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"Development of strategies to find inhibitors of HIV-1 Nef cellular interaction partners"

"Sviluppo di strategie per la ricerca di inibitori dei partner cellulari di interazione della proteina Nef di HIV-1"

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ABSTRACT

This Ph.D. project has been focused on the study of HIV-1 Nef protein.
Nef is a regulatory phosphoprotein and studies on animal models and seropositive patients have demonstrated its importance as virulence factor. Nef, in fact, plays a pivotal role in maintaining high viral load and in progression to AIDS, and is already object of interest of the HIV scientific community in the search of new targets to prevent or block HIV disease.
Nef exerts three main functions in HIV-1 target cells (*i.e.*CD4+ T-helper lymhpocytes, monocytes/macrophages, dendritic cells, microglial cells): 1) the modulation of cellular signalling pathways; 2) the enhancement of viral infectivity; 3) the regulation of expression of cell surface receptors, including the well-studied down-regulation of CD4 and certain class I major histocompatibility complex (MCH-I) antigens.
Altogether these effects increase viral infectivity and spreading contributing to render the virus able to escape the immune system response.
Nef presents a two-domains structure: a structurally flexible N-terminal membrane anchor domain and a well conserved and folded C-terminal core domain. In addition it is post-translationally modified by phosphorylation and by myristoylation which is important for the Nef signalling activity. Nef exerts its functions working as a molecular adaptor, interacting and influencing the activity of more than 30 intracellular partners and several Nef interaction sites have been identified. As a consequence of Nef ability to bind multiple targets certain Nef effects dominate over others in time- or cell type- dependent manner.
Macrophages are the first cells to be infected by HIV and represent the main reservoir of the virus. In these cells Nef induces the synthesis and the release of a specific sub-set of chemokines and cytokines able to recruit T-cells on the infectious site rendering them susceptible to HIV infection. Moreover, recent studies, carried out by the research group where it has been performed this Ph.D. project, have demonstrated that Nef is efficiently internalized by human non infected monocytes derived macrophages (MDMs) in *in vitro* cultures and mediates the NF-kB-mediated synthesis of specific cytokines and chemokines that in turn lead to the activation by phosphorylation of STAT (Signal Transducer and Activator of Transcription)-1, -2 and -3. Importantly, it has been demonstrated that the Nef interaction site designated as acidic cluster (A60QEEEE65) is required for this process. In addiction, modelling analysis and silencing experiments indicate that this region represents a putative binding motif for specific TNF receptor associated factor (TRAF) adaptor family members. It has been

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proposed, in fact, that in macrophages Nef intersects the CD40 signalling pathway and TRAF are involved in the signalling events downstream this receptor. Therefore, the study of the Nef interactome, of the molecular basis underlying the Nef acidic cluster/TRAFs interactions, and the finding of molecules able to block the main Nef interaction sites is certainly of great interest both to clarify the so far enigmatic biology of Nef and to open new perspectives in the field of HIV-AIDS therapy.

In detail, this project has been centred on the study of Nef anchor domain and of its acidic cluster with the aim to further investigate on the role of these regions in the Nef-mediated signalling.

To achieve these goals, the first part of the project has been focused on the production of a Nef target region designated as N-Term76-Nef that has been used first to carry out experiments on cellular systems, and secondly as target for phage display experiments in order to identify Nef binding peptides. Thus, an efficient protocol for the expression and the purification of the N-Term76-Nef has been setup and as result the production of a high level of purity of the target protein has been obtained.

During the second part of the work N-Term76-Nef has been used for the treatment of THP-1 cells in order to evaluate the role of the Nef anchor domain in the Nef signalling effects. It has been demonstrated that N-Term76-Nef region alone is able to affect the macrophages cell signalling as the Nef full-length protein does. This finding highlights on the importance of the Nef anchor domain in the Nef functions and opens new intriguing perspectives regarding the biology of Nef.

Finally, phage display of random peptide experiments have been performed to identify peptide to be used both for the characterization of the Nef interactome and for the search of potential Nef binding inhibitors.

