA amplification. Plasmids were purified in endotoxin-free conditions using the ZymoPure II Plasmid Maxiprep Kit (Zymo Research)

and sterile filtered at 0.22 µm (Millipore). Nucleic acid concentration was measured using NanoDrop One/One (ThermoFisher Scientific) and based on the absorbance at 260 nm.

Cell line, culture conditions, and transfection

The Expi293F cell line (ThermoFisher Scientific) was used for protein and VLP production. Cells were cultured in Expi293 Expression Medium (Gibco) at 37ºC, 8% CO_2, and under agitation at 125 rpm. All transfections were performed using ExpiFectamine transfection kit (Gibco) following the manufacturer's recommendation. Cells and supernatants were harvested 48 h after transfection. For SFE soluble protein, supernatant was harvested 96 h after transfection.

Design, production, and purification of SFE soluble protein

SFE soluble protein was designed based on a sequence alignment of HIV-1 Env and FeLV-A Env (Uniprot: P04578 and P08359, respectively). Sequence alignment was performed by Pairwise Sequence Alignment using the Needleman-Wunsch algorithm from EMBL-EBI (370).

Supernatant of SFE soluble protein expressed in transiently transfected Expi293F cells was purified by ion-metal affinity chromatography (IMAC). Culture supernatant, supplemented with 0.5 M NaCl and 5 mM imidazole, was incubated with Ni Sepharose™ excel beads (Cytiva) overnight at 4ºC with end-over-end mixing, then transferred to a PolyPrep Chromatography Column (Bio-Rad). Once the Ni Sepharose beads settled by gravity, the column was washed with 1x PBS, 40 mM imidazole, and 0.5 M NaCl. Next, protein was eluted from the column with 1x PBS and 500 mM imidazole. The eluted sample was concentrated, and buffer exchanged to 1x PBS using a 30 kDa Amicon Ultra-15 Centrifugal Unit (Merck). Protein concentration was measured using NanoDrop One/One (ThermoFisher Scientific), based on the absorbance at 280 nm.

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Size exclusion–high performance liquid chromatography (SE-HPLC) was performed to assess the molecular weight and oligomerization state of pure SFE soluble protein. The sample was subjected to SE-HPLC using an Alliance 2695 HPLC System (Waters) and an XBridge BEH 200 Å column (7.8 mm x 300 nm, 3.5 µm) (Waters), flow rate of 0.5 mL/min at room temperature (RTemp). Standard sample was BEH200 SEC Protein Standard Mix (Waters). Instrument control, data acquisition, and compilation of results were performed using Empower 2 software (Waters).

Analysis of VLP and protein production

Identification by Western Blot and Coomassie Blue

Samples (15 µg of total protein) were boiled for 5 min at 95°C and subjected to electrophoresis in NuPAGE Bis-Tris 4% to 12% acrylamide (ThermoFisher Scientific). Proteins were transferred to a PVDF membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 h at RTemp with blocking buffer (1x PBS, 0.05% Tween20 (v/v) and 5% (w/v) non-fat skim milk powder). Membranes were incubated overnight at 4°C with the primary antibody anti-FeLV p27 monoclonal antibody [PF12J-10A] (1:2000, Abcam), or anti-gp70 monoclonal antibody [C11D8] (1:2000, ThermoFisher Scientific). After washing with 1x PBS, 0.05% Tween20 (v/v), incubation with the secondary antibody, HRP-conjugated AffiniPure Donkey anti-mouse IgG (H+L) (1:10000, Jackson ImmunoResearch) was allowed for 1 h. Membranes were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) according to the manufacturer's instructions. Chemiluminescence was detected with ChemiDocTM MP Imaging System (Bio-Rad). For Coomassie staining, proteins were treated the same as Western Blot protocol. Protein gels were incubated in SimpleBlue SafeStain (ThermoFisher Scientific) for 1 h at RTemp on a rocker. Then, the excess was washed twice with dH_2O for 1 h and overnight at RTemp.

Flow Cytometry analysis of recombinant protein expression

Transiently transfected Expi293F cells were analyzed by flow cytometry. VLP-producing cells or soluble protein were stained with the primary antibody an anti-gp70 monoclonal [C11D8] (1:2000, ThermoFisher Scientific) and AlexaFluor647 goat anti-mouse IgG Fc as a secondary antibody (1:500, Jackson ImmunoResearch), and/or FITC anti-FeLV p27 Gag polyclonal antibody (1:100, ThermoFisher Scientific). For cell surface protein expression, cells were stained with the primary antibody an anti-gp70, and a secondary antibody AlexaFluor647, then fixed and permeabilized with FIX&PERM (Invitrogen) and stained with the anti-p27 antibody. For intracellular staining, cells were fixed and permeabilized before incubation with antibodies. Cells were acquired using a FACS LSRII Flow Cytometer (BD) with DIVA Software (BD). Flow cytometry results were analyzed using FlowJo™ v10.6.1 Software (BD).

Transmission (TEM) and Cryo-transmission electron microscopy (Cryo-EM)

Cells producing VLPs were visualized by TEM. Transiently transfected Expi293F cells were fixed with 0.1 M PBS and 2.5% glutaraldehyde for 2 h at 4ºC, post-fixed with 1% osmium tetroxide with 0.8% potassium ferrocyanide for 2 h and dehydrated in increasing

concentrations of ethanol. Then, pellets were embedded in epon resin and polymerized at 60ºC for 48 h. Sections of 70 nm in thickness were obtained with a Leica EM UC6 microtome (Wetzlar), stained with 2% uranyl acetate and Reynold's solution (0.2% sodium citrate and 0.2% lead nitrate), and analyzed using a JEM-1400 transmission electron microscope (Jeol Ltd.). All images were taken at 120 kV.

VLP extraction and purification was performed following the method described elsewhere(369). Purified VLP preparations were analyzed by Cryo-EM. VLPs were deposited on a carbon-coated copper grid and prepared using a Leica EM GP workstation (Leica). VLPs were observed with a Jeol JEM-2011 (Jeol Ltd.), equipped with a CCD 895 USC4000 camera (Gatan).

Mice immunization and immunogenicity analyses

All experimental procedures were done under the Spanish and European laws and by the Institutional Animal Care and Ethics Committee of the Center for comparative medicine and bioimage (CMCiB, Badalona, Spain). They were performed by trained researchers and approved by the regional authorities (Generalitat de Catalunya, Authorization ID: 10583). All experimental protocols were performed following the principles of the 3Rs, prioritizing the welfare of the animals used in the research.

DNA immunization was performed in groups of ten six-week-old C57Bl/6JOlaHsd mice (Envigo). Male and female were equally represented in each group. Two doses, at weeks 0 and 3, of sterile endotoxin-free DNA were electroporated intramuscularly at the hind leg (20 µg DNA in physiological saline). Electroporation protocol consisted of 8 pulses of 20 ms with a 1 s interval at 60 V. Prior to each immunization, blood was sampled via facial vein puncture. Serum was recovered from whole blood after coagulation by centrifugation for 10 min at 4000 x g and heat-inactivated for 30 min at 56°C. Euthanasia of all animals was performed at week 6 when blood samples and spleens of each animal were taken for *ex vivo* immune analysis.

Evaluation of humoral response by ELISA

The levels of antibodies against FeLV p27, p15E, and gp70 in mouse serum samples were determined by an in-house sandwich-ELISA.

For anti-FeLV p27 antibody quantification, Nunc MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 100 ng/well of recombinant FeLV p27 (ProSpec) and incubated overnight at 4°C in a wet chamber. Then, coated plates were blocked with 1x PBS, 1% BSA, and 0.05% Tween20 for 2 h at RTemp. Mouse anti-p27 monoclonal antibody [PF12J-10A] (Abcam) was used as standard. Blocking buffer-diluted serum

samples (1:100 and 1:1000) were added and incubated overnight at 4°C in a wet chamber. Total bound IgG was determined with a secondary HRP-conjugated AffiniPure Goat polyclonal anti-mouse IgG (1:10000) (Jackson ImmunoResearch). Plates were developed with o-Phenylenediamine dihydrochloride (OPD) (Sigma Aldrich) and stopped using 2N of H_2SO_4 (Sigma Aldrich). The signal was analyzed as the optical density (OD) at 492 nm with noise correction at 620 nm.

For anti-gp70 and anti-p15E antibody quantification, Nunc MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 100 ng/well of Pure goat anti-human IgG Fc (Jackson ImmunoResearch) and incubated overnight at 4°C in a wet chamber. Then, plates were blocked using 1x PBS, 1% BSA, and 0.05% Tween20 for 2 h at RTemp. After that, plates were incubated with SU-huIgG or TM-huIgG for 2 h at RTemp. Mouse anti-human IgG_1 Fc [HP6069] (Merck) was used as standard. The rest of the method followed the same scheme as for anti-p27 FeLV ELISA. TM-huIgG was produced and quantified as described in Ortiz et al (369).

