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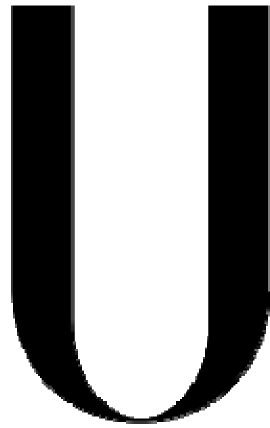

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Targeting Membrane Proteins of Latently Infected CD4+ T Cells Providing a Starting Point to the Eradication of HIV-1

Vasco Gonçalves

Dissertação de Mestrado

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS
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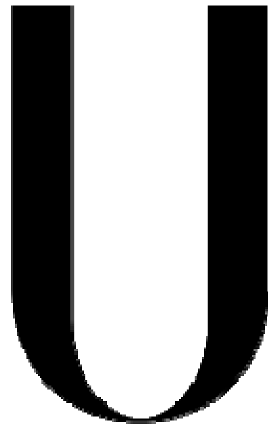

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Dissertação orientada pelo Profº Doutor João Gonçalves

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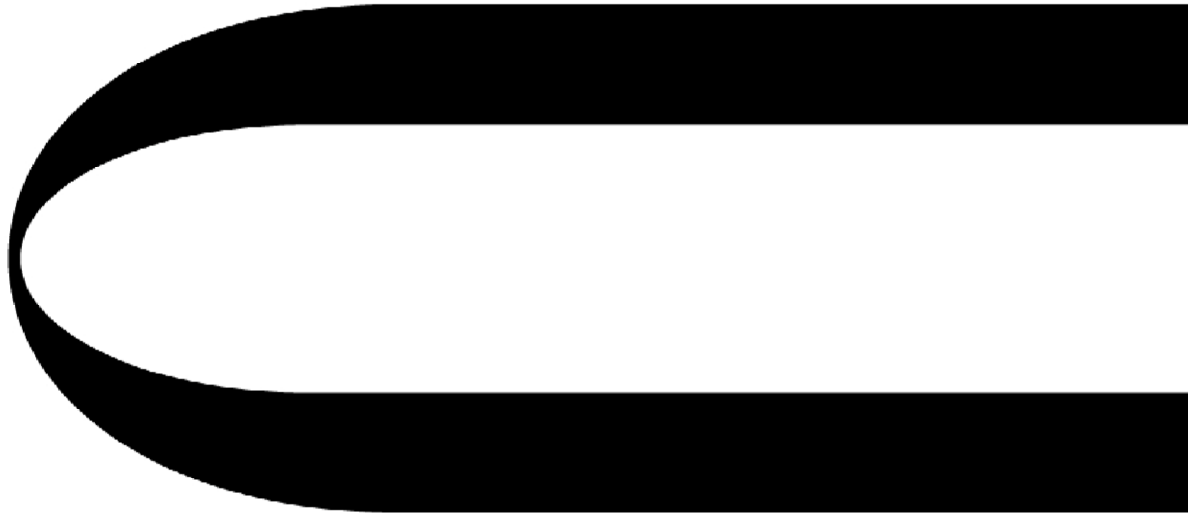
2013

"The greatest ideas are the simplest."
William Golding, Lord of the Flies, 1974



ABSTRACT

AIDS is characterized by an immunodeficiency developed due to an infectious disease caused by a world spread virus, the HIV-1. Infection with this virus culminates invariably in the death of the infected individual due to opportunistic infections that find a way into the organism. Today, HIV-1 is fought against with therapies based in antiretroviral drugs that, although efficient and able to stop or difficult the viral infectious cycle, do not provide a real cure for AIDS, this is, HIV-1 infection is still considered a chronic and fatal condition. The incapacity of such therapies to eradicate HIV-1 infection is in part due to the existence of viral reservoirs like the $CD4^+$ T cells population which are responsible for maintaining the HIV-1 in a latent state inside the cell, protecting it from the host immune system and administered drugs, waiting for some trigger capable to reactivate it. This project was born from the possibility to achieve a sterilizing cure upon finding that specific trigger, enabling the depletion of such reservoirs and subjugating the reactivated HIV-1 to the effects of the antiretroviral drugs. For this, we traced and objective in selecting antibodies through a run of the mill technology, the phage display technique, against unknown membrane determinants of the latently infected cells searching for agonist antibodies capable of reactivating the latent HIV-1 and possibly, bringing us one step closer to a cure for AIDS. scFv of human origin were selected against J-Lat 10.6 and their

agonist capacity in causing a phenotypic modification was evaluated resorting to flow cytometry.

The work here presented permitted us to identify four promising scFv with agonist HIV-1 latent reactivation capacities against J-Lat 10.6 cells and, additionally demonstrated the possibility for our simple approach to be an alternative methodology to the lentiviral agonist selection method developed by Hongkai Zhang and associates.

Key-Words: HIV-1 Latency, AIDS sterilizing cure, Agonist antibodies, scFv, Phage display.

A SIDA, ou síndrome da imunodeficiência humana adquirida desenvolve-se devido a uma doença infeciosa de incidência mundial causada pelo vírus da imunodeficiência humana, o VIH tipo-1. Este vírus tem um tropismo específico para células do sistema imunitário, o que leva à depleção desta população celular inevitavelmente debilitando as defesas naturais do organismo. Tal estado imunodeprimido contribui para que infeções oportunistas se instalem, causando complicações adicionais o que resulta invariavelmente na morte do individuo seropositivo. Até à data os doentes com VIH-1 são sujeitos a terapias que se baseiam em combinações de fármacos antirretrovirais e que, apesar de eficientes bloqueando ou dificultando o ciclo infecioso do vírus em algum momento, não constituem uma verdadeira cura para a SIDA. A infeção com VIH-1 continua a ser considerada uma condição crónica e fatal. A dificuldade que tais terapias têm em erradicar a infeção causada pelo VIH-1 é em parte devida à existência de reservatórios virais, como a população de células T CD4^{+}. Esta células inadvertidamente mantêm o VIH-1 num estado latente no interior da célula, protegido do sistema imunitário do hospedeiro e de qualquer terapêutica farmacológica, aguardando por algum evento desencadeador do processo de reativação. É derivado desta observação fisiológica que este projeto nasce. A partir da possibilidade de se obter uma cura esterilizante ao se encontrar um gatilho capaz de reactivar o VIH-1 latente e que permita então a depleção

dos reservatórios de latência viral e submeta o HIV-1 à mercê dos fármacos antirretrovirais. Para tal traçamos um objetivo baseado numa técnica laboratorial corriqueira, o phage display, para se selecionar anticorpos contra determinantes membranares apresentados pelas células latentemente infetadas. A identificação de anticorpos agonistas capazes de reativar o HIV-1 latente num modelo celular laboratorial, as J-Lat 10.6, aproxima-nos da possibilidade de encontrar uma cura para a SIDA. Tal identificação foi facilitada recorrendo à técnica de citometria de fluxo que permitiu avaliar a capacidade dos anticorpos selecionados desencadearem a alteração fenotípica desejada.

O trabalho aqui apresentado permitiu-nos identificar quatro scFv agonistas com capacidade de reativar o HIV-1 latente em células J-Lat 10.6 e, adicionalmente, demonstrou que pode ser utilizado como uma alternativa à metodologia utilizada por Hongkai Zhang na sua seleção de anticorpos agonistas através do uso de partículas lentivirais.

Palavras-Chave: Latência viral do HIV-1, Cura esterilizante da SIDA, Anticorpos agonistas, Phage display.

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"Tell me and I forget, teach me and I may remember, involve me and I learn."

Unknown

Uma viagem. É assim que, ao olhar para trás, reconheço este último ano. Não uma viagem que começou e acabou no espaço de um ano agora passado, mas uma viagem que se insinuava na eminencia desde a primeira vez que abri os olhos e vi com vontade de conhecer. Esta dissertação representa, para mim, não apenas um único período, o finalizar estéril de uma caminho, mas um quarto de século, uma vida, uma fértil viagem ainda a decorrer.

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“Can it be that I have not lived as one ought?" suddenly came into his head. "But how not so, when I've done everything as it should be done?”

Leo Tolstoy, The Death of Ivan Ilych, 1886

ABBREVIATIONS

Ab	Abtibody
Abs	Absorbance
ADCC	Antibody Dependent Cell Mediated Cytotoxicity
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
Amp	Ampicillin
APOBEC3G	Apolipoprotein B mRNA-editing Enzyme-Catalytic Polypeptide-like 3G
BSA	Bovine Serum Albumin
CA	Capsid
cART	Combination antiretroviral therapy
CDR	Complementary Determining Regions
C_H	Constant Heavy Chain
C_L	Constant Light Chain
dAb	Single Domain Antibody
DNA	Deoxyribonucleic acid

DR5	Death Receptor 5
E. coli	*Escherichia coli*
Env	Envelope
Fab	Fragment Antigen Binding
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Fc	Fragment Crystallizable
FcR	Fragment Crystallizable Receptor
FR	Framework Region
Gag	Group Specific Antigen
GFP	Green Fluorescent Protein
Glu	Glutamic acid
Gly	Glycine
HAART	Highly Active Antiretroviral Therapy
HAMA	Human Anti-Mouse Antobody
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HPV	Human Papilloma Virus
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgSF	Immunoglobulin SuperFamily
IN	Integrase
IPTG	Isopropyl-beta-D-Thiogalactopyranoside
Kan	Kanamycin
kDa	Kilodalton
LTR	Long Terminal Repeat
Lys	Lysine

MA	Matrix
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NC	Nucleocapsid
NF-*κ*B	Nuclear Factor kappa B
NK	Natural Killers
OD	Optical density
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PKC	Protein Kinase C
Pol	Polymerase
PR	Protease
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse Transcriptase
scFv	Single-Chain Variable Fragment
Ser	Serine
SU	Surface glycoprotein
Tet	Tetracycline
TM	Transmembrane glycoprotein
TNF-α	Tumor Necrosis Factor alpha
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TRAIL-R2	Tumor Necrosis Factor Related Apoptosis Inducing Ligand Receptor 2
TSA	Trichostatin A
U	Deoxyuridine
V_H	Variable Heavy Chain
V_L	Variable Light Chain
µg	Microgram
µl	Microlitre

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GENERAL INTRODUCTION

In 1901, Emil von Behring got his work recognized with a Nobel Prize in Physiology or Medicine. Such achievement was attributed to the effort he had done in developing the then known as serum therapy and its application against diphtheria. As it was stated, his work "*opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths"* ("The Nobel Prize in Physiology or Medicine", 1901). Being this weapon what we now know as antibodies. Since then, and until 1975, six more Nobel Prizes were attributed for advances related to such important proteins when Kohler and Milstein revolutionized biological research (Köhler & Milstein, 1975). They created a key tool for research, diagnosis and clinical applications that granted antibodies the place as the most important biomolecule in modern times. Understanding the structure and the genetics of such molecules granted two more Nobel Prizes to Baruj Benacerraf, Jean Dausset and George Snell (1980) and to Susumu Tonegawa (1987) and paved way to the development of important new technologies that enabled in vitro selection and the production of large quantities of new antibodies in new formats (Miersch & Sidhu, 2012). The great promise of antibodies led pharmaceutical companies everywhere to compile and exploit the knowledge acquired by all these years of research in the hope of creating new drugs or to generate new forms of diagnosis capable of fighting old diseases. This thesis started on this

idea, in the importance of antibodies, the importance of the new technologies used for selecting them and the possibility to use these molecules in innovative ways to fight diseases such like acquired immunodeficiency syndrome (AIDS).

1.1 The Antibody

In the microscopic biological realm entity recognition, binding and/or adhesion is imperial for the precise functioning of either a single cell or a complex organism (Kindt, Goldsby, & Osborne, 2007a). Such processes are made possible, in part, due to the existence of a super family of proteins, soluble or membrane-bound, called the immunoglobulin superfamily (IgSF). One of its integrants, the immunoglobulins (Igs), or simply putting it: the antibodies; are the main effectors for the natural process of adaptive immunity in complex organisms such as the human species. Antibodies by themselves form a family of Y shaped glycosylated proteins, produced by a specific class of lymphocytes, the B cells, and are characterized by their capability of binding to specific immunologic active regions of molecules. These molecules are given the designation of antigens and present one or more specific sites or antigenic determinants, the epitopes, to which the antibody’s paratopes bind to in a non covalent way. When membrane-bound, upon contact with the immunogen, antibodies act as triggers for B cell clone proliferation and differentiation into memory B cells and plasma cells, and when secreted in its soluble form, antibodies travel through the circulatory system acting as the effectors of humoral immunity. In normal conditions, they are responsible for identifying, binding, neutralizing or starting a cascade of events that leads to the neutralization and/or elimination of foreign particles that have found a way into our bodies. Deregulations of antibody activity and immune responses and their intervenients are in the base for the emergence of genetic or acquired immune deficiencies. Antibodies are therefore an important and powerful component of the immune system defending our organism from foreign menaces (Kindt, Goldsby, & Osborne, 2007b).

1.1.1 Antibody structure

Antibodies are not all the same and therefore form a rather significant family composed by similar but yet different members. One can say that the immunoglobulin family is complex and presents different classes that can be grouped and distinguished by their ability to perform several unique binding and effector functions directly related to their individual structures and formats. Although there are five major classes worth mentioning: IgG, IgA, IgM, IgE, IgD; this

work will only focus on the IgG class, as it is the most abundant in serum samples, representing about 80% of total immunoglobulins present in the serum.

In nature, human IgG is a protein of 150 kDa formed by four individual amino acid sequences illustrated in figure 1.1. Those are organized in a series of antiparallel β sheets that fold themselves into 110 amino acid long globular domains due to the existence of a conserved inter-domain disulfide bonds. They consist of two γ heavy chains (55 kDa) and two kappa (κ) or lambda (λ) light chains (22 kDa) arranged into two heterodimers connected by an intra-domain disulfide bond. The quaternary structure of the well known Y shape is given by two intra-domain disulfide bridges that connect the hinge regions of the two adjacent heavy chains and thus, dimerizes both heterodimers. Consequently, being a homodimer of heterodimers, an antibody can be represented in its molecular formula as $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$ (Kindt, Goldsby, & Osborne, 2007c).