To this purpose, a 50 amino acid long phage random peptide library has been designed and produced and N-Term76-Nef has been used as target for the affinity selection cycles that has been performed through bio-panning methodology. The selection cycles have been carried out until the obtainment of a final phage sub-library enriched for the interested phage. Sequencing analysis of the selected phage genomes have been performed while modelling analysis and pull-down experiments are currently under planning and design with the final goal to isolate peptides displaying the best binding affinity for Nef. Once found, the more suitable peptides will be tested in cell culture systems to verify their potential Nef inhibitory activity.

RIASSUNTO

La presente tesi di dottorato ha come argomento di studio la proteina retro virale Nef del virus dell'immunodeficienza umana (HIV)-1, agente eziologico della sindrome dell'immunodeficienza acquisita (AIDS).

Nef è un importante fattore di virulenza fortemente coinvolto nel mantenimento di un'elevata carica virale e nella progressione verso la fase conclamata della malattia, ed è attualmente oggetto di intenso studio da parte della comunità scientifica nella ricerca di nuovi target terapeutici per la prevenzione ed il contenimento della malattia da HIV.

Nef esercita sulle cellule suscettibili all'infezione (linfociti T CD4 positivi, cellule della linea monocito-macrofagica, cellule dendritiche e microgliali), tre principali funzioni: 1) regolazione di vie di trasduzione del segnale; 2) aumento dell'infettività del virus; 3) regolazione dell'espressione di alcuni recettori sulla membrana plasmatica, tra cui tra cui *down*-regolazione del recettore CD4 e di alcuni antigeni del complesso maggiore di istocompatibilità di classe I (MCH-I). Complessivamente gli effetti si traducono nell'incremento dell'infettività e della diffusione virale e contribuiscono all'evasione del virus dal sistema immune.

La struttura di Nef è caratterizzata dalla presenza di due domini principali: una regione N-terminale strutturalmente e geneticamente flessibile (*anchor domain*) seguita da una regione C-terminale altamente conservata e strutturalmente definita (*core domain*). Entrambi questi domini presentano numerosi siti funzionali coinvolti nell'interazione tra Nef e i suoi effettori molecolari. Nef è, infatti, un adattatore molecolare i cui effetti sono mediati dalle numerose interazioni tra la proteina e i suoi diversi partner cellulari. In questo contesto è di grande interesse, sia sotto un profilo di ricerca di base che applicata, lo studio dell'interattoma di Nef e l'identificazione di peptidi di *binding* ad azione inibitoria sui suoi siti di interazione. In particolare, il presente studio si è avvalso di precedenti risultati sperimentali, condotti su cellule umane non infette della linea monocito-macrofagica, ottenuti dal gruppo di ricerca dove è stato svolto il dottorato. Questi studi, hanno dimostrato che il trattamento esogeno di macrofagi con la proteina Nef miristoilata determina la produzione, NF-kB mediata, di uno specifico *sub-set* di citochine e chemochine pro-infiammatorie (MIP1α, MIP1β, IL-6, TNFα, IFNβ) che mediano l'attivazione dei trasduttori del segnale ed attivatori della trascrizione STAT(Signal Transducer and Activator of Transcription)-1, -2 e -3. In particolare, è stato dimostrato il coinvolgimento del cluster acidico (AC) presente nel braccio N terminale di Nef (A60QEEEE65) in questo processo e la possibile interazione della proteina

con alcuni membri delle proteine TRAF (adattatori molecolari coinvolti nella trasduzione del segnale, che parte da membri della famiglia dei recettori per il TNF).

Nel presente progetto la ricerca è stata incentrata sul *anchor domain* di Nef con particolare riferimento al *cluster* acidico nel tentativo di chiarire alcuni meccanismi alla base della complessa biologia di Nef e il significato biologico delle sue multiple interazioni.