Design and production of SU-huIgG

SU-huIgG is a fusion protein designed in-house. The SU fragment (residues 1 to 445 of Env FeLV) was cloned into a huCD4-huIgG plasmid(328). It was cloned using FastDigest *KpnI* and *NheI* restriction enzymes (Thermofisher Scientific). The supernatant of transiently transfected Expi293F cells was used directly for the in-house ELISA. The fusion protein was characterized by Western Blot using an HRP-AffiniPure Goat polyclonal anti-human IgG (Jackson ImmunoResearch) and an anti-gp70 antibodies and quantified by ELISA (123 µg/mL) as described elsewhere (328) (Figure 25).

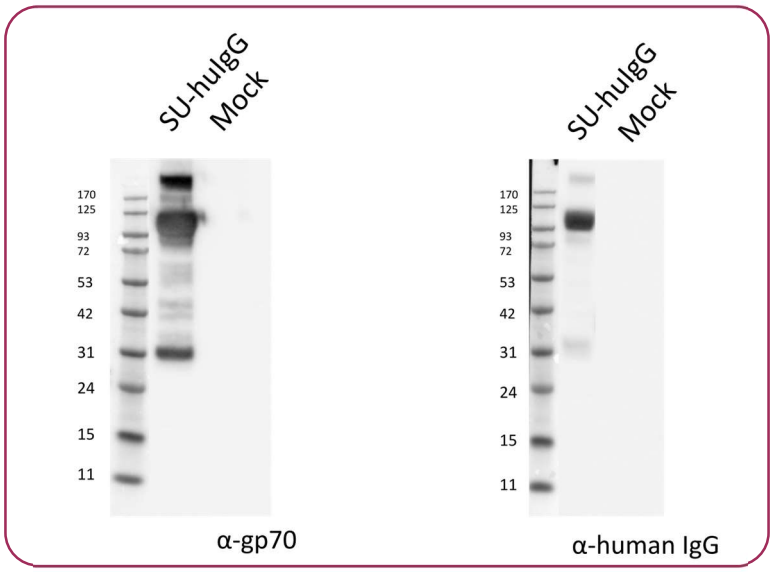

Figure 25. Western Blot of SU-huIgG. Western blot was developed with an anti-gp70 and anti-human IgG antibodies to analyze the expression of SU-huIgG fusion protein transiently transfected in Expi293F cells.

Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc.) and R v4.1.1. Comparisons in immunogenicity were tested including undetectable data (under LOD) using the Peto-Peto rank test for cross-sectional comparisons and Petro-Pentrice generalized Wilcoxon test for longitudinal ones. Multiple comparisons were adjusted using Benjamini & Hochberg method (FDR). For all analyses, a P-value of less than or equal to 0.05 was considered significant.

Results

Generation of a soluble and stable Env trimer.

Recently, we have developed a high-density platform of HIV-1 Gag-based VLPs, by directly fusing a small antigen of interest to the N-terminal region of HIV-1 Gag through a transmembrane domain and a linker (302,303). By analogy to that HIV-1 Gag-based VLPs, we designed FeLV Gag-based VLPs presenting a fragment of p15E on the surface (369). Even though the selected FeLV VLPs candidates induced a strong cellular and humoral response to FeLV Gag capsid (p27), we failed to generate neutralizing anti-p15E antibodies (369). Therefore, we decided to expose a more complex Env antigen on the surface of FeLV Gag-based VLPs.

Initially, we fused the complete SU sequence (region 1 to 445 of FeLV Env), including the signal peptide (SP) (residues 1 to 33), to the HIV-1 membrane spanning domain (MSD) (residues 684 to 707) and to full-length FeLV Gag. However, the expressed

protein after transient transfection of Expi293F cells showed massive fragmentation assessed by western blot (data not shown), ruling out further development and suggesting that FeLV Env required a more stable conformation.

In HIV-1, stable trimeric native-like Env called SOSIP is well described (357). SOSIP HIV-1 trimers contain i) an engineered disulfide bridge between gp120 and gp41 (mutations A501C and T605C); ii) a modification of the furin cleavage site by RRRRRR (R508 to R511) in order to favor cleavage; and iii) a stabilizing I559P point mutation (Figure 26A), conferring stability and similar antigenicity as membrane-bound mature viral trimeric Env (357). Translating this approach to FeLV, first, we aligned the two Env sequences (Figure 26A) and identified in FeLV Env the equivalent three-point mutations: XXXXZ, XXXXY, and XXXXZ; as well as the furin cleavage site (Figure 26A). These mutations were included in a novel SFE-FeLV soluble protein design containing the SP of Env FeLV (residues 1 to 33), the full gp70 sequence (residues 34 to 442, with a 6-R string in the furin cleavage site (residues xxx to xxx)), a fragment of the extracellular portion of p15E (region 448 to 572), and an 8-histidine tail (Figure 26B). Mutations XXXXZ / XXXXZ and XXXXY were introduced, to stabilize the trimeric protein through a disulfide bound and increased interactions after furin cleavage.

Expression of the novel SFE-FeLV soluble protein in transiently transfected Expi293F cells was analyzed by flow cytometry using an anti-gp70 antibody, confirming its expression not only intracellularly but also as an extracellular protein (Figure 26C). Supernatant of transiently transfected Expi293F cells was recovered 96 h after transfection and SFE soluble protein was purified by nickel affinity chromatography. Different fractions were recovered during the purification process: cell lysate (P), raw culture supernatant (SN), two fractions of flow-through (FT 1 and FT 2), wash fraction (Wash), and the eluted protein (Elution). All fractions were analyzed by western blot using an anti-gp70 antibody, confirming the expression and purification of the protein, which showed an apparent molecular weight of 90 kDa (Figure 26D). The same fractions were analyzed by Coomassie Blue staining showing an enrichment of the SFE soluble protein in the elution fraction compared to the starting material (SN fraction) (Figure 26E).

The oligomerization state of the purified SFE soluble protein was determined by size exclusion chromatography (SEC). Interpolated values of major peaks indicated a molecular weight of 619, 459, 340, and 168 kDa, with a % area of 12.85, 42.10, 32.15, and 12.13 respectively. This data indicates that SFE soluble protein oligomerizes in trimers, although lower (dimers) and higher order oligomers (pentamers and hexamers) are also present under our purification conditions (Figure 26F).