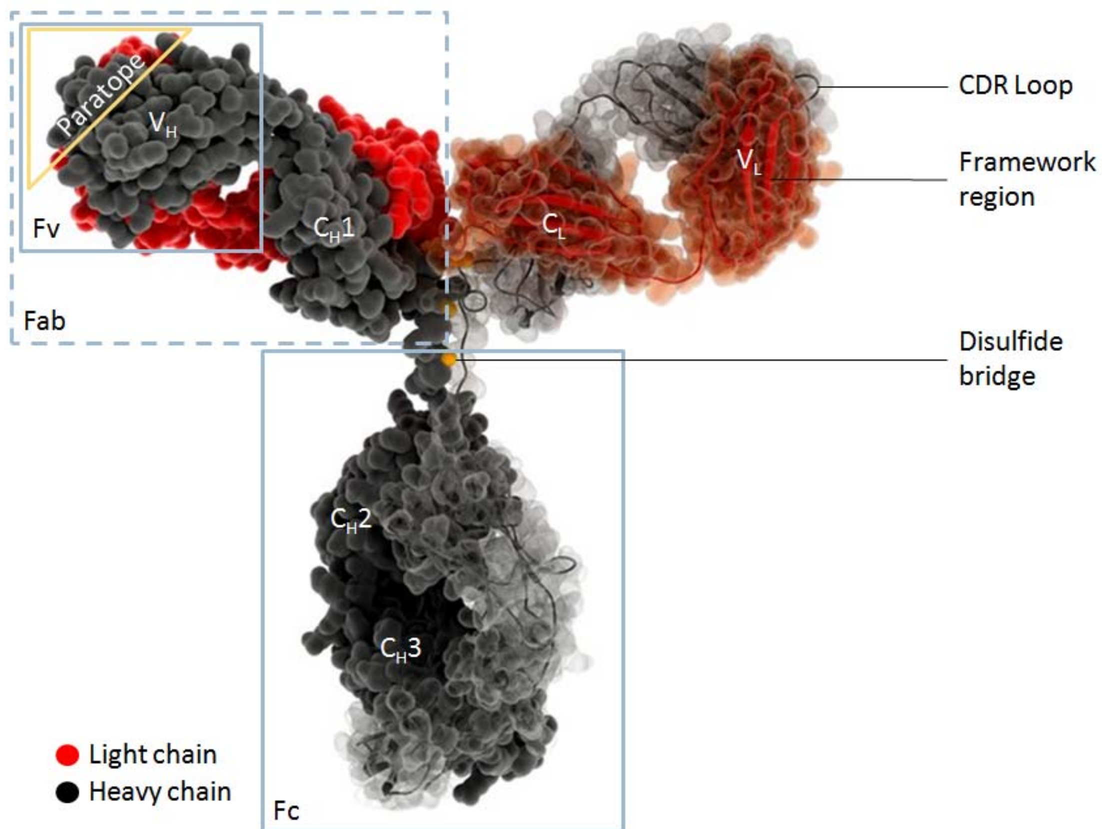


Figure 1.1 Typical immunoglobulin G format: this class of antibodies is composed by four chains grouped in identical pairs, two Heavy and two Light chains that maintain their quaternary structure due to disulfide bridges. Each chain is composed by globular domains named according to their sequence identity between different antigen binding antibodies. Heavy chains present three constant regions (C_H3 to C_H1) and one variable (V_H) while the light chains present one constant (C_L) and one variable region (V_L). Variable regions have peptide loops that extend outside of its frame work called CDRs and are responsible for forming the paratope.

On each monomer it is possible to identify several regions according to the globular domains. In the heavy chain four globular domains define three constant regions (C_H3 to C_H1 starting from the carboxyl-terminal region) and one variable region (V_H – amino-terminal), on the other hand, each light chain consists only in two regions, one constant (C_L) and one variable (V_L – also at the amino-terminal region). Constant regions acquired such name logically, as when compared with the same regions on other antibodies few differences in sequence are spotted. Variable regions on the other hand differ between antibodies with different antigenic determinants. Certain areas of these variable regions, called the complementary-determining regions (CDRs), exhibit a higher variability in amino acid sequences and are in fact responsible for forming the antigen binding site. Each variable domain is composed by three loops that correspond to three CDRs (CDR1 to CDR3) which in turn are responsible for connecting the conserved β strands that compose the so called framework regions (FRs). FRs are then responsible for maintaining a universal structure for variable regions acting as a scaffold upon which the six CDR loops are supported permitting them to undertake their hypervariable conformation unique to each monoclonal antibody (Kindt et al., 2007c).

Enzymatic digestion with the proteolytic enzyme papain cleaves the immunoglobulin at the hinge region producing three fragments. Two of those fragments (45 kDa each) presented antigen-binding activity, and thus were named Fragment antigen binding (Fab). The other one was named Fragment crystallisable (Fc) (55 kDa) as it was found to crystallize during cold storage. Fc corresponds to the portions C_H3 and C_H2 of both heavy chains explaining why it does not present antigen-binding activity. Functions for these regions will be analyzed further down the line (Kindt et al., 2007c).

1.1.2 Genetic organization of immunity

Considering the outside of our individual systems, the number of particles capable of being considered foreign is enormous. Therefore, in order to interact with as many foreign molecules as possible, there is need for an enormous number of antibodies as well. At this point it is important to mention that although response to an antigen can mobilize different B cell clones, one antigenic determinant mobilizes only one B cell clone and thus, a monoclonal antibody. It is estimated that the human organism is capable of, during its lifetime, producing between 10 to 300 million different antibodies. Such variability would mean a copious amount of genes. And looking at the size of our genome, one understands that all possible antibodies cannot be coded in individual genes. Actually, variability is assured by somatic recombination,

P and N nucleotide adition and hypermutagenesis of a small set of genes that culminates in a pool of circulating polyclonal B cells (Kindt et al., 2007a).

Ig genes are organized in three separate families: Heavy chains are coded from the IgH *locus* in chromosome 14 while light chains are coded either in the Ig or Igλ *locus*, in chromosome 2 or 22 respectively. As said before, human light chains can be either κ or λ, being κ more frequent than λ (60% to 40%), while heavy chains can be of five isotypes depending on the Ig class: γ (IgG); μ (IgM); α (IgA); δ (IgD) and ε (IgE). Germ-line DNA sequences are actually different for mature sequences and are composed of different segments, coding and non-coding alike. While the portions of these genes that correspond to the constant regions of Igs are called constant (C) segments, variable regions are coded by two or three segments, depending if they belong to light our heavy chains respectively. A variable (V) segment fallowed by a joining (J) segment for both regions and the addition of a diversity (D) segment in between both of them for the variable regions of the heavy chains. During lymphocyte maturation V(D)J segments are randomly combined in an event called somatic recombination which adds another layer for antibody diversity from the already so many possible sequences existent for antibody generation. DNA repairing enzymes active during somatic recombination are responsible for generating even more diversity by adding nucleotides during the joining of the coding sequences. If *palindromic* junctions are generated P nucleotides were added, otherwise *nontemplated* stretches with new nucleotides are generated (N nucleotides).

After lymphocyte activation upon contact with the antigen, two more diversity generating processes occur: hypermutagenesis and Ig class switch recombination. Somatic hypermutation or hypermutagenesis is responsible for random insertions of point mutations in V segments that leads to the selection of the lineage with the best affinity for the specific antigen – affinity maturation; and Ig class switch recombination, which only occurs in the C segments, is responsible for changing isotypes and therefore effector functions as well.

1.1.3 Antibodies and its effector functions

Besides antigen-binding activity, which by itself does not neutralizes or removes the foreign particle from our body, immunoglobulins play a determining role in humoral response. They are able to promote biological responses that lead to the effective removal or neutralization of the said foreign particle, this is, they have effector functions (Kindt, Goldsby, & Osborne, 2007d). Such functions are assured by interactions between the constant regions of the heavy chain and other soluble or membrane bound proteins and therefore are dependent upon

different immunoglobulin classes. One of those effector functions is opsonization, one of the most important defenses against bacterial invasions. It is characterized by antibody triggered phagocytosis of antigens by macrophages and neutrophils. Activation of this event is dependent on cross-linking of Fc receptors (FcR) presented on macrophages membranes upon binding to Fc regions of antibody-antigen complexes, setting off a signal-transduction pathway responsible for starting phagocytosis. Destruction of the infectious agent is carried out inside the phagocyte. Complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC) are other examples of antibody effector functions. The complement system is composed by a group of soluble proteins present in the serum capable of many actions such as the formation of pores in cellular membranes leading to their destruction or phagocytosis induction. Antibodies can act as cellular receptors as well, binding to target cells and exposing their Fc region to effector cells like natural killer (NK) cells activating a cytotoxic reaction – ADCC.

But for medical uses the most important feature of the antibodies is their antigen-binding activity. Antibodies can be used as a means of diagnosis passively marking target molecules or as therapeutic agents binding to certain molecules in the human system, sequestering them, or to their respective receptors, blocking them, and therefore preventing their natural interaction antagonizing the receptor. But they can also bind to a specific cellular receptors and act as an agonist triggering some form of cellular response. Present day research is avidly turning its efforts into the search for antibodies capable of causing an action in the cell whose receptor they bind to. Conatumumab (AMG 655) developed by Amgen Inc is an example of an agonist monoclonal antibody designed for the treatment of solid tumors of hematopoietic origin (Rosevear, Lightfoot, & Griffith, 2010). It targets the tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2 or death receptor 5 – DR5) mimicking its natural ligand and starting a caspase cascade that leads to the apoptosis of the tumor cell. Agonist antibodies can be studied and developed for use in other diseases rather than just neoplasic conditions. Syndromes and diseases caused by virus that do not have yet a cure, like the human immunodeficiency virus (HIV) or even the human papilloma virus (HPV), can possibly represent new target afflictions for agonist modulation.

1.1.4 Genetic engineering and the recombinant antibody

In the living organism, as it has been stated before, contact with an antigen causes proliferation and differentiation of a pool of B cell clones rather than just one specific clone with affinity for one singular epitope. This results in a mixture of antibodies directed for

different epitopes of the same antigen, this is, a polyclonal antibody mixture. Although advantageous for a living organism, for research or medical purposes this represents a difficulty. Thus monoclonal antibodies, with specificity to a single epitope, are preferred. Purification of a monoclonal antibody from a polyclonal mixture was not feasible until 1975 when Kohler and Milstein described a method that permitted monoclonal antibody production (Kindt, Goldsby, & Osborne, 2007e). Fusing a B-cell recovered from an immunized donor, like a mouse, with an immortal myeloma cell they created a hybrid cell, the hybridoma, capable of antibody production in large quantities with immortal properties and therefore with the possibility to be cultured indefinitely (Köhler & Milstein, 1975). Although this technology revolutionized the field of antibody research and application several difficulties were associated with it. B-cells used for generation of hybridomas were of murine origin and thus when used for medical purposes in humans created immunologic and hypersensitive reactions with human anti-mouse antibodies (HAMAs) limiting their use (Jaffers et al., 1986). Still, associated with their animal of origin, there was the problem of affinity. Different animals have different innate libraries of possible antibodies, which mean that high-affinity antibodies for a specific antigen are not always provided from mouse immunization. Other difficulties resided in the fact that the antibodies obtained, when used for medical purposes, did not stay in circulation for long periods of time. Also this technology is very laborious, time consuming and expensive (Jaffers et al., 1986). Cellular stability and storage costs, impossibility to immunize against toxic targets and difficulty in direct access to antibody genes are examples of some other problems for hybridoma technology.

1.1.4.1 New antibody formats: the advent of the single chain variable fragments

With recombinant DNA technology on the rise, new approaches to antibody production started to appear. Antibodies began to be designed rather than just produced. This genetic antibody manipulation contributed to furthering the knowledge about structure and functional organization of immunoglobulins which by itself permitted the emergence of new engineered antibodies for research, diagnosis, and therapeutic applications with new specificities beyond conventional antibody technologies. Coding sequences from Igs of different species started to be used to create chimeric or humanized antibodies trying to surpass some problems of hybridoma technology (Morrison, Johnson, Herzenberg, & Oi, 1984; Jones, Dear, Foote, Neuberger, & Winter, 1986). And not only molecules of different origins were added to the natural backbone of antibodies, but fragmentation also started to occur. Antibodies began to be divided into smaller fragments and their sizes were reduced considerably. Changes were

made in order to achieve specific goals such as increased protein expression, better biodistribution and longer circulation half-life, lower immunogenicity or even better tissue penetration. New formats like Fragment antigen binding (Fab) single chain variable fragments (scFvs) or single domain antibodies (dAbs) started to appear (figure 1.2).

The scFv format is a fusion protein of approximately 30kDa. It consists of a V_H and a V_L region connected by a flexible peptide linker of about 15 amino acids (Bird et al., 1988). Such linker contributes to protein stability allowing the variable regions to acquire their correct conformation and also facilitates expression in *E. coli* systems. Most common linkers are composed by stretches of Gly and Ser and/or Glu and Lys residues for flexibility or solubility respectively (Ahmad et al., 2012). scFv are one of the smallest portions of the immunoglobulin still retaining its antigen-binding activity and thanks to this characteristic associated with the easiness of expression in bacterial systems scFv have undertaken a central role in new antibody selection techniques like phage display. Also, scFvs can easily be transformed in bivalent or trivalent scFvs be linking this proteins together creating multimers. Such fusions can be between equal or different scFvs creating bi-specific diabodies.

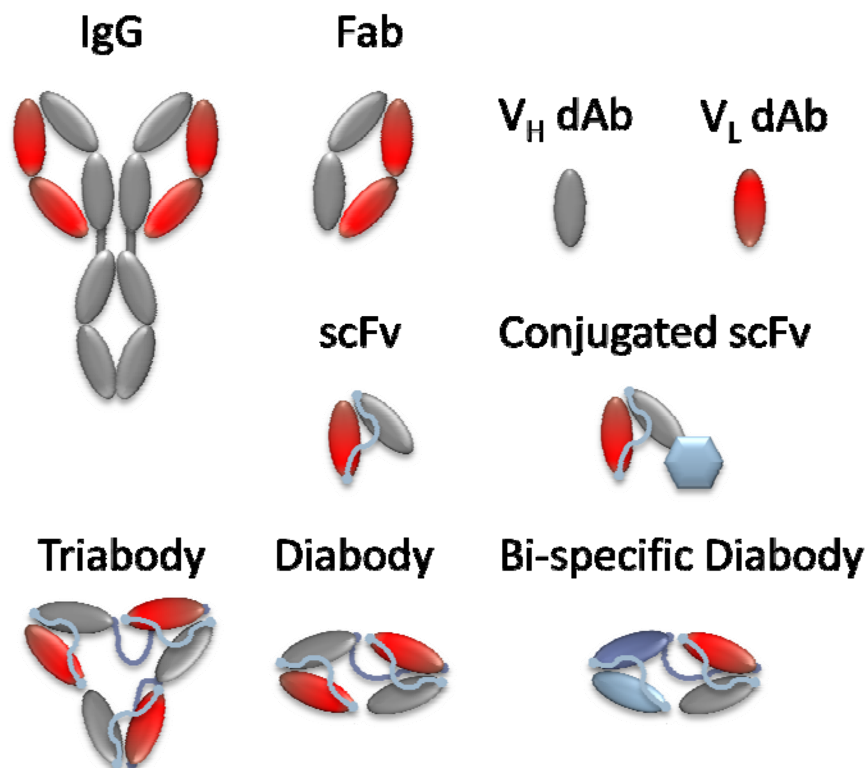


Figure 1.2 Representation of the natural occurring IgG and some of its derived new formats: scFv (30kDa) can be fused together to create diabodies (50kDa), triabodies (75kDa) and so forth. They can also be conjugated with other proteins of the same origin but with different affinity (bi-specific antibodies) or even chemical compounds.