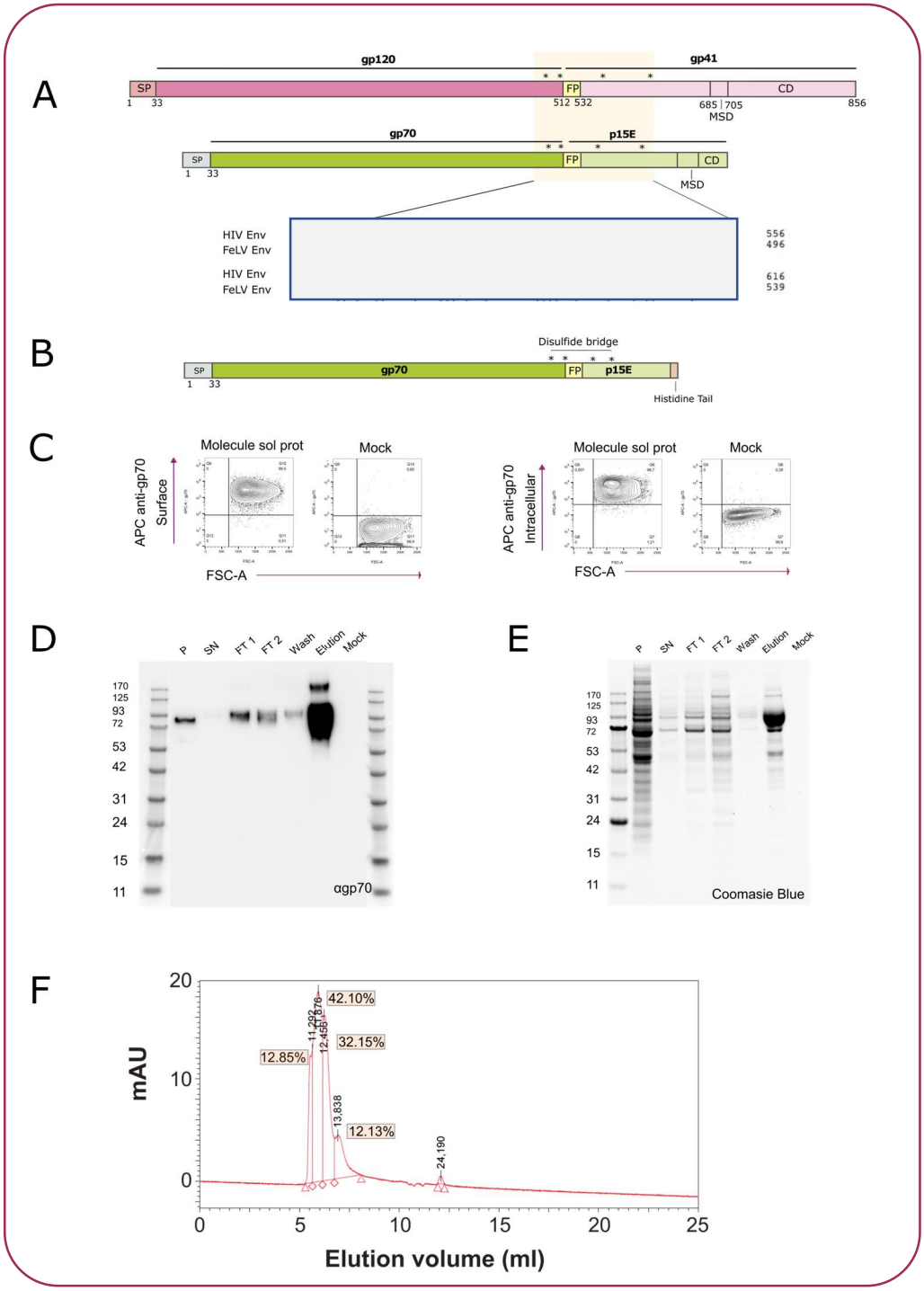

Figure 26. Generation of a soluble and stable Env trimer. (A) Alignment between HIV-1 and FeLV Env glycoproteins and mutations are denoted. **(B)** Schematic representation of the SFE soluble protein. Signal peptide (SP), mutations, disulfide bridge, and histidine tail are denoted. **(C)** Representative flow cytometry panels for extracellular and intracellular expression of SFE proteins detected with an anti-gp70 antibody. **(D)** Western blot analysis of the different fractions of SFE protein purification developed with an anti-gp70 antibody. **(E)** Coomassie blue analysis of the different fractions of SFE protein purification. **(F)** Chromatogram of SFE soluble protein.

Generation of SFE FeLV-based VLPs

Having confirmed the expression and oligomerization state of the SFE protein, we designed, produced, and characterized FeLV Gag-based VLPs exposing SFE protein on the surface of the particle. Three different recombinant fusion proteins were selected. All fusion proteins contained the SP of FeLV Env, the above described SFE without the Histidine tail, which was replaced by the Env FeLV MSD fused to different forms of FeLV Gag. Based on our previous work to optimize FeLV-based VLP formation (369), the following fusion proteins were tested: i) SFE, containing wild-type full-length FeLV Gag; ii) SFE-VA, containing two mutations at residues L199V and R200A of FeLV Gag; and, iii) SFE-ΔProR-VA, containing a deletion of a late domain containing region (residues 128 to 153 in FeLV Gag), in addition to the double VA mutation (Figure 27A). Moreover, a control VLP without extracellular FeLV antigens, but with human GM-CSF SP, a Flag-tag, the Env FeLV MSD, and full-length FeLV Gag was designed as a control for immunization assays (Figure 27A and Figure 28) (369).

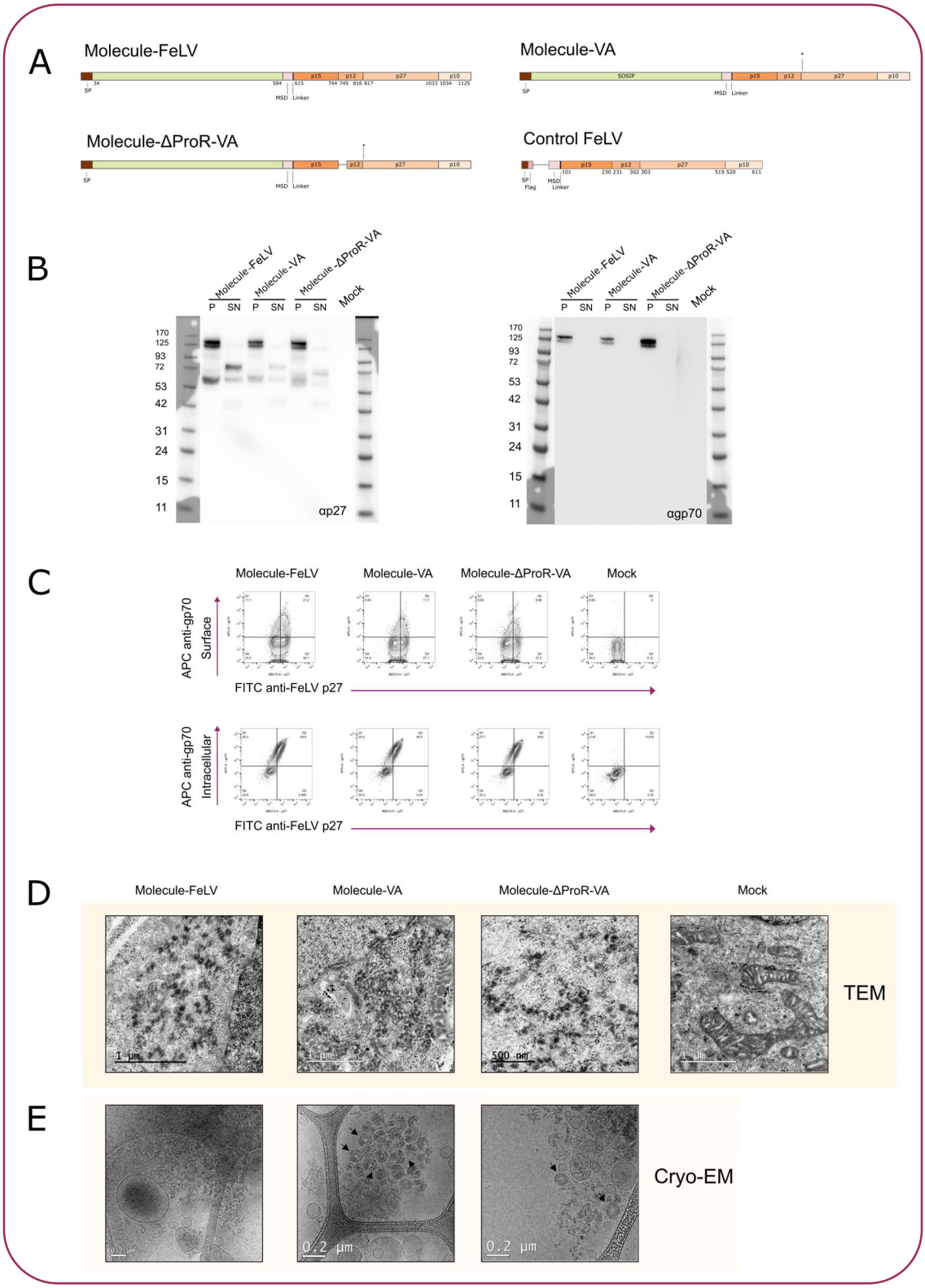

Figure 27. Design and characterization of SFE-Gag fusion proteins. (A) Schematic representation for each fusion protein. **(B)** Western blot membranes developed with an anti-p27 or anti-gp70 antibodies to analyze the expression of SFE-based fusion proteins transiently transfected in Expi293F cells. **(C)** Representative flow cytometry panels for extracellular and intracellular detection of the fusion proteins using anti-gp70 and anti-p27 antibodies. **(D)** TEM images of transiently transfected Expi293F cells expressing the indicated VLPs. **(E)** Cryo-EM images of purified VLPs.

Expression of recombinant fusion proteins in transiently transfected Expi293F cells was analyzed by western blot using anti-p27 and anti-gp70 antibodies. A prominent band was observed in cell lysates (P) compared to supernatants (SN, Figure 27B), indicating that the fusion proteins might be retained inside the cells and not secreted to the extracellular media. The expression of the proteins at their expected molecular weight (125 kDa) was only observed in the P lanes (Figure 27B). In order to confirm the possible retention of the fusion proteins inside the cells, we performed a flow cytometry analysis of transiently transfected Expi293F cells. The cells were stained at the surface or intracellularly with an anti-gp70 antibody and intracellularly with an anti-p27 antibody (Figure 27C). Anti-gp70 staining was detected mainly in permeabilized cells, confirming that the fusion proteins are retained inside the cells and are not reaching the plasma membrane, as indicated by the western blot analysis.

In order to confirm the formation of SFE FeLV Gag-based VLPs, transiently transfected Expi293F cells were visualized by transmission electron microscopy (TEM). A mock of Expi293F cells, transiently transfected with an empty vector, was included in the experiment as a control. All three recombinant proteins were producing intracellular FeLV Gag-based VLPs, showing a similar morphology and diameter (around 100 nm) to the control VLP (Figure 28C). Intracellular VLPs were extracted and visualized by cryo-EM. SFE-FeLV VLPs were not detected, but proper FeLV Gag-based VLPs were detected in SFE-VA and SFE-ΔProR-VA preparations (Figure 27E). Taken together, these data suggest that SFE-VA and SFE-ΔProR-VA fusion proteins promote the expression of morphologically correct FeLV Gag-based VLPs. Therefore, we retained both as vaccine candidates for subsequent analyses.

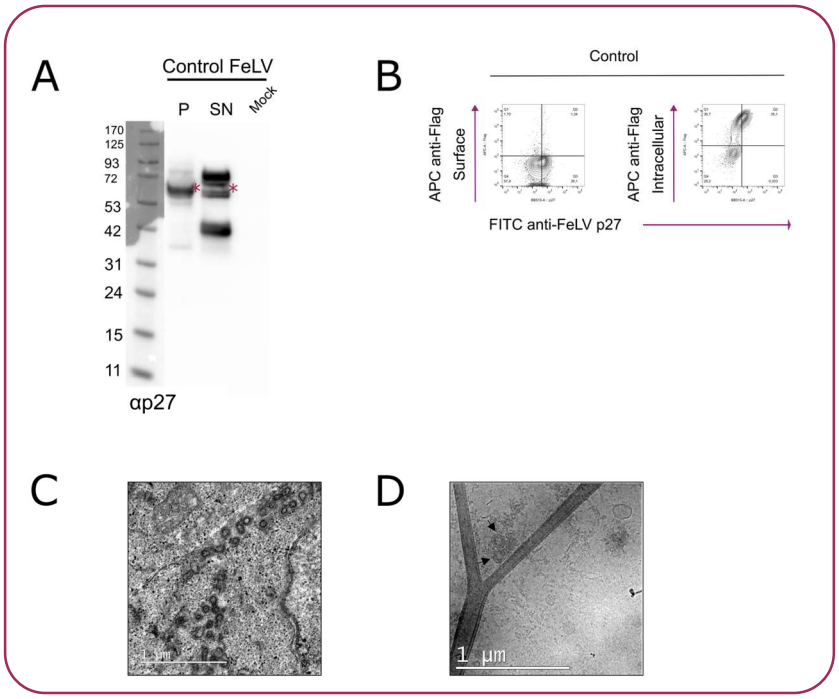

Figure 28. Design and characterization of Control-FeLV fusion proteins. (A) Schematic representation of the fusion protein. **(B)** Western blot membranes were developed with an anti-p27 antibody to analyze the expression of the fusion protein transiently transfected in Expi293F cells. **(C)** Representative flow cytometry panels for extracellular and intracellular detection of the fusion proteins using anti-Flag and anti-p27 antibodies. **(D)** TEM images from transiently transfected Expi293F cells expressing the indicated VLPs. **(E)** Cryo-EM images from purified VLPs.

Immunogenicity of SFE-FeLV VLPs in C57BL/6 mice.

The immunogenicity of SFE Gag-based VLPs was evaluated in C57BL/6 mice by intramuscular electroporation of plasmid DNA encoding the fusion proteins: SFE-FeLV, SFE-VA, SFE-ΔProR-VA, and Control FeLV. Even though SFE-FeLV VLPs were not detected by cryo-EM (Figure 27E), transiently transfected Expi293F cells were able to generate VLPs (Figure 27D). Consequently, this fusion protein was also selected for *in vivo* immunization assays as the delivery system is DNA, and once the muscular cells of the immunized animal uptake the nucleic acid, they would synthesize the SFE-FeLV VLPs which would be released upon cell death. Moreover, we included the sequence of SFE soluble protein and Control-FeLV Gag (lacking Env FeLV at the surface of the VLPs), as control experimental groups. Animals were vaccinated with two doses of plasmid DNA (20 µg/dose), at weeks 0 and 3, and the endpoint of the experiment was determined at week 3 post-second immunization (Figure 29A).

Humoral immune responses were evaluated against the TM, SU, and p27 antigens using an in-house ELISA. None SFE-FeLV Gag-based VLPs vaccinated animals generated detectable antibodies against TM (Figure 29B). Similarly, analysis of humoral response against SU showed that only the SFE-FeLV soluble protein group was able to generate

a low response with high heterogeneity (three animals were positive, Figure 29C). Finally, analysis of humoral responses against p27 (FeLV Gag capsid) showed a readily measurable response in Gag-VLPs vaccinated groups (Figure 29D). However, only the Control-FeLV VLPs were able to induce a high titer of antibodies against p27, reaching a plateau after a single dose (Figure 29D). In contrast, SFE-expressing VLPs induced a significantly lower response against p27 (P-value = 0.0011, Figure 29D).

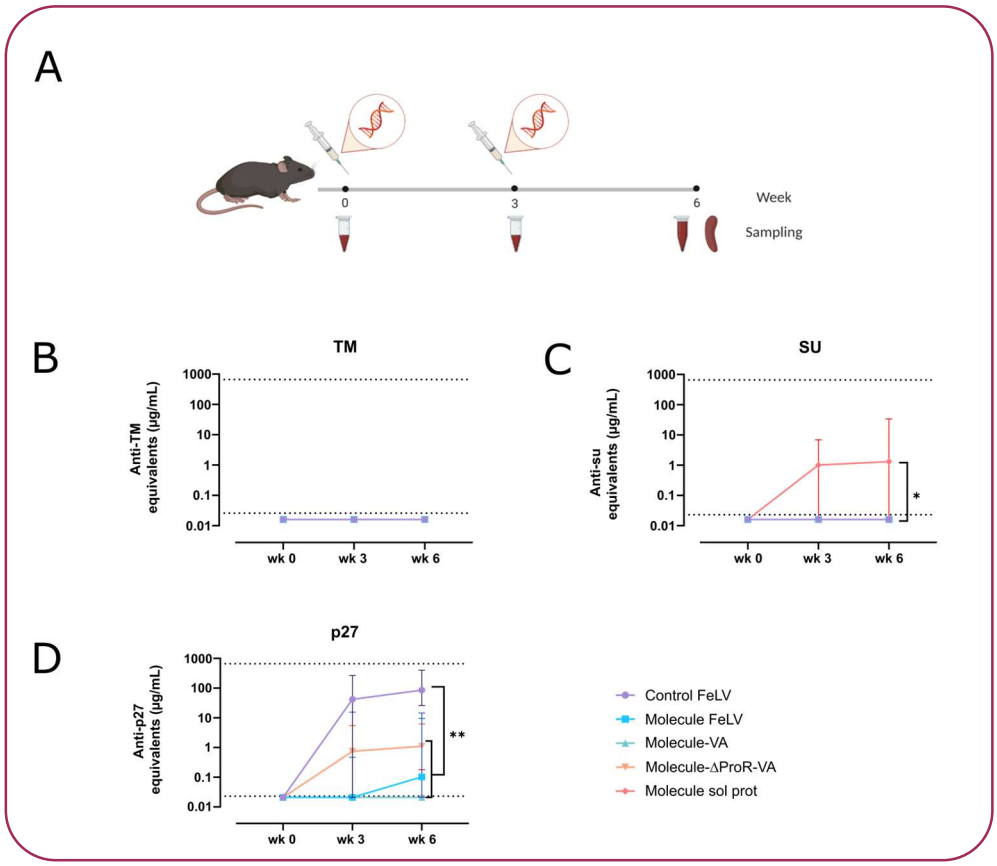

Figure 29. Immunogenicity of SFE FeLV Gag-based VLPs in C57Bl/6. (A) Graphical scheme of the experimental procedure. C57Bl/6 mice were electroporated twice with 20 ug of DNA. Antibodies titters against TM **(B)**, SU **(C)**, and p27 **(D)** are shown. Data is presented as Median with 95% CI of antibody concentration in mouse sera. Statistical differences were found using the Peto-Peto rank test for wk6 and Paired Prentice-Wilcoxon test for longitudinal comparison (*p<0.05; **p<0.01; ***p<0.001).

Discussion

A fully protective vaccine against retroviruses, such as HIV-1 or FeLV, is still unavailable. To improve vaccine efficacy, in the HIV-1 field, different strategies have been tested. Currently, one of the most promising prototypes of subunit vaccine is SFE (269), which presents trimeric Env in a stable native conformation. HIV-1 SFE trimers have demonstrated their immunogenic potential in non-human primates preclinical experiments (371). Therefore, we hypothesized that a SFE approach could be also

applied to FeLV. In this field, there have been many efforts to design a vaccine able to induce both humoral and cellular responses (214,223,317). Currently, three vaccine formulations against FeLV are commercially available: i) classical inactivated virus vaccines (245,246); ii) subunit vaccines based on the FeLV Envelope protein gp70 (372), and iii) infectious recombinant canarypox virus engineered to express FeLV genes (248,249). Unfortunately, none of them provide full protection (255,256,318). Therefore, new efforts are needed to design more efficient vaccines against FeLV.