Yet, the smallest possible antibody format is the dAb with 15kDa (Putnam, Liu, & Low, 1979). They are formed by a sole variable domain either from the heavy or light chain of immunoglobulins and thus only present three CDRs against one specific epitope (Ward, Güssow, Griffiths, Jones, & Winter, 1989). Like scFvs, their small sizes give them special attributes like higher tissue penetration and lesser immunogenic activity, besides the facility of expression in bacterial systems.

1.2 New antibody selection techniques

As we have seen before, antibody diversity in higher organisms is assured by combinatorial recombination of germ-line antibody genes associated with somatic hypermutation. This means that each individual organism caries a library of all its possible antibodies with the majority of them presented in its circulating B cells. Hybridoma technology was based on this natural occurring library using it as a form of *in vivo* selection. Since then, antibody generating technology evolved due to impending medical necessity and new forms of *in vitro* antibody selection arouse such like phage, microbial or cell display or even cell free technologies like ribosome, DNA or mRNA display (Oliveira, 2013). Such technologies rely in the possibility to present a protein conjugated to a particle able to genetically identify it. Ribosome, mRNA and DNA display require for *in vitro* transcription and translation and are by themselves variants of each other. Translation can be stopped by the addition of antibiotics like rifampicin which permits the generation of protein-ribosome-mRNA complexes presenting the basis for ribosome display. If mRNAs are treated to present at their 3’ end a puromycin oligonucleotid which is incorporated at the end of the translation process, a protein-mRNA complex is formed and mRNA display is made possible. DNA display was developed to circumvent the fast degrading mRNA usage and uses a mixture of reversed micelles where individualized DNA fragments, marked for example with biotin, are transcribed and translated to streptavidin-fused proteins. Cell dependent displays work in a similar way. Cells provide the machinery necessary for our protein production and present them is their surface therefore forming a protein-cell-DNA conjugate. Phage display will be further explained below as it is the mains focus in this work.

But for *in vitro* selection techniques to work the huge diversity of antibodies observed in humans had to be duplicated in manmade libraries. Presently it is possible to create several different types of libraries that differ in their origin depending on the selection technique and purpose of the intended result. Obviously each immune library has its own limitations that

have to be equated depending on the type of antigen or the affinity and quantity of antibodies expected from the selection procedure.

scFv libraries can be divided into three categories, immune, naïve and synthetic (Ahmad et al., 2012; Oliveira, 2013), explained hereafter. Immune libraries derive from the variable domains of antibody genes collected from enriched pools of activated B cells of immunized animals like mice (Xu, Jin, & Fan, 2003) or camels (Rahbarizadeh, Rasaee, Moghadam, Allameh, & Sadroddiny, 2004). Meaning that previous animal injection with the desired antigen is required in order to construct such libraries. Due to the previous in vivo affinity maturation, in vitro selection is biased. Selection procedures result in greater pools of specific binders against the pre-immunized antigen and selected antibodies usually present a wider range of higher binding affinities. Immune libraries are constructed for specific uses and therefore, when considering another antigen, a new library needs to be made. Naïve libraries also derive from live donors, but lack the pre-immunization procedure and therefore are not biased against any particular antigen. They are constructed from samples of non activated B cells (Vaughan, Williams, & Pritchard, 1996). This feature allows them to be used in various selection procedures against non related antigens without the need to construct a new library and most importantly, they overcome the inability to select antibodies against toxic, self or non-immunogenic antigens. One important aspect of such libraries is their size and its relation to selection success. Libraries with larger sizes, usually maxing at 10^{9}-10^{10} individual scFv, present better chances to select more antibodies with higher affinities than smaller libraries (Griffiths et al., 1994; Vaughan et al., 1996; Winter, Griffiths, Hawkins, & Hoogenboom, 1994). All these characteristics contributed for the choice to work with this kind of libraries in this experimental work. The last category is the synthetic libraries. These libraries are also not biased against any particular antigen as they derive from non-immunized samples prepared resorting to manmade combination of germ-line gene sequences and randomized CDRs (Hoogenboom & Winter, 1992). Being CDR3 the CDR with the most diversity and the one that is essentially responsible for antigen binding it is understandable that most synthetic libraries focus on randomizing this particular CDR and therefore are created specifically to yield high affinity antibodies.

1.2.1 Phage display technology

Derived from the work developed by George Smith in 1985, phage display technology is based in the possibility to present a determined protein on the surface of a filamentous bacteriophage (Smith, 1985). Smith demonstrated that DNA from foreign origin could be fused

together with the gene for a coat protein of a non-lytic filamentous phage and thus, expressed and displayed at its surface as a fusion protein without affecting the normal infectious cycle of the bacteriophage. In 1990 and with the help of recombinant DNA technology, McCafferty created a recombinant phage that successfully displayed a functional scFv (McCafferty, 1990). He demonstrated that the fusion scFv maintained the same antigen-binding activity as the original scFv and that, even diluted in a mixture with 10^6 different phages, it was possible to re-isolate it. Additionally, access to the gene sequence of the fusion protein was made possible due to phenotype genotype conjugation, this is, scFv displaying particles have associated to them the DNA for the respective fusion scFv displayed. This became one of the most important characteristics of phage display technology permitting quick and easy access to the gene sequence of any selected scFv amongst an enriched and diverse library (Griffiths et al., 1994). This technology was further perfected in the following years (Barbas, 1991; Clackson, 1991; Winter, 1994) and now it is a widely used procedure presenting proteins other than scFv. Phage display technology was then born and permitted the fast and efficient selection of antibodies isolated from large populations of recombinant phages based on their affinity against a specific target.

In more detail, the phage display technique relays in filamentous bacteriophage biology. Phages like M13 and its relatives are non-lytic bacteriophages that present a rod-shaped capsid composed mostly by the major coat protein, pVIII, with approximately 2700 copies, that encapsulates its single strand circular DNA genome. On one tip of the phage particle there are approximately five copies of each minor coat protein pVII and pIX and on the other tip also around five copies of each minor coat protein, pVI and pIII, being the last responsible for the infection of the host bacteria by binding its n-terminal region to the F pilus (Smith & Petrenko, 1997). Infection ends with the viral genome being injected unto the bacterial cytoplasm where it remains as a separate entity from the bacterial genome being transcribed and replicated. But, in order to create

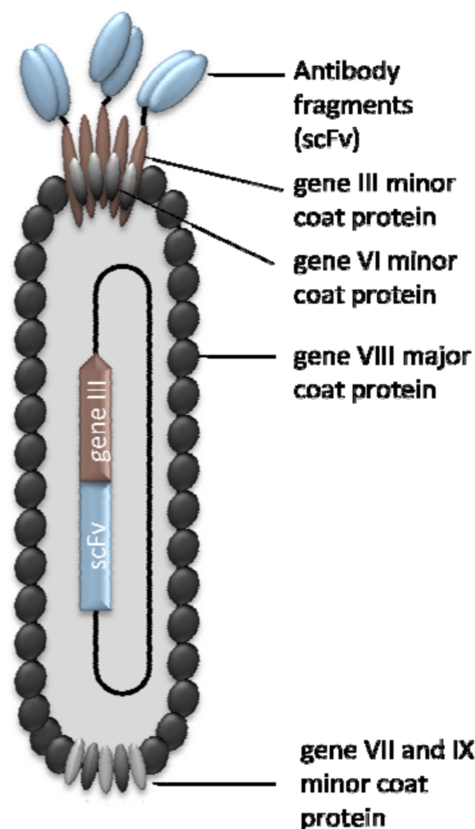


Figure 1.3 Generic structure of a recombinant phage: A phagemid coding for a fusion protein between pIII and a scFv (or any other displayable protein) is the basis of phage display.

recombinant phages expressing or protein of interest, one has to first clone it in a phasmid (Figure 1.3). Phasmids are vectors derived from small coding plasmids with a phage origin of replication which code for only one phage coat protein preceded by a multiple cloning site. Therefore, a phasmid by itself is not capable of producing phages (Garrard, Yang, O'Connell, Kelley, & Henner, 1991) and only when complemented with helper phages like VCSM13, with a complete viral genome, recombinant phage production is possible (Vieira & Messing, 1897). Proteins of interest cloned in such vectors, like the pCOMB vector family (Barbas, Burton, Scott, & Silverman, 2001), can be fused to any desired coat protein (Barbas et al., 1991) but most common practices clone the protein of interest in fusion with pIII or pIX. Such choices depend on the desired final valence. Phasmid may also present leader sequences enabling export of the synthesized protein to the oxidative environment of the periplasm and an amber stop codon separating the cloned protein and the phage coat protein (Figure 1.4). The ambar stop codon, *glnV,* is especially important for the production of the cloned protein in its soluble form. This is made possible resorting to an ambar non-suppressor strain like *E.coli* TOP10F' where *glnV* is read as a stop codon (Hoogenboom et al., 1991) supplemented with IPTG.

Figure 1.4 Detail of a phagemid: pCGMT is a phagemid derived from pCOMB that codes for a FLAG epitope before the ambar stop codon facilitating the screening protocols of the purified scFv. A lacZ promoter makes scFv production inducible by the presence of IPTG when in ambar non-suppressor strains like TOP10F'.

For recombinant phage scFv selection to be achieved, inquiry of a built library against a target antigen of interest is necessary (figure 1.5). Multiple rounds of panning of the phage library against the antigen are performed with multiple washing steps in order to remove unbound recombinant phages. Due to the washing steps antigens need to be immobilized in a solid support like small peptide high binding microtiter plates, or streptavidin coated beads (for biotinylated antigens) per example. At the end of each panning specifically bound phages are eluted and reamplified allowing to continue the selection process serving as the input of the next panning. Normally three rounds of pannings are performed, allowing specifically bound phage enrichment (Smith & Petrenko, 1997), ending with the infection of an ambar non suppressor *E. coli* strain allowing the production of soluble proteins for further characterization. When performing this technique it is expected to select a high affinity clone against the chosen antigen from a large number of nonspecific recombinant phage clones.

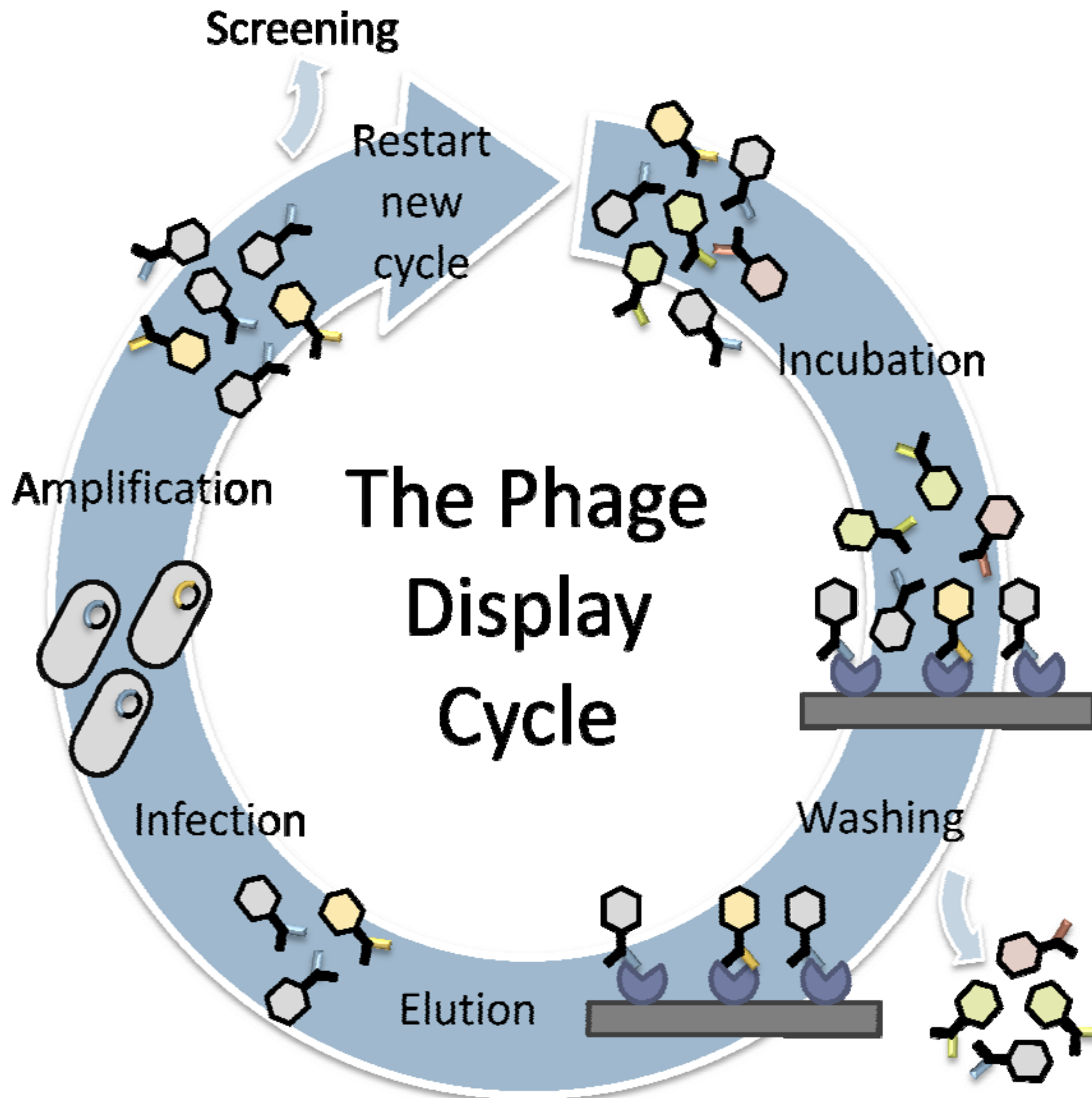


Figure 1.5 The phage display cycle: After phage library amplification and recombinant phage production the phage pool is incubated against the immobilized antigen for a determined period of time before any washing steps, responsible for removing unbound recombinant phages, and posteriorly eluted to recover the binding phages. Eluted phages are in turn used for creating a new library biased against the antigen by infecting an *E. coli* strain and are then reamplified to produce a new pool of recombinant phages, which can be the starting point of a new selection round, or directed for screening purposes.