Taking HIV-1 as a reference, we have designed a SFE-FeLV soluble protein. To our knowledge, this is the first study to describe a SFE strategy for the FeLV Env. After aligning both Env sequences, we identified the equivalent amino acids where we could introduce mutations intending to stabilize the FeLV Env trimer. Then, we analyzed in vitro the expression and the oligomerization state of the SFE-FeLV soluble protein. Even though we confirmed that our SFE protein was produced in Expi293F, further optimization is needed for the purification process. Certainly, production in Expi293F cells generated a heterogenous oligomerization profile (from hypothetical dimers to hexamers). Reducing this heterogeneity can be achieved by modifying culture conditions, purification protocol, and formulation buffers. However, to easily test the potential immunogenicity of our SFE prototype, we decided to express it on the surface of our previously described FeLV Gag-based VLPs (369) and to exclusively test DNA delivery in immunogenicity assays (avoiding the purification process).

To present our SFE antigen on the surface of VLPs, it was fused to FeLV-Gag through the FeLV Env transmembrane domain and a linker (GS). Different N-terminal modifications and mutations (VA and ΔProR-VA) on FeLV Gag were included. These mutations were previously described and confirmed to produce intracellularly FeLV Gag-based VLPs (369). Therefore, after ensuring that all designed fusion proteins were capable to form intracellular VLPs, an immunogenicity assay in C57BL/6 mice with two doses of electroporated DNA was performed. The soluble form of SFE was also included in the assay. Unfortunately, no response against the SFE subunits, SU nor p15E, was observed in any of the VLP vaccinated groups. Only a minor response was observed for the soluble protein vaccinated group. Also, the humoral response against p27 was low or not detected in SFE Gag-based VLP vaccinated groups when compared with the titer levels of Control-FeLV expressing only Gag proteins.

These unfavorable results could be explained by a lower *in vivo* production of SFE Gag-based VLPs compared to Control-FeLV or soluble SFE-FeLV form. This could explain the lower humoral responses observed for both Env and Gag antigens in VLP-immunized

groups compared to the respective controls (control FeLV Gag and SFE-FeLV soluble form). Another possible explanation of the low humoral response could be a potential immunosuppressive activity of SFE FeLV, despite having mutated the main immunosuppressive domain of p15E (346). Indeed, humoral anti-Gag responses were negatively impacted by SFE expression. This was surprising, as we have previously generated high titers against p27 (FeLV Gag capsid) (369).

Therefore, we describe a new potential FeLV Env immunogen applying SFE technology described for HIV. Preliminary characterization of its expression and immunogenicity suggests that further optimization in both aspects is required to evaluate its potential. Particularly, an upstream and downstream production process and an optimal immunogenic delivery (including adjuvants) need to be defined.

Conflict of Interest

ABarrreiro, AG, MC, and LF are employees of HIPRA. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Outside this work BC, JB and JC are founders and shareholders of AlbaJuna Therapeutics, S. L.

Author Contributions

RO wrote the paper and performed research. RO, ABarajas, BT, FT-F, CR, and CA-G collected the data and performed the analysis. AP-G performed the animal handling during immunization experiments. VU performed the statistical analysis. ABarreiro, AG, MC, and LF performed the vaccine formulation. CA-G, JC, and JB conceived and designed the analysis. All authors contributed to the article and approved the submitted version.

Funding

This work was partially funded by an institutional grant from HIPRA (Spain) to conduct the work.This work has also been funded by Secretaria d'Universitats i Recerca of Generalitat de Catalunya and the European Social Fund through the project 2022 FI_B 00698.

Acknowledgments

We are grateful to CMCiB staff for their excellent technical help with animal care. We would also like to thank the staff from the Servei de Microscòpia of Universitat Autònoma

de Barcelona. Finally, thanks to the Plataforma de Proteómica y Metabolómica (IGTP) for the SEC analysis.

DISCUSSION

Retroviruses cause a wide range of infections in humans and other species. The knowledge of these infections, their pathogenesis, and protective immune responses are crucial to understand virus-host interactions and defining vaccine design and public health strategies.

Among retroviruses, HIV has focused most of the research efforts since it is a major global health issue. HIV is a blood-borne virus that can infect CD4-expressing immune cells, especially $CD4^+$ T helper lymphocytes, and destroys them during its life cycle, weakening the host's immune system (59). If not treated properly with ART, HIV infection can lead to AIDS, during which many opportunistic diseases can arise and may prove fatal for the infected individual. Unfortunately, HIV is one example of pathogen in which the development of a protective vaccine has been elusive for more than 40 years (373).

A deeper understanding of the natural course of HIV infection, the immune response against the virus, and the virus/host immune system adaptation are essential to the successful development of a vaccine candidate. Equally important is the development of appropriate animal models of HIV to better understand the pathogenesis of the retrovirus and to develop and test vaccine candidates and new therapies (374). The only animals thus far found susceptible to infection with HIV-1 are the chimpanzee, gibbon ape, and rabbit, but disease has not yet been reported in these species (374). Despite this, some retroviruses can cause disease and immunosuppression in a wild range of animals (Table 1) providing potential models for HIV-1 infection.

For instance, the domestic cat is afflicted with multiple viruses that serve as models for human diseases including cancer, SARS, and AIDS. Cat viruses that cause these diseases have patterns of evolution, virulence, and pathogenicity that offer strong parallels to related viruses in humans (67) (Table 10).

Feline Virus	Human homolog
Feline Leukemia Virus (FeLV)	Human T-Cell Leukemia Virus (HTLV)
Feline Immunodeficiency Virus (FIV)	Human Immunodeficiency Virus (HIV)
Feline Coronavirus (FCoV)	SARS-Coronavirus
Feline Sarcoma Virus (FSV)	~20 Human oncogenes
Feline Herpes Virus (FHV)	Cytomegalovirus (CMV)
Feline Foamy Virus (FFV)	Human Foamy Virus (No pathology)
Feline Calicivirus (FCV)	Human Calicivirus
Feline Parvovirus (FPV)	Human B19 Parvovirus
Feline Morbillivirus (CDV)	Human Morbillivirus

Table 10. Examples of domestic cat viruses with human homologs. Adapted from (67)

Specifically, two retroviruses causing immunosuppression are of major importance in domestic cats: FeLV, a gammaretrovirus, and FIV, a lentivirus (75,375). Understanding the immune response of these feline retroviruses after natural infection or vaccination might provide insights into developing strategies for vaccines against HIV-1 (376,377).

In this thesis, we have focused on FeLV. FeLV was discovered in the 1960s and its ability to recombine with host cellular oncogenes resulted in a better understanding of numerous feline and human malignancies (378). Importantly, FeLV is not a public health threat, although it can replicate in human cell lines in culture, no conclusive evidence of natural infection with FeLV has ever been detected in humans (379).

FeLV is one of the few retroviruses for which commercial vaccines exist (in addition to FIV) (243,255,380–384). Consequently, the analysis of immune responses facilitating complete recovery in FeLV-exposed cats with abortive infections, and those protecting cats following FeLV vaccination, are important to develop effective vaccines against other retroviruses, such as HIV-1 (375,385). It is important to highlight that not only the knowledge of FeLV can influence HIV-1 research, but HIV-1 research can also help to understand and improve FeLV vaccines.

As explained above, different types of vaccines are available for FeLV, and it is relevant to continue vaccinating domestic cats to avoid a fatal disease outcome after infection (151). The main aim of this project is to generate a novel vaccine strategy against FeLV.

Our group has been working on HIV-1 based VLPs. They are enveloped particles that mimic the structure of immature HIV-1 virions but are non-infectious and non-replicative, hence they are a good and safe vaccine platform to induce an immune response following vaccination (274). HIV-1 enveloped VLPs are an attractive vaccine platform since they can accommodate Env at their surface while containing Gag at their core, therefore, triggering a balanced adaptive immune response. However, a drawback of this platform is that since they mimic immature virions, they poorly incorporate Env-derived immunogens at their surface. In this sense, our group has recently worked to achieve higher densities of antigen at the VLP surface by fusing Env-derived HIV-1 immunogens to Gag through a transmembrane domain and a linker (303).

By analogy to HIV-1, our first purpose was to generate FeLV Gag-based VLPs as a novel vaccine strategy against this retrovirus. We designed different strategies to face this objective. We had first to adapt the HIV-1 Gag-based VLP platform to a FeLV Gag-based VLP. Results from section 1, showed that when producing and characterizing our initial fusion proteins, we could detect truncations of Gag in the cell culture supernatant. We hypothesized that this instability may be originated from the similarity of our fusion proteins with a naturally occurring glycosylated form of FeLV Gag. It has been described that FeLV Gag has two starting codons for the *gag* gene. One of them encodes for the polyprotein precursor of the internal capsid protein and the other encodes for an alternative type II transmembrane glycoprotein form, known as glycogag, which has an expected molecular weight of 80 kDa and a soluble cleavage product of 40 kDa. Additionally, TEM confirmed that our fusion proteins were capable to produce VLPs, but they were retained inside the cell, not reaching the cell surface (results section 1). Nevertheless, after screening several deletions and mutations of Gag, we could avoid its truncation and optimize the FeLV Gag-based VLP platform. Furthermore, FeLV Gag-based VLPs could be successfully extracted from transiently transfected Expi293F cells.

Our VLPs were produced in Serum-free (SF) and Animal-derived component-free (ADCF) medium and purified by ultracentrifugation (UC) in a 70% and 30% double sucrose cushion, which separates particles according to physical properties, mainly density. Unfortunately, this method is hardly scalable. This is an important point because it would be difficult the escalation to industrial production. Therefore, although our purification protocol led us to obtain a purified VLP preparation, it could be a drawback when escalating to larger volumes.

Also, VLP-producing cells released extracellular vesicles (EVs) that had similar size to our VLPs, as observed by cryo-EM, these EV contaminants were not efficiently removed

by UC. To overcome this limitation, further work is necessary to develop an optimized VLP purification protocol. Affinity strategies could be pursued to achieve higher purity yields. For instance, VLP separation could be managed by a combination of tangential flow filtration, ultrafiltration steps, and size exclusion chromatography (386–388).

Still, our protocol allowed the isolation of pure and homogeneous VLPs, enabling us to proceed to *in vivo* immunogenicity assays.

Once we optimized the FeLV-based VLPs platform different key points were relevant for the research progress: i) selection of the antigen presented on the VLP surface, ii) antigen delivery, and iii) selection of the adjuvants.

i. Antigen on the surface

By analogy to our previous work on HIV-1, we first, selected a fragment of p15E (so-called TM) as the antigen presented on the VLP surface. This protein is known to be the target of some neutralizing antibodies (200,316). Unfortunately, after testing the immunogenicity in two different murine models, using different vaccination delivery systems /DNA and VLPs), and, in the presence or absence of adjuvants, no response against the TM antigen was detected (results section 1). Even though, a potent humoral and cellular response against FeLV Gag was elicited.

Different reasons could explain these results. On one hand, FeLV, like other retroviruses, has developed mechanisms to interfere with the host immune response and establish persistent infection. In particular, a region of p15E contains an immunosuppressive domain (ISD) which is conserved among gammaretroviruses (231,346,348). This ISD consists of 17 conserved amino acids (389). However, this region was excluded from the tested antigen. Moreover, although the exact mechanism is still not known, gammaretroviral TM have been shown to promote evasion from anti-tumor cytotoxicity (390–392). On the other hand, it is possible that our antigen it is not correctly presented on the VLP surface. This possibility seems unlikely, as in our previous work with HIV-1 Gag-based VLPs we presented a gp41 miniprotein (Min) equivalent to the fragment selected for FeLV, and antibodies against Min were elicited (393).

To overcome these results, a new strategy presenting SU on the VLP surface was developed. Sadly, *in vitro* experiments showed a truncation of the antigen (data not shown). Therefore, and to overcome these results, we designed a new potential FeLV Env immunogen applying Envelope technology described for HIV-1 (results section 2).

Even though we were capable to produce and characterize the expression of SFE antigen, the preliminary immunogenicity assay suggested that further optimization in both aspects is required to evaluate its potential. SFE antigen was only capable to induce a humoral response when delivered as the soluble protein. Therefore, an optimization in the production process to generate a preparation of pure native trimeric forms and an in-depth analysis of immunogenic delivery (including adjuvants) needs to be performed.

ii. Antigen delivery

A relevant aspect of our fusion-protein VLP platform is that they can be produced and purified *in vitro* and delivered as VLPs, or they can be delivered as a DNA-based vaccine. In this current work, we have tested both antigen deliveries

On one hand, subunit proteins or immunogens presented through multivalent platforms, as in our case, will directly stimulate APCs and B cells to promote an efficient generation of humoral responses (394). On the other hand, nucleic acid vaccines are considered to be particularly fit to trigger cellular responses, since they will enter the target cell and produce the encoding protein while also presenting fragments of this protein through MHC-I molecules (395)

We have also delivered our fusion-protein VLPs as a DNA vaccine. *In vivo* DNA administration, however, faces some issues like the inefficient uptake of plasmid DNA by somatic cells. To bypass this hurdle, DNA electroporation is a convenient delivery technique since purified DNA can be directly administered without the need for other processing steps like liposomal formulation and encapsulation (396). Our group previously confirmed that electroporation resulted in a 3-log higher production by bioluminescence after administering a luciferase-coding plasmid delivered with and without electroporation (393). Moreover, the expression of luciferase was stably maintained for at least three months after electroporation, indicating that DNA vaccination has the potential to produce antigen for a long period of time, or at least until the immune system completely cleared the antigen (393).

Nucleic acid vaccines can be categorized into two major groups: mRNA vaccines, and DNA vaccines. Recently, RNA vaccines have been the focus of interest, due to the SARS-CoV-2 pandemic (397). The unprecedented fast design, production, and approval of mRNA-based SARS-CoV-2 vaccines and their proven efficacy globally is paving the way for many more RNA-based vaccines to reach advanced clinical trials shortly (398). One possibility of our current work would be to adapt our fusion-protein VLPs to an RNA-

based vaccine and analyze whether the immunogenicity increase regarding DNA-based vaccines.

It is important to highlight that we studied homologous regimens VLP/VLP or DNA/DNA. Future work could consider trying a heterologous regimen of DNA/VLP to induce a more complete immune response (393,399), this would allow us to vaccinate animal models with the same immunogen but using two different strategies, achieving more complete responses.

iii. Adjuvants

Aluminum salts have a long history in vaccine formulations; they were the first-approved vaccine adjuvant in humans and have been in use for over 80 years (305). They have been used successfully in vaccines against pathogens where antibodies provided the primary mechanism of protection. However, they have limitations exerting effect on Th1-type or cytotoxic T-cell responses. Therefore, one approach to overcome the limitations of alum is to use it to co-deliver it with additional adjuvants.

Adjuvant system AS04 combines aluminum hydroxide or aluminum phosphate with the immunostimulatory molecule Monophosphoryl lipid A (MPLA) (400). MPLA is a modified version of lipopolysaccharide (LPS) that is significantly less toxic but still remains a TLR4 agonist (311)., capable of inducing a Th1 and cytotoxic CD8+ T-cell response (305,311). Therefore, by including MPLA with aluminum hydroxide, both a Th1 and Th2 response can be induced (312).

Different adjuvants were studied in this thesis with the objective to elicit an immune response against the selected antigen (results section 1). Even though high titers of antibodies against FeLV Gag were elicited, none of them was capable to induce an immune response against the antigen exposed on the VLP's surface.

Among the different adjuvants tested, two adjuvant formulations, MPLA + $Al(OH)_3$ and MF59 were eliciting higher titers of antibodies against p27 FeLV Gag. Regarding the cellular immune response, MPLA + $Al(OH)_3$ and QuilA + cholesterol +DDABr were the ones inducing a higher immune response. Therefore, results in section 1 suggested that the best formulation includes MPLA + $Al(OH)_3$ in the final vaccine, capable to induce both a Th1 and Th2 response.

In summary, with this work our group has successfully tested the versatility of our fusion-protein VLP platform, adapting it from HIV-1 Gag-based VLP to FeLV Gag-based VLP. We not only provided the novel concept of a FeLV Gag-based VLP but also optimized the production and purification processes, tested the immunogenicity of different

immunogens displayed at the VLP surface with the presence or the absence of adjuvants, and adapted the VLP concept into a DNA-based vaccine strategy.

Future research should move towards the identification of a relevant immunogen that could induce a potent and neutralizing humoral response. We have proved that our FeLV Gag-based VLPs were capable to induce a cellular response against Gag, which could be relevant to a progressive outcome after infection but probably insufficient to protect from infection. One possibility to increase humoral protective responses would be to continue optimizing the SFE soluble form, as it was able to induce humoral response against gp70. Once an optimized antigen is defined, it can be exposed on our versatile VLP platform to generate a combined humoral and cellular response. The versatility of our high-density antigen VLPs suggests that they could be adapted to other pathogens of interest and become a vaccine platform prepared for future emerging pathogens.

CONCLUSIONS

Objective I: To adapt the HIV-1 Gag-based VLP platform to a FeLV Gag-based VLP.

The versatility of HIV-1 Gag-based VLP platform was confirmed. A novel fusion-protein FeLV Gag-based VLP expressing p15E immunogen on the VLP surface was successfully developed (TM-FeLV).

Objective II. To optimize the production of FeLV Gag-based VLPs.

Optimization of the initial fusion protein candidate TM-FeLV was performed by screening Gag N-terminus modifications and mutations to avoid the formation of glycogag. Two final candidates TM-VA, and TM- ΔProR-VA were selected.