For the presentation of scFvs this technique exhibits several advantages over hybridoma technology. First of all, animal immunization is easily avoided, and if necessary to library creation, no hybridomas need to be produced. Selection of antibodies against difficult or toxic antigens becomes more accessible and feasible. Additionally, phages are more stable and therefore can be stored at 4ºC for long periods of time and if needed are also easily, rapidly and inexpensively produced. And finally, scFv genes are easily obtainable and thus more easily manipulated by mutagenesis as affinity or specificity capacities of the selected scFv, although normally sufficient for research usage, might be unsatisfactory for medical applications.

1.2.2 Lentiviral selection method

Although the vast amount of selection technologies at hand, new purposes and intentions pave the way for new selection techniques to be imagined and developed every day. The lentiviral selection method developed by Hongkai Zhang and its associates was designed to answer impending new needs (H. Zhang et al., 2013; H. Zhang, Wilson, & Lerner, 2012). As we mentioned before, great efforts are being made in order to discover antibodies that present agonist capacities, but selection techniques are mainly focused in binding rather than function. This group states that the generation of antibodies capable of perturbing cellular functions is hampered by a secondary screening against antigens in their natural environment presented by eukaryotic cells while the first screening was performed under different conditions against a highly purified antigen *in vitro*, and for a different function – binding. Also, they state that the success of the first screen is of upmost importance affecting the complexity and success of the second. Therefore they designed a method where scFv can be produced by the eukaryotic model cells themselves bypassing the first screening and selecting for function right from the start. For this scFv libraries are constructed in an eukaryotic infectious agent, the lentivirus, which is actually a problem for this method as lentiviral libraries reach at maximum 10^7 elements against the 10^{10} in phage libraries (H. Zhang et al., 2012). But on the other hand, one advantage of this method is the possibility for multiple lentiviral infection of one cell creating a possibility to study combinatorial synergy maximizing the size of the library. After insertion of the foreign scFv DNA in the cellular genome due to lentiviral infection cells need to be incubated with a methylcellulose based medium in order to confine the secreted scFv to the vicinity of the cell producing it permitting the phenotype genotype conjugation. After a pre-determined incubation period necessary for the agonist scFv to act cellular samples are prepared and sorted resorting to flow cytometry in order to individualize them for screening and sequencing purposes.

1.3 The HIV problem

Since the very beginning, the human race has always fought against all adversities. Predators, self-imposed wars, lack of food or plagues. And, since that very beginning, and always with costs associated, the human race recovered, learned and prevailed. One of our most notable achievements against the natural predators of our own body, the infectious microorganisms, was accomplished during the twentieth century. This was due to the discovery and commercialization of natural antibiotics. The war against infectious diseases had therefore officially begun. During most part of the previous century we considered ourselves on the

upper hand. Eradication of infectious agents was soon to be seen (Fauci, 2001; Pier, 2008; Spellberg, 2008). But our foresight was blinded by success. In 1981, the syndrome we now know as acquired immunodeficiency syndrome was for the first time described (Gottlieb, 1981; Masur et al., 1981; Siegal et al., 1981). It is characterized by a severe trauma to the patient immune system making him prone to opportunistic fatal illnesses. Some years later, in 1983, AIDS was associated with an infectious agent, a virus – the human immunodeficiency virus (Barré-Sinoussi et al., 1983). This date marked and scared our society as we witnessed our own limitations. In 2010, and according to data from UNAIDS, it was estimated that 2,6 million new infected individuals arise each year and another 1,8 million die due to the infection. Thirty years later the identification of HIV, the true cure for AIDS still elope us.

HAART, or highly active antirretroviral therapy (also known as cART - combination antiretroviral therapy) was proposed in 1996 during the XI International AIDS Conference in Vancouver. This therapy encompasses a cocktail combination of three or more antiretroviral drugs that target different essential stages of the HIV-1 lifecycle (Richman et al., 2009), reaching more than 5 million infected individuals (WHO, UNAIDS, & UNICEF, 2010). Although science has managed to greatly reduce morbidity and mortality rates for those with access to care (Braitstein et al., 2006; Mocroft et al., 2003; Palella et al., 1998), viremia does not disappear during treatment. Plasma viral RNA levels are only dropped below 20 copies per mL (Dornadula et al., 1999; Geeraert, Kraus, & Pomerantz, 2008). As of the moment therapy is discontinued, viremia rebounds to measurable levels in about two weeks (Harrigan, Whaley, & Montaner, 1999). This means that, in the present day, even though forty six compounds were developed and at some time and place commercialized (data compiled from www.fds.org and www.ema.europa.eu/ema/), HIV infection passed only from fatal illness to chronic disease (Pomerantz & Horn, 2003; Richman et al., 2009). Also, patient adherence to this therapeutic strategy is mined due to the costs related to a lifelong medical treatment, long-term side effects, social stigma, and more importantly due to the fact that HAART is not sufficient to eradicate HIV-1 from circulation. Reasons for this inefficiency possibly relay on a cryptic ongoing replication, poor drug penetration (L. Zhang, Ramratnam, & Tenner-Racz, 1999) or, most importantly, on the persistency of the virus in cellular reservoirs due to viral latency. So, paraphrasing, no efficient way to eradicate this virus from the human body as yet been found and new ways to arrest infection and to stop viremia rebound are needed.

1.3.1 Characterization of HIV-1

Although the HIV family is divided into two sub types both infectious the human species, HIV type 1 (Barré-Sinoussi et al., 1983) and type2 (Clavel et al., 1986), focus is taken mostly on HIV-1 as it is responsible for a more severe syndrome associated with higher infectious rates that resulted in the AIDS pandemic. Therefore, during this thesis, focus will be on HIV-1 only.

HIV-1 belongs to the Lentivirus genus of the Retroviridae family, meaning that it is an RNA virus, and one of the most complex of its family (Wagner, Hewlett, Bloom, & Camerini, 2008a). Its genome (figure 1.6) is composed of two positive single strain RNA molecules of about 9kb that code for 9 open reading frames (ORFs): *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env* and *nef*; flanked by long-terminal repeats (LTRs). The promoter for viral transcription is found on the 5' LTR. As in other retrovirus proteins are transcribed in polyproteins that are subsequently proteolyzed into their individual components (Wagner, Hewlett, Bloom, & Camerini, 2008b) (figure 1.7 for HIV-1 structure). The Gag (group specific antigen) polyprotein is cleaved into four other proteins: matrix (MA), capsid (CA), nucleocapsid (NC), and p6; while Env (envelope) is cleaved into two: glycoprotein gp120 (surface or SU) and glycoprotein gp41 (transmembrane or TM) (Allan et al., 1985; Veronese et al., 1985). These six proteins are structural components that build the core of the virion and outer membrane envelope. Pol (polymerase) polyprotein is proteolyzed into three proteins – protease (PR), reverse transcriptase (RT) and integrase (IN). These are responsible for providing the essential enzymatic functions for infection and are also encapsulated within the viral particle. The remaining proteins are called accessory proteins. One is responsible for indirectly assisting in the assembly of the virion – Vpu; while two of them are essential to gene regulation – Tat, a transcription transactivator and Rev, a protein responsible for the regulation of viral protein production. The remaining proteins Vif, Vpr, and Nef are the only accessory proteins encapsulated and are responsible for modulating cellular events like blocking APOBEC3G action, arresting the cell cycle and downregulating CD4 and the major histocompatibility complex (MHC) expression respectively (Wagner et al., 2008a).

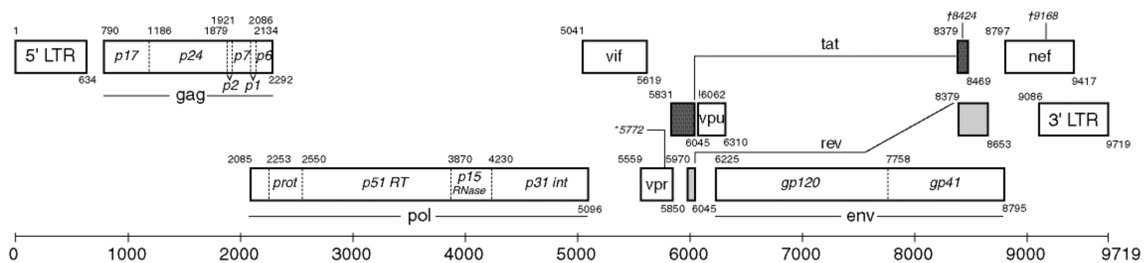


Figure 1.6 The HIV-1 genome: the HIV-1 genome is composed by three structural genes: *gag*, *pol* and *env*; three regulator genes: *tat*, *rev*, *nef*; and three accessory genes: *vif*, *vpr*, *vpu*; all flanked by LTRs coded in one single strand of RNA. Proteolyses of the polyproteins is necessary for individual proteins to perform their action. (adapted from http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html)

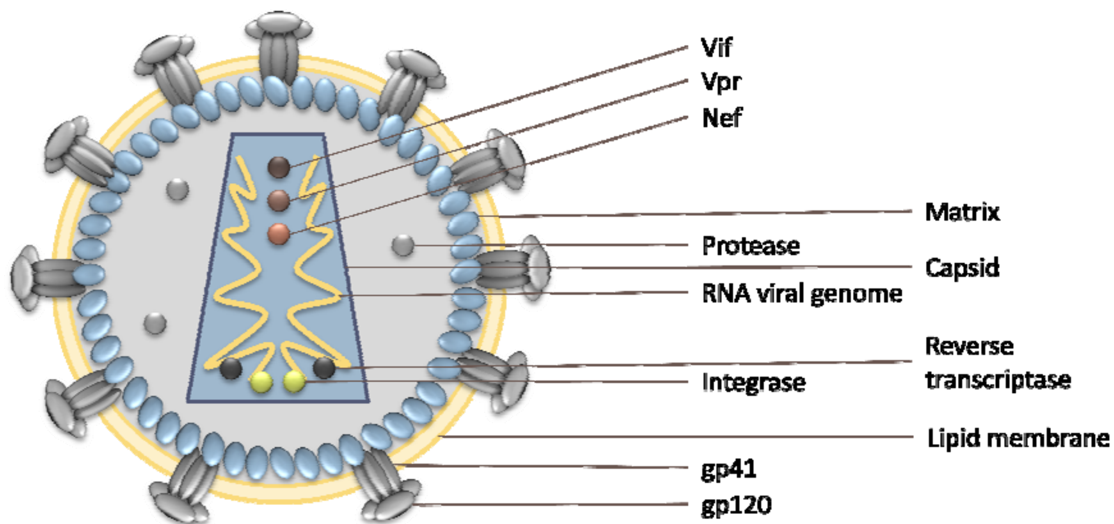


Figure 1.7 HIV-1 structure: This virus is composed by two positive RNA single strands coated by a nucleocapsid (not present in the figure) encapsulated together with Vif, Vpr, Nef, Integrase and Reverse transcriptase in a particle of close to 100nm in diameter. Right below the lipid membrane there is the matrix that surrounds the viral capsid.

HIV infects CD4 positive cells like $CD4^+$ T cells, macrophages and dendritic cells, by fusing with their membrane. This is due to the viral proteins gp120 and gp41 presented in the outer membrane envelope responsible for the targeting and fusion process. gp120 initially binds to the cellular receptor CD4 and subsequently to a group of CC and CXC chemokine receptors like CCR5 and CXCR4, a coreceptor. At this point gp41 undergoes conformational changes in order to expose a fusion peptide that triggers the membrane fusion process. Once in the cytoplasm, the viral core suffers an uncoating process by dissociation of the CA important for RT to copy the RNA genome into a double stranded linear DNA genome which in turn is delivered to the nucleus and intregrated onto the cellular genome with the help of IN. The provirus is transcribed by the host RNA polymerase II into spliced and unspliced mRNA transcripts. In an early phase Tat, Rev and Nef are translated from small multiple spliced mRNAs. While Tat enhances viral mRNA transcription by binding to the trans-activating response element (TAR) in association with other host proteins, Rev is responsible for halting viral mRNA cleavage allowing the multiplication of the viral genome and for Gag and Pol polyproteins to by synthesized. The Env polyprotein is produced in the endoplasmatic reticulum and cleaved and glycosylated into gp120 and gp41 (McCune et al., 1988) and transported to the plasma membrane where in association with Gag and Pol polyproteins, protease and genomic RNA form the immature virions. Upon release by gemulation the viral protease triggers the maturation of the viral particles with a drastic reorganization of the core and gain of infectious capacity.

Alternatively the integrated provirus can be maintained in a dormant state avoiding the immune system for long periods of time being casuistically reactivated and restarting the infectious cycle with ease. This property is universal to other kinds of virus, like the herpes virus, and it is called viral latency, representing an important problem for viral eradication.

1.3.2 HIV-1 and viral latency

The first evidence relating HIV-1 with proviral latency was obtained *in vitro* in 1989 where HIV-1 infected T cells, surviving the cytopathic effects of infection, showed a decreased expression of viral genes and afterwards, in the presence of different and specific stimuli (as the ones causing T cell activation) restarted HIV-1 production (Duh, Maury, Folks, Fauci, & Rabson, 1989). *In vivo* evidence of HIV-1 latency was only obtainable in 1995 when, recurring to inverse PCR, it was shown that HIV-1 genome, stably integrated and capable of replication only after cellular activation, was present in highly purified populations of resting CD4+ T cells (Chun et al., 1995). This was the turning point proving the existence of reservoirs in infected individuals. Since then plenty of studies tried to explain why and how HIV-1 latency was achieved and maintained. Several hypotheses substantiated by experimental evidence started to appear related to various factors such as the chromatin environment that surrounds the viral promoter upon integration which could cause transcriptional interference or viral silencing due to heavily packed chromatin; or the level of host cell activation (Colin & Van Lint, 2009) lacking key cellular factors for transcription, like NF-κB and STAT5, or presenting host transcription repressors, like DSIF and c-myc. Epigenetic modifications also appear to be related to the maintenance of proviral latency. Analysis furthering latency onset mechanisms goes beyond the intent of this work and one has only to bear in mind that latency is caused due to the genetic and epigenetic environments of the host cell that can be disturbed from the outside.