Objective III. To test the immunogenicity (humoral and cellular responses) of FeLV Gag-based VLPs in murine models

The immunogenicity of the selected candidates was tested in C57BL/6 and BALB/c mice. A potent humoral and cellular response against p27 (FeLV Gag capsid protein) was elicited, but no response against TM antigen was detected.

Objective IV. To maximize humoral and cellular immune responses by identifying optimal adjuvants and antigens.

Two different adjuvant formulations, MPLA + $Al(OH)_3$ and MF59, improved the cellular and humoral immune response against Gag p27 capsid protein. A novel FeLV antigen, based on HIV-1 SOSIP, was designed and produced correctly. Unfortunately, the humoral response elicited by this antigen when administered in a DNA/DNA regimen was poor.

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DISSEMINATION

Publications related to this thesis project

Ortiz, R., Barajas, A., Pons-Grifols, A., Trinité, B., Tarrés-Freixas, F., Rovirosa, C., Urrea, V., Barreiro, A., Gonzalez, A., Ferrer, L., Clotet, B., Carrillo, J., Aguilar-Gurrieri, C., Blanco, J. (2022) **Exploring FeLV-Gag based VLPs as a new vaccine platform. Analysis of production and immunogenicity.** *In preparation*

Raquel Ortiz, Ana Barajas, Anna Pons-Grifols, Benjamin Trinité, Ferran Tarrés-Freixas, Carla Rovirosa, Victor Urrea, Antonio Barreiro, Anna Gonzalez, Maria Cardona, Laura Ferrer, Bonaventura Clotet, Jorge Carrillo, Carmen Aguilar-Gurrieri and Julià Blanco. **"*Production and immunogenicity of FeLV-based VLPs exposing a stabilized envelope glycoprotein.*"** In preparation**.**

Publications related to other projects

Tarrés-Freixas, F., Aguilar-Gurrieri, C., Rodríguez de la Concepción, M., Urrea, V., Trinité, B., **Ortiz, R.,** Pradenas, E., Blanco, P., Marfil, S., Molinos-Albert, L., Pons-Grífols, A., Ávila-Nieto, C., Varela, I., Cervera, L., Gutiérrez-Granados, S., Segura, M., Gòdia, F., Clotet, B., Carrillo, J., Blanco, J. (2022). An engineered HIV-1 Gag-based VLP displaying high antigen density induces strong antibody-dependent functional immune responses. *Submitted.*

Pérez-Yanes, S., Pernas, M., Marfil, S., Cabrera-Rodríguez, R., **Ortiz, R.,** Urrea, V., Rovirosa, C., Estévez-Herrera, J., Olivares, I., Casado, C., Lopez-Galindez, C., Blanco, J., & Valenzuela-Fernández, A. (2022). The Characteristics of the HIV-1 Env Glycoprotein Are Linked with Viral Pathogenesis. Frontiers in Microbiology, 13(March). https://doi.org/10.3389/fmicb.2022.763039

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beyond 1 year after infection. Cell Reports Medicine, 3(2). https://doi.org/10.1016/j.xcrm.2022.100523

Trinité, B., Tarrés-Freixas, F., Rodon, J., Pradenas, E., Urrea, V., Marfil, S., Rodríguez de la Concepción, M. L., Ávila-Nieto, C., Aguilar-Gurrieri, C., Barajas, A., **Ortiz, R.,** Paredes, R., Mateu, L., Valencia, A., Guallar, V., Ruiz, L., Grau, E., Massanella, M., Puig, J., … Blanco, J. (2021). SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity. Scientific Reports, 11(1), 1–10. https://doi.org/10.1038/s41598-021-81862-9

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frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 March 2022
doi: 10.3389/fmicb.2022.763039

The Characteristics of the HIV-1 Env Glycoprotein Are Linked With Viral Pathogenesis

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The understanding of HIV-1 pathogenesis and clinical progression is incomplete due to the variable contribution of host, immune, and viral factors. The involvement of viral factors has been investigated in extreme clinical phenotypes from rapid progressors to long-term non-progressors (LTNPs). Among HIV-1 proteins, the envelope glycoprotein complex (Env) has been concentrated on in many studies for its important role in the immune response and in the first steps of viral replication. In this study, we analyzed the contribution of 41 Envs from 24 patients with different clinical progression rates and viral loads (VLs), LTNP-Elite Controllers (LTNP-ECs); Viremic LTNPs (vLTNPs), and non-controller individuals contemporary to LTNPs or recent, named Old and Modern progressors. We studied the Env expression, the fusion and cell-to-cell transfer capacities, as well as viral infectivity. The sequence and phylogenetic analysis of Envs were also performed. In every functional characteristic, the Envs from subjects with viral control (LTNP-ECs and vLTNPs) showed significant lower performance compared to those from the progressor individuals (Old and Modern). Regarding sequence analysis, the variable loops of the gp120 subunit of the Env (i.e., V2, V4, and mainly V5) of the progressor individuals showed longer and more glycosylated sequences than controller subjects. Therefore, HIV-1 Envs from virus of patients presenting viremic control and the non-progressor clinical phenotype showed poor viral functions and shorter sequences, whereas functional Envs were associated with virus of patients lacking virological control and with progressor clinical phenotypes. These correlations support the role of Env genotypic and phenotypic characteristics in the *in vivo* HIV-1 infection and pathogenesis.

Keywords: HIV, viral envelope, functional defect, elite controllers (HIV-1 EC), functional envelope, lack of virological control

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Edited by:
François Villinger, University of Louisiana at Lafayette, United States

Reviewed by:
João Mamede, Rush University, United States
Judd Hultquist, Northwestern University, United States

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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *23 August 2021*
Accepted: *31 January 2022*
Published: *24 March 2022*

Citation:
Pérez-Yanes S, Pernas M, Marfil S, Cabrera-Rodríguez R, Ortiz R, Urrea V, Rovirosa C, Estévez-Herrera J, Olivares I, Casado C, Lopez-Galíndez C, Blanco J and Valenzuela-Fernández A (2022) The Characteristics of the HIV-1 Env Glycoprotein Are Linked With Viral Pathogenesis. Front. Microbiol. 13:763039. doi: 10.3389/fmicb.2022.763039

Cell Reports Medicine

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Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection

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https://doi.org/10.1016/j.xcrm.2022.100523

SUMMARY

To understand the determinants of long-term immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the concurrent impact of vaccination and emerging variants, we follow a prospective cohort of 332 patients with coronavirus disease 2019 (COVID-19) over more than a year after symptom onset. We evaluate plasma-neutralizing activity using HIV-based pseudoviruses expressing the spike of different SARS-CoV-2 variants and analyze them longitudinally using mixed-effects models. Long-term neutralizing activity is stable beyond 1 year after infection in mild/asymptomatic and hospitalized participants. However, longitudinal models suggest that hospitalized individuals generate both short- and long-lived memory B cells, while the responses of non-hospitalized individuals are dominated by long-lived B cells. In both groups, vaccination boosts responses to natural infection. Long-term (>300 days from infection) responses in unvaccinated participants show a reduced efficacy against beta, but not alpha nor delta, variants. Multivariate analysis identifies the severity of primary infection as an independent determinant of higher magnitude and lower relative cross-neutralization activity of long-term neutralizing responses.

INTRODUCTION

Immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection involve an undefined balance of innate and adaptive pathways[1] resulting in the development of a seemingly long-lasting immunological memory.[2,3] Although there is a general consensus on the key role of both T and B cells in the protection against SARS-CoV-2 infection and the development of coronavirus disease 2019 (COVID-19), the specific contribution of each arm of the immune system is still unclear.[1] Neutralizing antibodies mediate their protective effect by binding to the spike (S) glycoprotein of SARS-CoV-2 and by blocking viral entry into target cells; however, additional effector functions promoting viral clearance or natural killer (NK)-mediated infected-cell killing seems to be also relevant in SARS-CoV-2 and other viral infections.[4] Nevertheless, abundant experimental and epidemiological studies on SARS-CoV-2 indicate that neutralizing antibodies can serve as surrogate markers of protection,[5–7] as they do for other viral infections.[8,9]

Given the relevance of antibodies, the early (1–3 months) and mid-term (3–12 months) humoral responses after SARS-CoV-2 infection have been thoroughly described.[10–14] Current data outline a heterogeneous scenario in which infected individuals generate a wide range of neutralizing antibodies (from no seroconversion to rapid development of high titers) with no definitive association to age, gender, or disease severity.[15–17] Various authors have also suggested complex kinetics of neutralizing activity decay.[3,18,19] This is particularly relevant in the current context of viral evolution, as several variants of concern (VOCs) have shown total or partial resistance to neutralizing antibodies and partial resistance to polyclonal humoral responses elicited by infection or vaccination.[20]

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SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity

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The protective effect of neutralizing antibodies in SARS-CoV-2 infected individuals is not yet well defined. To address this issue, we have analyzed the kinetics of neutralizing antibody responses and their association with disease severity. Between March and May 2020, the prospective KING study enrolled 72 COVID-19+ participants grouped according to disease severity. SARS-CoV-2 infection was diagnosed by serological and virological tests. Plasma neutralizing responses were assessed against replicative virus and pseudoviral particles. Multiple regression and non-parametric tests were used to analyze dependence of parameters. The magnitude of neutralizing titers significantly increased with disease severity. Hospitalized individuals developed higher titers compared to mild-symptomatic and asymptomatic individuals, which together showed titers below the detection limit in 50% of cases. Longitudinal analysis confirmed the strong differences in neutralizing titers between non-hospitalized and hospitalized participants and showed rapid kinetics of appearance of neutralizing antibodies (50% and 80% of maximal activity reached after 11 and 17 days after symptoms onset, respectively) in hospitalized patients. No significant impact of age, gender or treatment on the neutralizing titers was observed in this limited cohort. These data identify a clear association of humoral immunity with disease severity and point to immune mechanisms other than antibodies as relevant players in COVID-19 protection.

In December 2019, a novel severe acute respiratory disease was reported in China[1]. Following the early identification, in January 2020[2], of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the etiologic agent of the Coronavirus disease-19 (COVID-19), the new virus rapidly spread to generate a pandemic with a deep impact in global human health. The virus has caused more than 32,800,000 infections and more than 990,000 deaths (as of September 27th, 2020) despite worldwide restrictions in economic activities and mobility.

This massive impact has prompted an unprecedented research taskforce to define the epidemiological features of SARS-CoV-2 transmission, to identify new antivirals and to develop new vaccines able to generate protective immunity against the virus[3,4]. To guide vaccine development, the understanding of the interplay between the virus and the immune system as well as the definition of protective mechanisms have also been established as research priorities[5]. The current knowledge indicates that COVID-19 patients elicit a rapid humoral response

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Previous SARS-CoV-2 Infection Increases B.1.1.7 Cross-Neutralization by Vaccinated Individuals

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Citation: Trinité, B.; Pradenas, E.; Marfil, S.; Rovirosa, C.; Urrea, V.; Tarrés-Freixas, F.; Ortiz, R.; Rodon, J.; Vergara-Alert, J.; Segalés, J.; et al. Previous SARS-CoV-2 Infection Increases B.1.1.7 Cross-Neutralization by Vaccinated Individuals. *Viruses* **2021**, *13*, 1135. https://doi.org/10.3390/v13061135

Academic Editors: Luis Martinez-Sobrido and Fernando Almazan Toral

Received: 2 May 2021
Accepted: 10 June 2021
Published: 12 June 2021

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Abstract: With the spread of new variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there is a need to assess the protection conferred by both previous infections and current vaccination. Here we tested the neutralizing activity of infected and/or vaccinated individuals against pseudoviruses expressing the spike of the original SARS-CoV-2 isolate Wuhan-Hu-1 (WH1), the D614G mutant and the B.1.1.7 variant. Our data show that parameters of natural infection (time from infection and nature of the infecting variant) determined cross-neutralization. Uninfected vaccinees showed a small reduction in neutralization against the B.1.1.7 variant compared to both the WH1 strain and the D614G mutant. Interestingly, upon vaccination, previously infected individuals developed more robust neutralizing responses against B.1.1.7, suggesting that vaccines can boost the neutralization breadth conferred by natural infection.

Keywords: SARS-CoV-2; humoral response; pseudovirus; neutralization; B.1.1.7 variant

1. Introduction

Early in the COVID-19 pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants started to develop regionally and globally. Currently, the rapid spread of the B.1.1.7, or 501Y.V1, variant [1], first reported in the UK, casts doubts on the

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ACKNOWLEDGEMENTS

28 de septiembre de 2022

Esta mañana sobre las 7, he enviado la tesis a mis directores, sé que aún le faltan cosillas y habrá correcciones, quizás faltan algunas referencias y el formato final habrá que pulirlo…pero noto que casi esta, esto se acaba. Ahora le toca a Julià y a Carmen corregirla, (¿por cuarta vez?) y darme su feedback, que menos mal de ellos que me han guiado estos años a sacar este proyecto adelante. He aprendido tanto…a nivel científico por descontado, pero he aprendido el trabajo en equipo y a organizar el trabajo, entre otras cosas. No solo he aprendido de ellos, todas las personas de aquí me han ayudado y enseñado, además de todo se aprende, incluso durante las comidas, que pueden derivar a temas de conversación muy dispares.

Hoy es el día de después de mi cumple y he traído unas galletas, básicamente de chocolate, y algún café. Mi representación, no? Carlos y Ana estaban esperando impacientes y ansiosos a que llegara, como niños con la comida!! Estábamos en el office varios predocs, junto con Silvia, Mariona y Marisa, que se hacen las mayores, pero solo se lo hacen. En 20 minutos escasos, las galletas han arrasado. Las felicitaciones no paraban y Miguel me ha dado un medio-abrazo. He abrazado a Edurne, y he notado lo fuerte que es. Mientras, Ana se comía un paquete de galletas (ya volveré a esta mujer). Le he dado un kit kat a Chiara y me ha dado las gracias por acordarme de ella, pero cómo olvidarme? Acabo de ver a Luis, descansado después de haber defendido la tesis hace dos días, me alegro mucho por él. Y en dos días le toca a Ify, my company in the evening at P3! Good luck Ify, I'm so proud of you!!

Después de empacharnos con Filipinos y Principes, Julià ha venido a mi sitio para mirar la tesis y asegurarse que la figura del glycogag estaba correcta. El resto de la mañana ha sucedido en los siguientes actos:

- Miguel ha ido corriendo detrás de Carlos porque tiene un gossip, creo.
- Ana: "joer he comido un montón no tengo nada de hambre".
- Miguel quiere poner a crecer una maxi pero Carlos tiene todo el incubador ocupado con 13 flascos (xD).
- Miguel y Ana no paran de pelear como hermanos, mientras Anna y yo miramos divertidas a ver cuánto faltará para que se peguen.

Durante la comida hemos hablado de marruecos y también de que es importante lavar la ropa del revés y que yo plancho la ropa, al decir esto, Luis me ha dicho que he envejecido (más?!?!). Sin darme cuenta, he acabado siendo actriz para el vídeo de Ify.

Por la tarde, Amaya ha ofrecido su casa para hacer un afterwork y celebrar mi cumple, ha habido empache de vino, guacamole y un tiramisú final <3

Este solo es uno de mis días en IrsiCaixa, hay personas con las que no he coincidido hoy, como Lucía, que está de mudanza y en una nueva etapa, mucha suerte y a tope contigo! Francesc y Ester los albajunos majísimos, creo que estaban en P3, Ángel que está en su ordenador ocupado, Lidia y sus consejos, siempre reunida! Óscar que estará pintando cuadros preciosos, Edwards que está confinado ahora mismo, ah, porque no olvidemos que, en estos años, además de una tesis, hemos vivido una pandemia y todo lo que ha conllevado: las rondas de Barcelona vacías, P3 horas y horas, los turnos? Pero ahora ya podemos comer juntos, divertidísimo.

To sum up, acabo de cumplir 29 y empecé en IrsiCaixa con 24, no sé cómo han pasado los años tan rápido, pero en esta etapa no solo me llevo ciencia, me llevo a la gente que la constituye. Gracias a todo el equipo de IrsiCaixa, a lo/as IPs, lo/as postdocs, lo/as técnicas, Project managers, las chicas de muestras y comunicación, el team Penélope, Arnau y Cris…Todos y cada uno de vosotros me habéis ayudado y he pasado momentos “de lo mejorcito” con vosotros. Quiero agradecer especialmente a Ventura y a Lourdes por la oportunidad que me han ofrecido. A mis directores Julià y Carmen por su enseñanza y apoyo durante estos años. A Jorge y a Ben por preguntarme siempre qué tal voy y ayudarme en todo lo posible. A Ana, a tope contigo, menos mal de las risas, amiga, una frase se queda corta para agradecértelo todo. Al tri-grupo VIC-IgG-Albajuna, por todo. Y a todo el grupo de predocs (algunos ya postdocs), por el buen ambiente, las quedadas, el apoyo mutuo y las risas, con vosotros todo ha sido mucho más fácil.

Fuera de Irsi, me gustaría agradecer a mis padres, mi familia, amigas, amigos y a Alejandro por la paciencia y el apoyo inigualable.

Esto solo ha sido un día de toda esta etapa, para describir los casi cinco años necesito otro libro...Y como una imagen vale más que mil palabras os dejo varias (para que no os olvidéis, y yo lo recuerde siempre). Gracias a todos!!

Un besazo <3