It is also important to mention that most of the studies for HIV-1 latency have been made in CD4+ T cells since it is the most representative reservoir but other latency reservoirs exist, like myeloid dendritic cells or macrophages. There is a strong possibly that different reservoirs present different mechanisms that lead to viral latency (Van Lint, Bouchat, & Marcello, 2013).

Synthesizing, HIV-1 latency is a complex event, most probably a multifactorial process that works all together to effectively stop viral replication. HIV-1, in exploring the preexisting and necessary mechanisms of immunity – the immunological memory; has found a way to truly gain an advantage in survival. Memory $CD4^+$ T cells are naturally long lived, and stay in circulation for long periods of time. This is imperial to our capacity to rapidly react to

reencounters with some antigens. Infecting these cells and inadvertently establishing latency, HIV-1 found a way to persevere itself in the organism for long periods of time escaping the host immune system and manifesting itself in a state insensitive to HAART. Until recently one could say that the pool of latently infected T cells was very small, but the time estimated for the latent provirus to be reactivated is too long. It was estimated that, during HAART, the half-live of the $CD4^+$ T cell reservoir was of 44 months (Finzi et al., 1999; Siliciano et al., 2003). This means that, during therapy and in the absence of other reservoirs, it would take approximately 60 to 70 years for a complete eradication of the virus. But recent studies by Robert Siliciano and his team at the John Hopkins University show that the size of such reservoirs was in fact underestimated ("Latent HIV not as defective as once hoped," 2013). They decided to sequence the HIV-1 genome from a pool latently infected cells that did not reactivate the provirus under laboratory conditions, previously believe to harbor a defective proviral genome and therefore, and found out that 12% of that pool presented the intact and fully functional HIV-1 genome. This study results were published on October 24 in Cell under the name "Replication-competent non-induced provirus in the latent reservoir increase barrier to HIV-1 cure". Simply putting it, and repeating, HIV-1 guaranties a life time infection (Strain et al., 2003). Again we find reinforcement in the need for new therapies, especially ones that purge latent HIV-1 infections and provide a sterilizing cure. Stopping HAART in order to reactivate latent provirus is not viable as associated there could be the establishment of new reservoirs with hypothetical new mutations that could mine future return to antiretroviral therapy. So, the need for methods in dealing with HIV-1 latency is of the upmost importance. Never the less, this new drugs and therapies are difficult to find due to the highly complex and variable way of establishing latency, the heterogeneity of reservoirs and their location in terms of accessibility.

1.3.2.1 Therapeutic strategies against viral latency

Present day researches, on one hand, try to stop latency from establishing. Such feat would require therapeutics to start immediately or even before the first contact of the patient with the virus. This is simply too complex to be ever achieved. Another approach for fighting latency, on the other hand, relies on the purging the HIV-1 provirus by reactivating it and thus providing a sterilizing cure. As an example of this last approach we have a recent study that shows Disulfiram as a good drug for reactivating HIV-1 integrated provirus through depletion of PTEN via the Akt signaling pathway (Doyon, Zerbato, Mellors, & Sluis-Cremer, 2012). In fact, plenty of other examples exist. Since epigenetic modifications are related at some point in the establishment of HIV-1 latency it is logical that drugs which act as epigenetic modulators, such

as valproic acid (Archin et al., 2010; Matalon, Rasmussen, & Dinarello, 2011), although some controversy exists about this drug (Sagot-Lerolle et al., 2008) or the more potent SAHA (Archin et al., 2009) have long been under scrutiny. Although efficient, these drugs need to be directed to their specific targets as otherwise their effects will be felt by any cells they find along the way. This is, they have an indiscriminate action and therefore mechanisms for specific delivering have to be imagined. Trichostatin A (TSA) and Tumor necrosis factor-alpha (TNF-α), a histone deacetylase inhibitor and a NF-κB inducer respectively, are also good drugs at reactivating latent HIV-1 infections but saw their possibilities for therapeutics mined due to their toxicity (Yang, Shen, Siliciano, & Pomerantz, 2009). TNA-α is actually a very common product used as an additive to provide positive controls for latent HIV-1 reactivation in laboratorial context. Other recent studies indicate that protein kinase C (PKC) agonists lacking tumor promoter activities are the best class of drugs for reactivating the latent HIV-1 infection without polyclonal activation of non-infected cells (Sanchez-Duffhues, Vo, & Perez, 2011). Such as prostratin (Korin, Brooks, Brown, Zack, & Korotzer, 2002), macrolide bryostatin (Kovochich, Marsden, & Zack, 2011; Mehla et al., 2010; Pérez et al., 2010) and jatrophanes (Bedoya et al., 2009). But none is to date commercialized for this indication.

So, as we can see from these last studies, events outside of the cytoplasm can cause alterations on the transcriptional state of the cell. More specifically, compounds capable of acting like agonists for certain receptors can promote latent HIV-1 reactivation. But to date, we are faced with little knowledge about what specific receptors promote proviral reactivation, therefore there is an impending need to question all the possible receptors in $CD4^+$ T cells in order create new agonist molecules directed specifically to the ones with the capacity to deplete this HIV-1 reservoir.

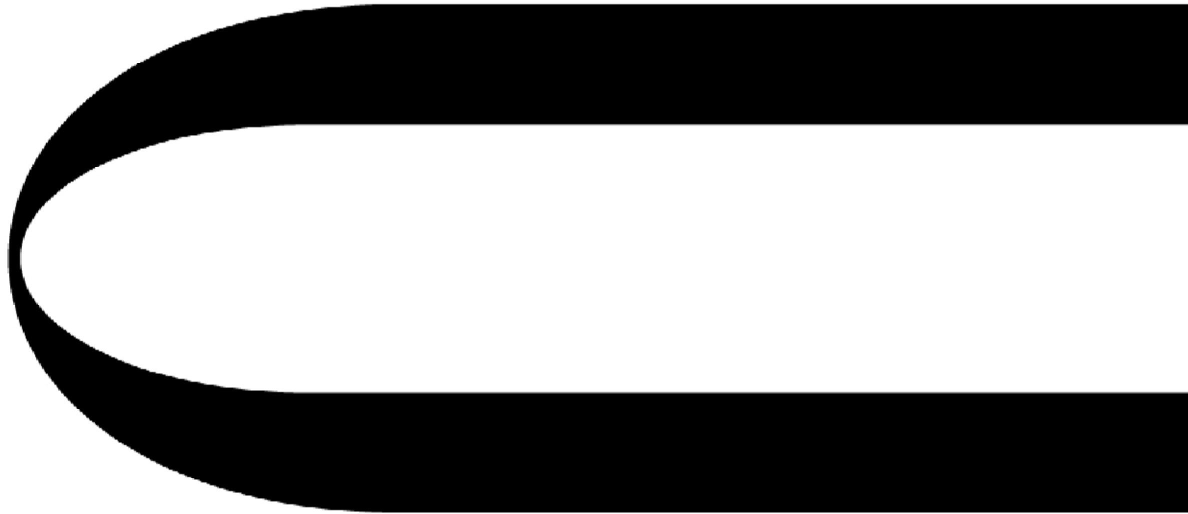
AIMS OF THE PRESENT WORK

Besides the vast amount of already commercialized antibodies and the ones undergoing research, the discovery of not only new antibodies but new targets as well will always be imperial for the development of new therapies. Nowadays, as it been mentioned before, antibody selection is performed resorting to a great deal of different techniques, each one with its own advantages as well as limitations associated, and therefore it is up for the researcher to choose the best one to meet his needs. Phage display technology is a simple technique universally used for selecting antibodies against well known antigens, either display on a silica or cellular surface. For this project we hypothesized the possibility to use this run of the mill technique as a starting point in order to selected antibodies based on phenotype alteration.

With this in mind and using HIV-1 as the disease model for this study the overall objective of this thesis is linked with the possibility to find a cure for AIDS. Here, we aim to find agonist antibodies capable of purging latent HIV-1 from infected $CD4^+$ T cells. We believe, as does other researchers that the cure can be achieved by depleting the HIV-1 reservoirs present in the human body and thus, eradicating the most representative reservoir, the $CD4^+$ T cells, we are putting ourselves one step closer to the cure of AIDS.

In order to achieve our overall objective, specific objectives have to be traced:

1. To select a library of scFvs against a laboratory perpetuated T cell model through phage display technology;
2. To identify agonist scFv capable of reactivating HIV-1 latent infection in the same T cell model.



METHODS

3.1 Recombinant phage library reamplification and recombinant phage purification:

Two naïve libraries were chosen for this work differing in their origin. One built in a pCGMT vector (Gao et al., 1997) of human origin originally built within CPM-URIA, belonging to João Gonçalves unpublished work, with a diversity of 10^9 different recombinant phages; and the other built in pComb3H of rabbit origin with a size of 10^8 kindly provided by TechnoPhage. Both libraries were maintained in *E. coli* K12 ER 2738 aliquots and stored at -80ºC. For recombinant phage production libraries were grown in SOB growth medium complemented with ampicillin and tetracycline until reaching an optic density close to 0,6 (Abs_{600}=0,6-0,7) and them infected with helper phages VCSM13 (Stratagene). After adding kanamycin cultures were incubated for 16 hours over night for optimal recombinant phage production. Phages were then purified resorting to PEG-8000 centrifugation, resuspended in BSA1% in 15% glycerol (PBS1x) and filtered in order to remove contaminants. Recombinant phage production and purification protocol was adapted from Phage display: A laboratory manual (Barbas et al., 2001).

3.2 Recombinant phage panning against eukaryotic whole cells in suspension:

J-Lat 10.6 cells were cultured in RPMI 1640 10% FBS at 37ºC in a humidified atmosphere containing 5% CO_2. Upon reaching 0,5-1x10^6 cells/mL, the cells were washed with PBS1x and resuspended in serum free RPMI 1640 in a final concentration of 4x10^6 cells/mL (Hoogenboom et al., 1999). Individual microcentrifuge tubes were prepared with 1mL of the washed cell suspension and incubated for 1 hour at room temperature with 0,5 mL of the recombinant phage library (1x10^{12} phages/mL) (Y. Zhang et al., 2007). Cells were washed 5 times to remove unbound phages with 1mL of PBS-Tween 0,05%, PBS-Tween 0,025% or NaCl 200mM depending on wanted stringency. After the last wash cells were transferred into a new microcentrifuge tube in order to discard plastic binding phages. Phage elution was performed with the addition 0,5 mL of trypsin (0,25%) for 10 minutes at room temperature (Jakobsen, Rasmussen, Laenkholm, & Ditzel, 2007; Y. Zhang et al., 2007). Eluted phages were then added to 2 mL of *E. coli* K12 ER 2738 (Abs_{600}=0,6-0,7) and incubated for 15 minutes at 37ºC without shaking. The eluted phage infected culture was then prepared according to the literature in order to calculate the selected phage titer and amplify the selected recombinant phage library (Barbas et al., 2001).

3.3 Recombinant phage J-Lat reactivation assay and flow cytometry analysis:

This method was applied to two different situations: a preliminary and a single clone screening assay; carried out before or after the sorting procedure respectively, differing in the kind of multi-well plate used and consequently volumes. One as to remember that J-Lat cell lines are laboratory perpetuated cultured cells derived from Jurkat cells with the special feature of being prone to HIV-1 latency onset. Due to this fact, they are used as a model for the study of HIV-1 latency. Some J-Lat lines have been modified in order to code for a fluorochrome gene inserted in the defective latent proviral genome like the ones used for this work. Therefore, viral reactivation causes the flourochrome, GFP for J-Lat 10.6, 6.3, 8.4 and 9.2, to be transcribed causing a phenotype alteration measurable by FACS. Therefore, cytometry is made possible by the GFP gene encoded in the latent provirus genome present in J-Lat lineages. Measurements were made at OD 525nm for green fluorescence and OD 575nm for the red fluorescence, in order to exclude cellular auto-fluorescence, using Millipore's GUAVA easyCyte HT system and the analysis InCyte software.

3.3.1 Preliminary selected recombinant phage library J-Lat reactivation assay:

J-Lat cells were cultured as previously described until reaching 0,5-1x10^6 cells/mL. Before starting the assay cells were washed and resuspended into fresh medium. Each well of the 24 well plate was seeded with 1x10^5 cells in 400 µL of RPMI 1640 and 100 µL of the selected recombinant phage library. Three individual negative controls were incubated with 100 µL of RPMI, BSA 1% in 15% glycerol (PBS1x) or helper phages instead of phage libraries. The positive control was seeded in 500 µL of RPMI containing TNF-α in a concentration of 10ng/mL. Plates were incubated for 24 and 48h at 37ºC in a humidified atmosphere containing 5% CO_2. Preparation for flow cytometry included two PBS1x washes and resuspension in formaldehyde 1% (PBS1x) to preserve the GFP signal.

3.3.2 Single clone recombinant phage J-Lat reactivation screening assay:

The previous protocol was adapted in order to perform assays in 96 well plates as the volume of samples increased. Therefore volumes were diminished but recombinant phage/J-Lat cells proportions were maintained. The same protocol was also used when evaluating agonist capacities of a selected pool of scFvs against J-Lat 6.3, 8.4 and 9.2.

3.4 Sorting of GFP$^+$ J-Lat cells and single clone individualization:

3.4.1 Recombinant phage recuperation and individualization:

Sorting procedures were performed at IMM in a BD Biosciences FACSAriaIII cell sorter with the same parameters used for flow cytometry for recovery of GFP$^+$ cells. Samples were prepared the same way as for the reactivation preliminary assay without cell fixation. GFP$^+$ cells were recovered into a FACS tube previously filled with PBS1x. recombinant phages attached to this population of cells were eluted according to the elution procedure previously mentioned and added to 2 mL of *E. coli* K12 ER 2738 (Abs_{600}=0,6-0,7) – incubation of 30 minutes at 37ºC without shaking. Bacterial cells were then diluted and plated into ampicillin LB Petri dishes allowing the growth of individualized colonies. Each colony or single clone was then grown individually in four 96 well plates and stored at -80ºC. From here, and for the sake of time, 40 clones were chosen to be screened. Recombinant phages from each clone were prepared as previously mentioned and according Barbas, 2001.

3.5 Sequencing of the selected scFv clones:

The sequencing procedure was done resourcing to external companies like Stabvida and Macrogen using a forward primer design specifically for sequencing purposes called pComb1F (5'-GTTGGCCGATTCATTAATGCAG-3').

4.1 Recombinant phage panning against eukaryotic whole cells in suspension

To specifically select scFvs that present an agonist effect directed to a desired phenotype, one first needs a pool of antibodies capable of binding to membrane components of the desired target cells. For achieving this, recombinant phages from two scFv phage display libraries of different origins were prepared and panned against J-Lat 10.6 cells. Reamplification from the human naïve recombinant scFv phage library produced a solution with $1,06x10^{12}$ phages/mL which corresponds to our input value, and the phage solution from rabbit naïve recombinant scFv phage library had a concentration of $5,56x10^{14}$ phages/mL. Output samples were labeled according to their origin (H – Human, C – Rabbit) and stringency parameters ($_5$ – T-PBS 0,05%, $_{25}$ – T-PBS 0,025%, $_{na}$ – NaCl 200mM) as shown in figure 4.1 and 4.2. The first pannings were done with the human library for the three different stringency conditions which resulted in three singular samples with their own phage concentration: H_5 – $1,25x10^2$ phages/mL; H_{25} – $5x10^3$ phages/mL; H_{na} – $1,4x10^4$ phages/mL. As it become evident, T-PBS 0,025%, represented an intermediary stringency force and was for that reason ignored when performing the

subsequent pannings with the rabbit library. Both stringency conditions caused the selection of two samples with 10^5 phages/mL (C_5 and C_{na}). Because output values dropped immediately after the first round of panning and because we are not interested in extraordinary binder-antigen affinity, no further rounds of selection were preformed as they would only decrease scFv diversity. Additionally, the trypsin treatment to release membrane bound recombinant phages also lysated J-Lat cells assuring the recovery of possible internalized phages, so further treatment with a lysis buffer was redundant.

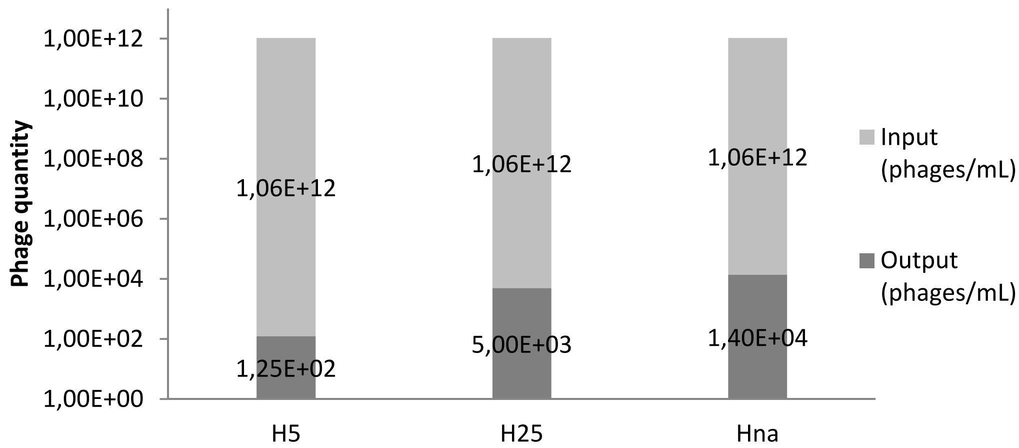


Figure 4.1 Human scFv recombinant phage library panning against eukaryotic whole cells in suspension: Input values for each panning are the same ($1{,}06 \times 10^{12}$) with outputs being progressively lower according to increases in stringency.

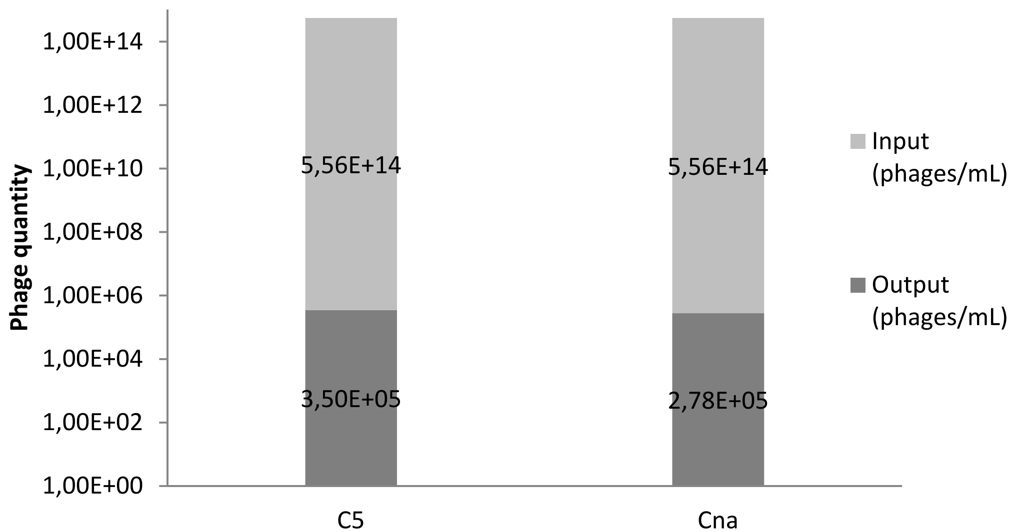


Figure 4.2 Rabbit scFv recombinant phage library panning against eukaryotic whole cells in suspension: Pannigns using the rabbit scFv naïve library against J-Lat 10.6 resulted in the samples with higher recombinant phage counts when compared to scFv of human origin.

4.2 Preliminary selected recombinant phage library J-Lat reactivation assay

During our preliminary reactivation assay our five different pools of selected recombinant phages were tested alongside a previously selected anti-IL7R library labeled IL-7R. As our scFvs of interest are presented by recombinant phages which in turn are diluted in BSA 1% in 15% glycerol (PBS1x), negative controls also encompass the effects of BSA and Helper phages as well. Flow cytometry data (figure 4.4) showed that J-Lat 10.6 presented a basal level of reactivation, with approximately 1% of the population manifesting green fluorescence. These values were replicated for the other negative controls meaning that neither BSA 1% nor helper phages interfere in the assay.

Flow cytometry data was converted into percentages of GFP^+ cells, directly proportional to the percentage of cells where the latent provirus was reactivated, and compiled into a chart (figure 4.3). Analyzing the data it is evident that, for the 24 hour mark, H_{25} and IL-7R samples showed the lowest proviral reactivation results, presenting values similar to the negative controls, while the sample with the higher reactivation percentage was H_5 with 3,52%. The remaining samples showed some capacity for proviral reactivation, with phenotype alterations on 2% of the analyzed population. Interestingly all samples presented higher values of reactivation during the second time point measurement, the 48 hour mark, again with H_5 being the most capable of causing proviral reactivation with 8% of GFP^+ cells.

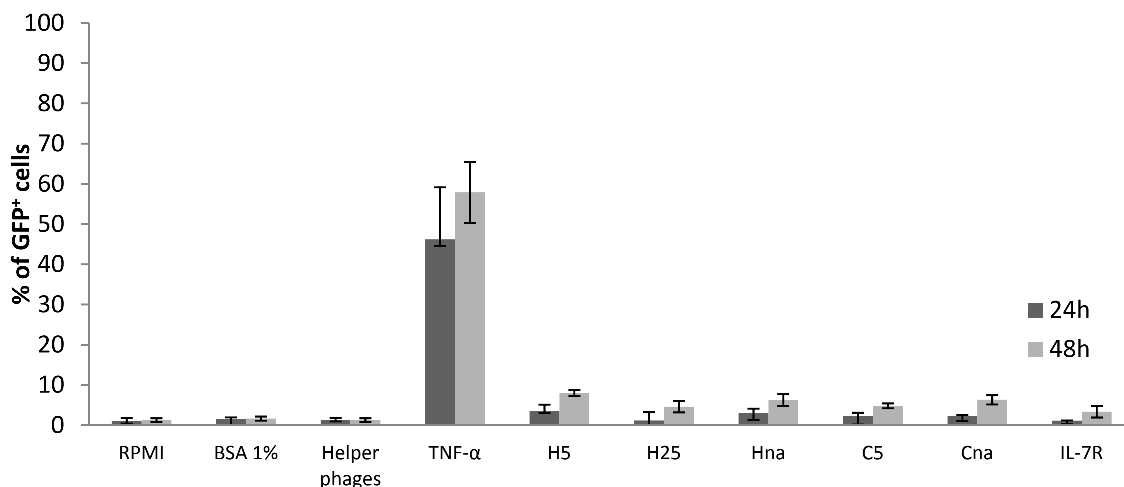


Figure 4.3 Preliminary selected recombinant phages library J-Lat reactivation assay: J-lat 10.6 cells where incubated for 24 and 48 hours with recombinant phage samples selected against their membrane proteins. Phage displayed scFv of human origin selected with the higher value of stringency resulted in the most promising sample. n=3

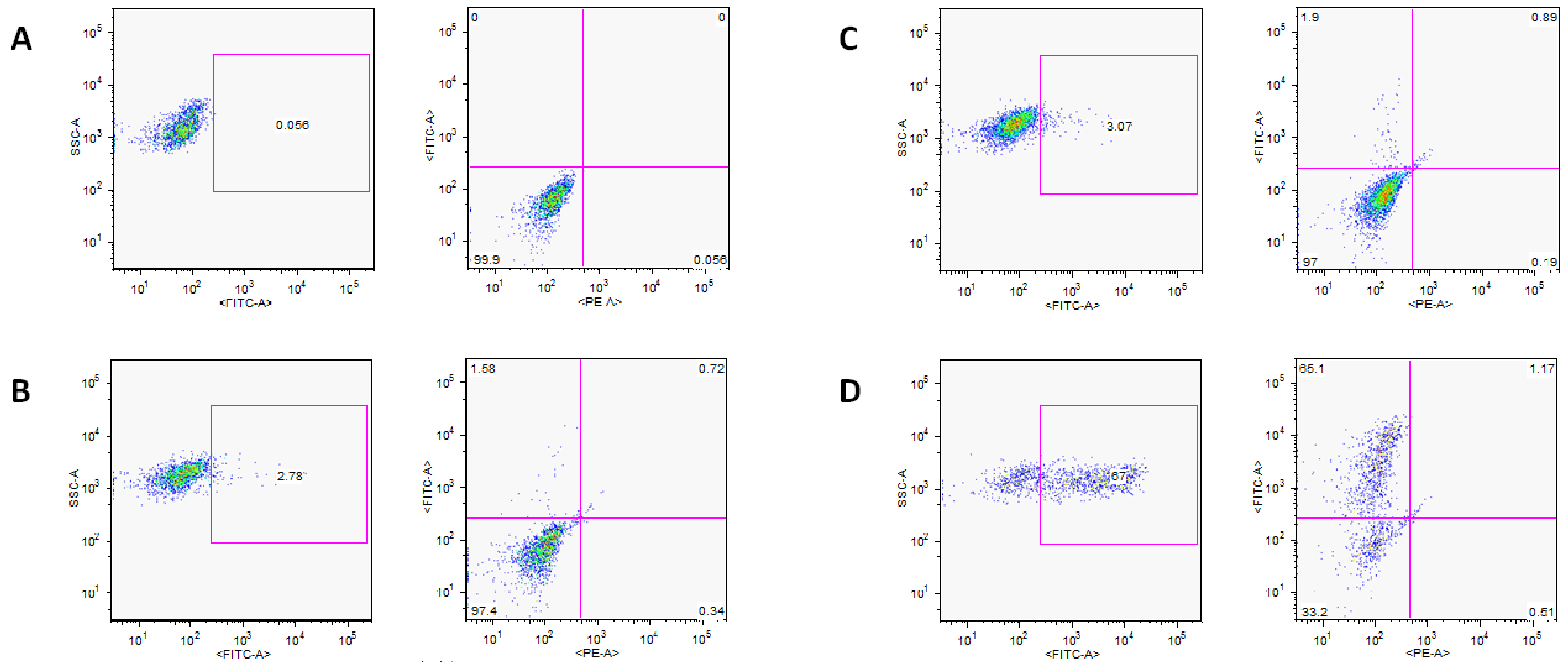


Figure 4.4 Flow cytometry preliminary evaluation of negative controls: J-lat 10.6 cells where incubated for 24 and 48 hours with **A** supplemented RPMI **B** BSA1% **C** Helper phages and **D** TNF-α. This served as confirmation that both helper phages and BSA 1% did not contributed to adding noise to posterior assays.

4.2.1 Sorting of GFP+ J-Lat cells and single clone individualization

As H_5 presented itself as the sample with the higher values of reactivation it was decided to run it through the FACSAriaIII cell sorter at IMM in order to individualize the recombinant phages capable of causing the desired phenotype alteration. For the first time point, we were able to recover nearly 6000 recombinant phages from 473 GFP+ cells meaning that in average there were roughly 12 phages binding to each individual cell. For the 48 hour mark we retrieved 4469 GFP+ cells, which would be equivalent to $5,6x10^4$ phages. For the sake of time and ease of handling we decided to select 40 clones (labeled with a two letter code from A-H and 1-5) from the first time point to proceed with the study.

4.3 Single clone recombinant phage J-Lat reactivation screening assay

To evaluate the agonist ability of our pool of 40 scFv clones, and to screen them, a preliminary single clone reactivation assay was prepared with an incubation of 24hours and a threshold set for 50% of GFP^+ cells. As it was previously observed, the 24hour mark gave us the lowest values for HIV-1 reactivation, so a GFP^+ subpopulation representing more than half of the analyzed initial population would represent a promising agonist scFv. This assay permitted the identification of 4 clones, D1, H5, C1 and G1 (10% of the initial pool) as visible in the data shown in the figure 4.5. Although D1 reactivated 49% of the latent provirus, being below the threshold for one percentage point, it was still considered as a good candidate for standing out among the other clones. G1 recorded the highest value for proviral reactivation, 56,73%. It has to be noted that these four selected clones represent 10% of the screened scFvs.

In order to have a bigger picture and consider the 48h mark as well, this assay was repeated after two months using the same samples with the results presented in figure 4.6. Reactivation percentages were diminished but the same pattern of action amongst the clones was visible. The same four clones standing out from the others with G1 being the most effective one. But surprisingly reactivation values did not increase for the 48hour mark as demonstrated in previous assays.

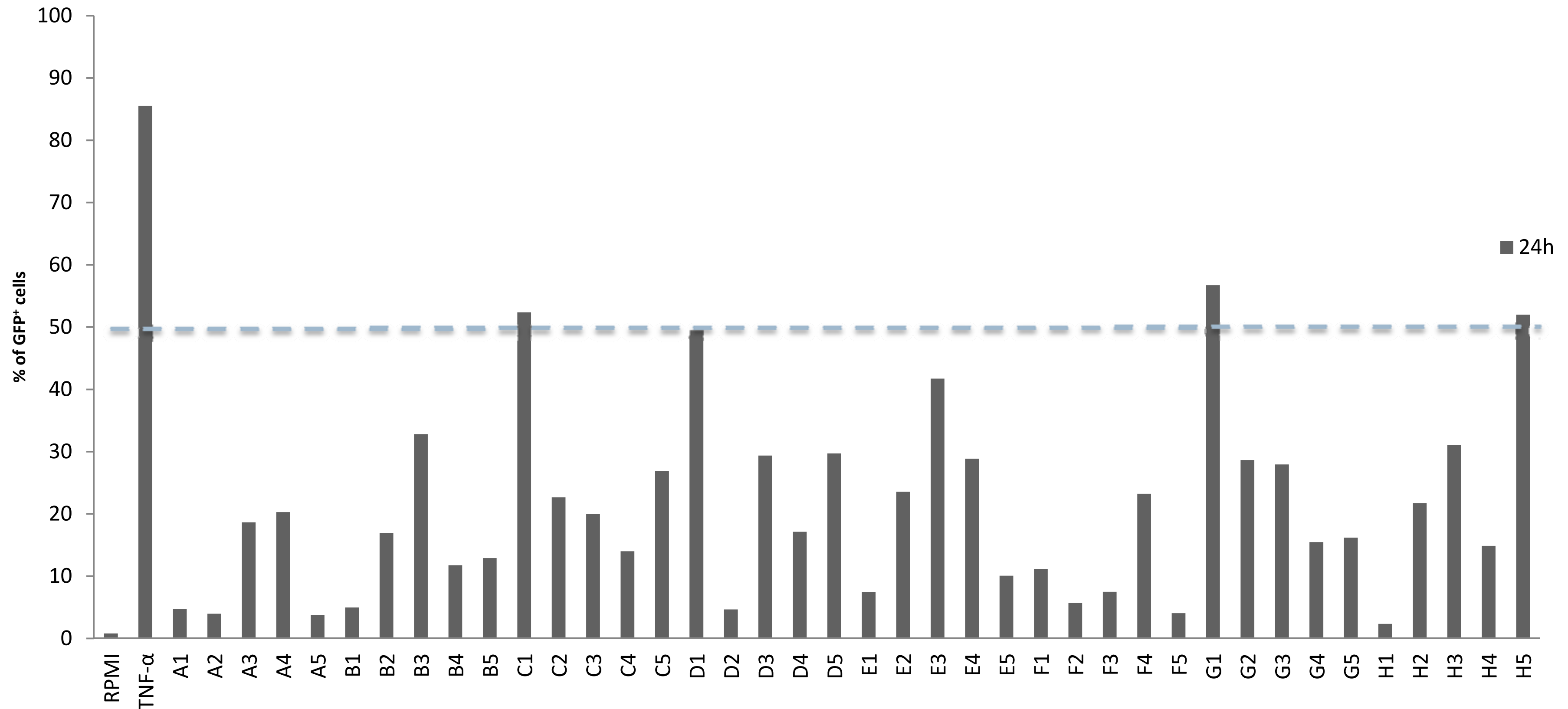


Figure 4.5 Preliminary single clone recombinant phage J-Lat reactivation screening assay: This assay was performed in order to evaluate which of the single clones recovered after cell sorting could more efficiently act as an agonist against an unknown membrane determinant of J-Lat 10.6. a threshold of 50% was set and four clones surpassed it: C1, D1, G1 and H5. This assay was done for only one time point (24h) n=1

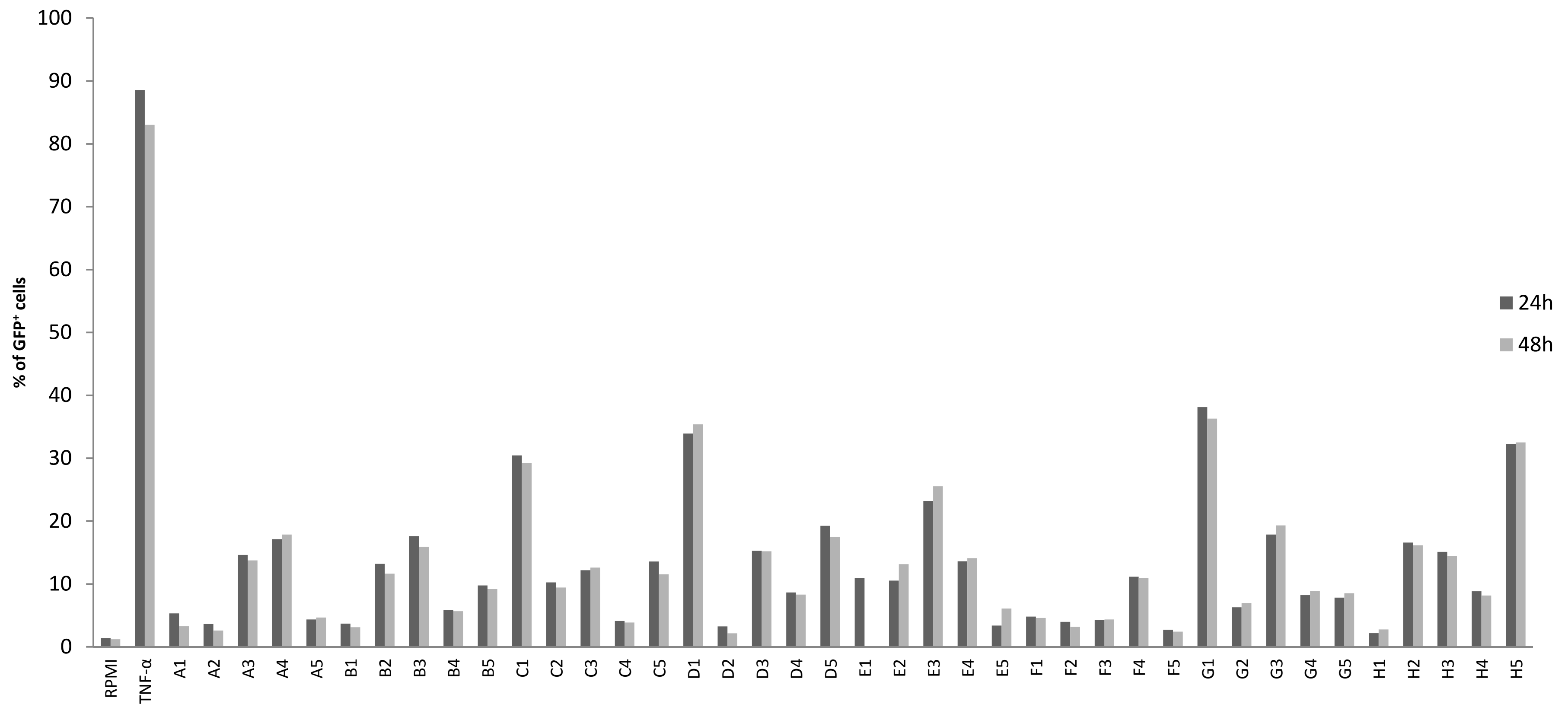


Figure 4.6 Single clone recombinant phage J-Lat reactivation screening assay: Repetition of the preliminary single clone recombinant phage J-Lat reactivation screening assay for both time points, 24 and 48 hours. This assay was performed with the same samples, stored at 4ºC for two months. The same reactivation profile and %GFP+ is evident but values were decreased. Still, C1, D1, G1 and H5 stand out amongst the 40 screened clones. Surprisingly GFP+ values were maintained between measurements at 24 and 48 hours. n=1

4.3.1 Single clone recombinant phage screening reactivation assay against multiple J-Lat lineages

The capacity of the selected clones to reactivate the latent provirus in other cell lines was also evaluated. For this we used three other J-Lat cell lines, J-Lat 6.3, 8.4 and 9.2, which have different responses of latent proviral reactivation even to the compound used as positive control, TNF-α (being 9.2 the most reactive of them all). On the previous assay we observed that reactivation values from these clones carried on to the 48 hour mark equal to the 24hour so it was decided to measured the GFP signal after 48 hours of incubation. Alongside D1, H5, C1 and G1, four other scFvs were also tested in order to evaluate if the same pattern of reactivation was visible for other cell lines. We have chosen three clones, G4, D3 and D5, which shown a reasonable capacity in proviral reactivation, ranging from 15% to 30% of GFP+ cells, and one that performed poorly, F5 with 4% of cells expressing GFP. Results are shown in figure 4.7 and although D1, H5, C1 and G1 were capable of reactivating the latent provirus on some extent results do not seem as promising when carried to these lineages. All of them but C1 showed to be more reactive against J-Lat 9.2 than to the other lines with reactivation values ranging from 5% to 7%, again with G1 appearing to be the most effective. From the additional four clones only one clone maintained similar values of proviral reactivation and that was F5. Interestingly G4 caused GFP production in ~5% of the population of J-Lat 9.2 and 6.3.

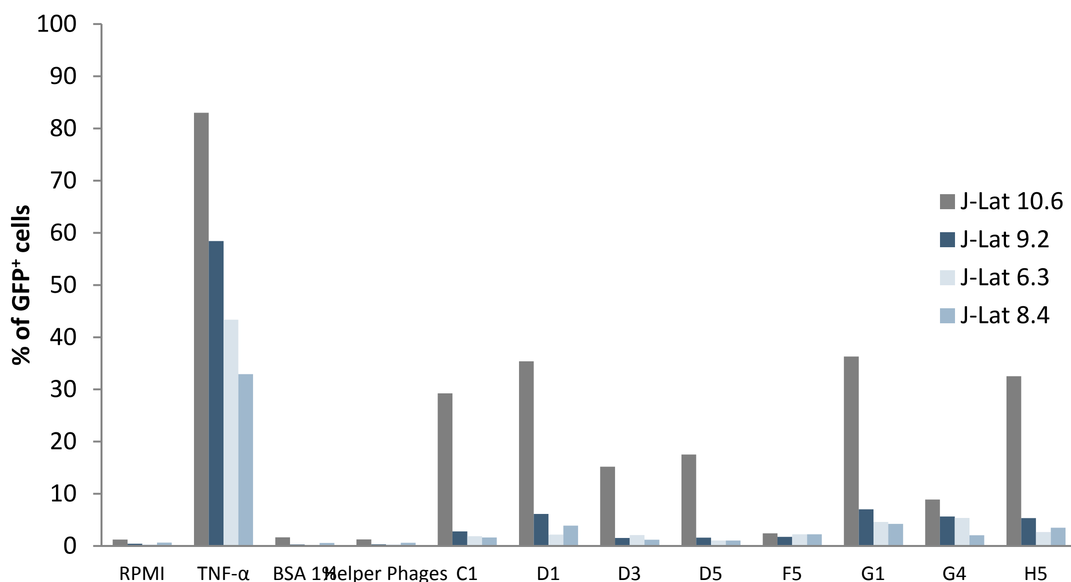
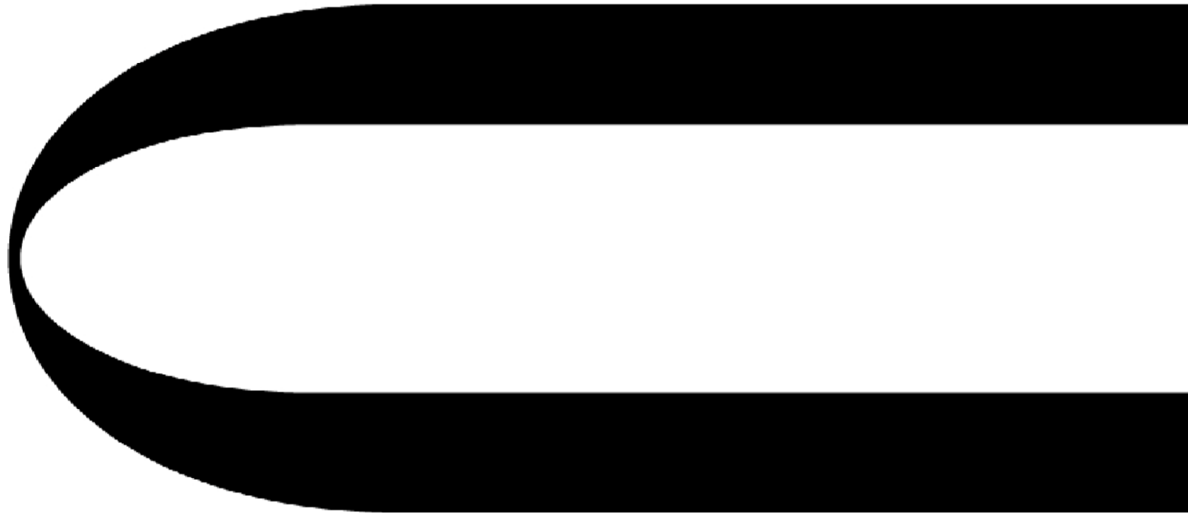


Figure 4.7 Single clone recombinant phage screening assay against multiple J-Lat lineages: C1, D1, G1 and H5 were screened alongside with scFv candidates previously discarded against multiple J-Lat lineages. Note that recombinant phages were, unintentionally, once again incubated for two months previous to this assay. The reactivation pattern do not seem to replicate against other J-Lat lineages other than the one used for scFv selection. n=1

4.4 Sequencing results

Preliminary sequencing results permitted the conclusion that C1, D1, H5 and G1 are in fact four different scFvs as DNA sequences were all different between them. For reasons of patent protection and submission, DNA or peptide sequences for each scFv are not shared in this work.



DISCUSSION

The aims set at the beginning of our study were all successfully accomplished: we have efficiently used phage display technology to screen for scFvs that present and against action in reactivating the latent HIV-1 provirus upon contact with unknown targets presented by a cellular model for laboratorial latency study.

Particularizing, two specific aims were established. First, and specifying, selection of a library of scFv against a laboratory lineage of T cells through phage display technology was performed and productively completed. We were able to generate 5 different pools of scFvs against membrane determinants of J-Lat 10.6 cells from 2 distinct naïve libraries with different animal origin, human and rabbit. Decreases in stringency during the pannings gave us higher counts of recombinant phages as it is expected when performing this technique. Surprisingly this did not happen for the rabbit library as C_{na} and C_5 samples presented both outputs of 10^5 phages/mL. Such concentration values also make them the two samples with the higher count of recovered recombinant phages per mL. This anomaly can be explained by the fact that it is expected that evolutionary pressures not only decrease the number of self against self occurrences but antibody affinity against self determinants as well. Thus, when selecting human scFvs against human targets it is expected for few scFvs to be selected with observable

decrease in their quantity if stringency is to be augmented. The same is not expectable for non-human scFvs against human targets, as the case of rabbit scFvs. Therefore, in comparison, more scFvs of rabbit origin were efficiently selected when using such low stringency parameters as the ones used during the course of this work. There is also the possibility for the rabbit scFvs selected to present similar values of affinity as increases in stringency do not seem to decrease the quantity of recovered recombinant phages.

Those five recombinant phage samples were characterized for their potential in reactivating the latent provirus and H_5 was identified as the most promising sample for the possibility of containing a scFv capable of acting as an HIV-1 reactivating agonist. One particularity observed during the preliminary reactivation assay was that the sample of human origin selected with the intermediary stringency force, H_{25}, was revealed as the least probable sample for containing a scFv agonist for HIV-1 reactivation. Related, it is also interesting that H_5, being the sample selected with the highest value of stringency, revealed itself as the most promising when it is expected that recombinant phages that make up this sample should also be contained in the other human samples selected with lower stringency values. This can hypothetically be explained by causality with recombinant phage combinations that present different proportions of each individual clone in each sample. This is directly related to the fact that only one panning round was preformed. It is possible that such curious events could be minimized if at least one more panning was done in order to enrich recombinant phage samples. Has it has been stated before, we chose to perform only one round of selection in order to obtain the highest scFv variability possible, as otherwise, if enrichment was done with further pannings, we could be losing potential agonists and directing our selection for binding purposes instead. Another possible explanation is related to recombinant phage to J-Lat ratio. Diluting the recombinant phage samples or increasing J-Lat quantities could increase GFP signal.

Parallel to our scFv samples we decided to test a library previously selected against a specific portion of the IL-7 receptor (unpublished work) for the possibility of finding a agonist scFv that replicated IL7 activity. This choice was made due to the fact that the natural ligand, IL-7, has the capacity to promote CD4+ T cell survival and proliferation, therefore genetically activating it, and was at the time being tested for is capacity to effectively reverse latency in clinical trials (Van Lint et al., 2013). Our results showed reactivation values close to the negative controls and therefore a decision was made to drop this sample for further investigation. Interestingly, reactivation values were higher for the 48 hour mark but still showed a very low viral reactivation capacity. Supporting our data, two groups have in fact shown that IL-7

administration to virally suppressed patients resulted in an 70% increase in circulating latently infected CD4+ T cells (Vandergeeten et al., 2013) and that it leads to the partial reactivation of latent HIV-1 with no impact in the size of the reservoir (Bosque, Famiglietti, Weyrich, Goulston, & Planelles, 2011). Both concluded that IL-7 actually contributes to the maintenance of viral latency. Together with our results, this supported our decision to drop the search for a scFv agonist specifically against IL-7R. Finally, H_5 was chosen for further tests based on its obvious potential for HIV-1 reactivation. Associated with this decision was the fact that H_5 is of human origin, and thus, immunogenicity problems for eventual future researches would be minimized.

Another particularity that became evident during this reactivation assay was that the percentage of J-Lat cells expressing GFP increased between the first and the second time point and thus two possible questions arouse. Does scFv agonist activity increase with longer exposures or is it possible that some scFvs only have a visible effect after 48 hours?

The sorting procedure done in order to individualize single clone scFvs revealed the possibility for the reactivation effects of antibodies to be based on combination as it became evident that each individual cell was coated with close to 12 recombinant phages. Still individual clones were screened individually as initially proposed as possible combinatorial tests would extend beyond the purpose of this work and could not be completed in the defined timeline. It is important to comment that during the single clone screening process it was intended to screen close to 400 individual clones. For this to be achieved, bacterial growth would be performed in 96 well microtiter plates according to existent protocols for phage ELISA. These protocols lack the phage purification step and thus, recombinant phages remain in the supernatant after cellular lysis. This represented the first problem for our screening as we observed that not only the supernatant but the lysate from ER 2738 *E. coli* cells as well, were both capable of reactivating the J-Lat provirus more efficiently than TNF-α itself. Therefore we concluded that for our specific assay, phage purification was needed. We maintained the 96 well microtiter plates and adapted the recombinant phage library reamplification protocol for smaller volumes. The first problem that we faced when growing our clones in these plates was cross-contamination. Growth control wells consistently appeared contaminated with Tet, Amp and KanA resistant bacteria, which meant that our clones were not staying confined to their specific well. This could be explained by the quantity of manipulations and centrifugations needed for this specific protocol to be completed, but no further conclusions were taken as another problem arouse mining this protocol. The final step for phage purification consists in passing the recombinant phage solution through a 0,22µm filter, which has a dead volume of

about 500µL, to remove any residual PEG left after the centrifugation step and to assure its sterility. Because we were growing our clones in 96 well microtiter plates, our maximum volume per well was 300µL which would be lost during any filtration. To confirm that the filtration step could be ignored J-Lat incubations with a PEG solution with the correct dilution were prepared and evaluated through FACS after 24h. Cellular death was evident with only 5% or less of cellular viability. These final results made us drop the intention for bacterial growth in 96 well plates. And as growing 400 individual clones each in its own flask was just time and resource consuming the decision to screen only 40 clones was made.

During the single clone reactivation screenings we also faced a minor problem. Results from the second reactivation assay for the 40 clones showed a decrease in viral reactivation capacity when compared to the previous one. This is easily explained by the fact that between the first and the second assay, recombinant phages samples were stored at 4ºC for approximately two months. If scFvs had been produced separately and purified no results would be visible for this last assay, as purified V_H and V_L samples when stored as 4ºC only keep their viability for about a month. Such phenomenon is also extended to the reactivation assay performed for the other three J-Lat lines, done simultaneously with the second reactivation assay and, although it has become obvious that the selected scFvs do not behave in the same manner for other J-Lat lineages as they do for J-Lat 10.6, these results become inconclusive. Nonetheless, from the data presented in the first single clone reactivation screening assay, and supported by the fact that the reactivation pattern was maintained when repeated, we were able to identify promising agonist scFvs for HIV-1 reactivation. Thus, the second specific aim was also successfully accomplished. From the initial 40 scFvs screened, we have identified 4 different scFvs capable of reactivating the latent proviral genome from J-Lat 10.6 and therefore 4 different scFv with an agonist effect: C1, D1, H5 and the most promising scFv G1. Although results didn't carry on to other J-Lat lineages they still reveal some very small residual effect. It is also interesting to observe that scFv previously discarded for their low capacity in reactivating the provirus in J-Lat 10.6, like G4, show different viral reactivation patterns in other lineages. This only comes to confirm the upmost importance in choosing the correct model to perform this type of selection. Laboratory perpetuated cells although good for understanding and evaluating many aspects of some diseases do not present themselves as optimal targets for drug development.

Also, analyzing the data from the two time points from this single clone reactivation screening assay we observed that the percentage values of GFP^+ cells were consistently maintained between them, this is, no increase in the 48 hour signal was observed as the one evident

during the preliminary reactivation assay. This, associated with the fact that we only screened the sample sorted for the 24 hour mark, leads us to conclude that our screening excluded possible scFv agonists which action takes longer than 24 hours to become evident.

On a more broad consideration, we have successfully used a simple two step method for antibody selection that enabled us to identify agonist scFvs against unknown membrane targets capable of causing a desired phenotypic alteration. Consequently, we present a possible alternative to the lentiviral selection method; although we can confirm that the creation of a library of scFv against membrane determinants of J-Lat through phage display dictates the complexity of the function selection step.

CONCLUDING REMARKS AND FUTURE WORK

The most significant obstacle in fighting HIV-1 and eradicating it is its ability to establish a viral latent infection. But which is the best way in dealing with HIV-1 reservoirs: does one target and eliminate the latently infected cells or target the latent provirus for reactivation? Although recent developments may pose a barrier for the so-called “shock and kill” approach we believe that a sterilizing cure can be achieved by the reactivation of the fully functional dormant provirus to expose it to the efficient effects of the antiretroviral drugs and therefore preventing the perpetuation of the infection. Our approach has its innovation in the search for biopharmaceuticals oriented against the latently infected cell. We believe that agonist antibodies, in the likeness of conatumumab, could act externally to the cell creating the necessary genetic changes that culminate in the latent proviral reactivation. Here, and resorting to a simple technique of phage display, we have successfully identified 4 different scFvs – C1, D1 H5 and G1 – against J-lat 10.6 unknown membrane proteins capable of functioning as agonists for the reactivation of the latent HIV-1 provirus in these specific cells.

But many promises born from this work still remain unanswered. In the field of HIV-1 latency, it is imperial to repeat of the single clone reactivation screening assay with fresh samples. This should be done not only against J-Lat 10.6 but against the other lineages as well as it was observed that the reactivation patterns of the 40 selected scFv are lineage dependent. Initially discarded scFv clones can hypothetically present a better agonist capacity reactivating HIV-1 in other J-Lat lineages when compared to C1, D1, H5 and G1. Also, another logical step for this line of work would be to produce the scFvs in solution for further testing, this is, purifying them individually and not associated to recombinant phages. At this point, reactivation assays should be repeated, with the incorporation of primary cultures as well to access scFv effects on models closer to humans. On a more fundamental aspect of this investigation, there is need to identify their targets and associated pathways that lead to proviral reactivation. For this last task to be possible new techniques are required like mass spectroscopy. One has also to bear in mind that we have selected considerable large libraries of scFv against J-Lat cells and tested only 40 of them for their capacity in causing a desirable phenotype alteration. Plenty more scFvs remain to test not only for their agonist effects but for their differential binding power. If some of the scFv target specific membrane proteins present only on latently infected cells (biomarker) then they can be used as vectors for specific drug delivery or for diagnosis purposes as quantifiers of latently infected pools and patient disease evolution. Such biomarkers could also be used for different approaches in dealing with HIV-1 latency being antibody-dependent cell-mediated cytotoxicity the key term here.

An important difficulty that we faced during this work was highlighted when performing the reactivation assay against other J-Lat lineages. This confirmed the upmost importance in choosing the correct model to perform this type of selection. A way to surpass the problem of model and reality discrepancies is to use the least reactive laboratory model (J-Lat 6.3) or latently infected cells form live donors. While a successful screening against J-Lat 6.3 would give us an agonist scFv for reactivating HIV-1 in the least reactive laboratory latency model there is still the possibility that such effects do not transfer to other models or even to clinical usage. Using primary cells for a study like this, although harder to obtain, could prove itself more fruitfull. Acquiring a population composed exclusively of latently infected T cells has been made possible by a group of investigators that developed a dual-color HIV reporter which enable their purification resorting to a sorting procedure similar to the one described and used during this work (Calvanese, Chavez, Laurent, Ding, & Verdin, 2013). Pannings made against this sorted primary cultures would diminish the possibilities of selecting scFv ineffective outside of the laboratory environment. Such appreciation about the correct model is also

extended to the technology itself, as the selection of the appropriate cellular model is of great importance.

On a more broad consideration, the investigation for new antibodies can be cost effective. Knowing the target, one has only to screen a pre-fabricated library against it in order to identify new antibodies with binding capability. This is also true for chemical compound library screenings as well. Posterior development and optimization of the selected proteins for a specific function and effect form the bulk of the expenses. On the other hand, investigation for not only new antibodies but new targets as well becomes much more expensive. Big pharmaceuticals can afford to spend the resources needed for the discovery of new and important targets but, as any commercially quoted company, the profit/benefit balance reduces their risk taking initiative. In this thesis, we present a simple and cost effective technique, when compared to the lentiviral library screening methodology, capable of identifying new targets associated with their respective agonist scFvs for a wide array of diseases depending on the cellular model chosen. Identification of new targets, that upon interaction with antibodies are capable of start a signal cascade in direction of a desired phenotype, can shift the way we now look at diseases and therapies presenting new and important information both for fundamental or applied investigation. Talking about the methodology used here, although it has proven successful and a possible alternative to the creation of lentiviral libraries, remember that 10% of our pool of 40 selected scFvs was identified as promising, further assessments need to be made. One can say empirically that the lentiviral approach can be more expensive and time consuming (H. Zhang et al., 2013) since a successful screening can rapidly be made once individual phage samples are produced. There is no need for lentiviral library creation, lentiviral infection protocols, immobilization of eukaryotic cells to minimize scFv migration or eukaryotic cell individualization after sorting procedures. Recombinant phage samples are easily obtained and incubated against eukaryotic cells and single clone individualization is easily achieved by *E. coli* infection. Also, library sizes presented for inquiry are different; phage display libraries can present 10^{10} different scFv against 10^{7} from lentiviral libraries. And although combinatorial synergy was a feature of the lentiviral selection method, we have shown that it is also a possible reality for our approach. Still, during this work no further considerations were taken about screening capabilities. In order to create a basis for such comparison a relatively small library, easily screened by hand, should be used. On this note, pannings from the H5 sample should be repeated to create a basis to really compare the lentiviral agonist technique against ours. As this sample showed the lowest value of recombinant phages recovered it should be easier to screen all of them

resorting to the sequence of protocols here presented. At the same time, the DNA from H5 sample should be collected for the creation of a lentiviral library to be tested and screened. Then, by comparing reactivation assays from both techniques it would become possible to access and compare screening capacities: number of selected promising agonist scFvs and reactivation profiles for the multiple clones. Production and purification of soluble scFv from phage display selection is also important for this assessment as scFv produced from lentiviral libraries are always soluble. This becomes important to evaluate if scFv valence is a variable to take in account as in phage display valence ranges from one to five while lentiviral libraries have a valence of one (H. Zhang et al., 2013).

Another interesting assessment to be investigated is if there is the possibility for pannings with a non-biased library against the cellular model chosen to be made for periods longer than 1 hour. As we demonstrated recombinant phages can be incubated against our chosen cellular model for periods of 24 or 48 hours in RPMI 1640 10% FBS at 37ºC in a humidified atmosphere containing 5% CO_2 and posteriourly recovered, therefore there is the possibility to resort instantly to FACS and select immediately for function rather than initially screen for binding to a target. Possibly this would require a previous negative selection against FBS to minimize unspecific interactions.

In conclusion, the results of this work made possible the page display selection and identification of 4 agonist scFvs against unknown membrane targets capable of purging the latent HIV-1 infection from the selected latency laboratory model, J-Lat 10.6. Furthermore, this thesis, although not intentionally, comes to evidence a possible new methodology based on a simple and overused technique alternative to the lentiviral selection method with the successful identification of 4 scFv capable of acting as agonists causing a desired phenotypic alteration.

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