Per la realizzazione di questi obiettivi la prima parte del lavoro ha richiesto la produzione di una specifica regione della proteina definita come N-Term76-Nef comprendente l'*anchor domain* e la regione del cluster acidico. A tale scopo, è stato messo a punto un efficiente protocollo di espressione/purificazione per l'ottenimento della regione target altamente purificata. La regione N-Term76-Nef così ottenuta è stata quindi utilizzata sia per esperimenti su colture cellulari che come target per esperimenti di *display* fagico. A questo scopo N-Term76-Nef è stata utilizzata per il trattamento di cellule THP-1 ed è stato dimostrato che questa regione è in grado di indurre la fosforilazione in tirosina di STAT-1 nei macrofagi così come la proteina *full-length*, suggerendo che N-Term76-Nef attivi un processo di trasduzione del segnale identico a quello innescato dalla proteina intera. Questi risultati evidenziano l'importanza del *anchor domain* in questo processo e aprono nuove interessanti prospettive sull'ancora enigmatica biologia di Nef.

L'ultima parte del progetto è stata focalizzata sull'identificazione di peptidi, tramite esperimenti di *display* fagico di librerie peptidiche casuali, per la caratterizzazione dell'interattoma di Nef e la ricerca di potenziali inibitori di interazione. A tale proposito, è stata prodotta una libreria fagica di peptidi casuali utilizzata per cicli di selezione diretti contro N-Term76-Nef realizzati tramite la metodologia del *biopanning*. I cicli di selezione sono stati portati avanti fino all'ottenimento di un'ultima sotto-libreria fagica arricchita per i fagi esibenti le caratteristiche attese. Il processamento di tale libreria, che è attualmente in corso, prevede l'isolamento di peptidi che presentano alta affinità di legame per i principali siti di interazione di Nef tra cui il *cluster* acidico, tramite analisi di *modelling* ed esperimenti di *pull-down*. Una volta isolati, questi peptidi saranno testati in sistemi cellulari per la valutazione della loro potenziale attività inibitoria.

1. INTRODUCTION

1.1. Human immunodeficiency virus-1 (HIV-1) and its involvement in AIDS.

1.1.1. HIV-1 structure and genomic organization.

Human immunodeficiency virus-1 (HIV-1) is a primate complex retrovirus (Goff, 2001) belonging to *Lentivirus* genus (Ratner et al., 1985; Wain-Hobson et al., 1985) and, together with HIV-2, is the etiological agent of the acquired immunodeficiency syndrome (AIDS) (Barrè-Sinoussi et al., 1983; Popovic et al., 1984; Sarngadharan et al., 1984).

HIVs enveloped mature virions are about 100-120 nm in size and present a cone-shaped cylindrical core (capsid) (Fig. 1.1).

HIV-1 genome consists of two copies of linear single stranded RNA molecules with positive sense that are linked at the 5' end through a self-complementary sequence called *dimer linkage structure* (DLS). The genomic organization shares *gag, pro, pol*, and *env* structural genes with the other retrovirus members while presents, in addition, six overlapping open reading frames (ORFs) of regulatory and accessory genes such as *vif, vpr, vpu, tat, rev* and *nef*. The products of the structural genes (*gag, pro, pol and env*) are initially translated as polyprotein precursors. The *gag* gene leads the expression of the 55-Kda Gag precursor that, after cleavage, generates matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins; Gag-Pol precursor is a 160-Kda polyprotein and after processing generates protease (PR), reverse transcriptase (RT) and integrase (IN); the Env precursor (gp160) gives rise to the envelope proteins of surface (SU) gp120 and trans membrane (TM) gp41 (Fig. 1.2).

The products of accessory genes are generated through mRNA alternative splicing (Freed and Martin, 2001) and are classified in structural (present in the virion) and non-structural proteins (founded exclusively in the infected cells). Below their known functions will be described, even if their role is still partially solved. However it is known that these accessory proteins manipulate host biology to promote the viral life cycle.

The main regulatory proteins that are indispensable for virus replication are Tat and Rev. Tat (from *Trans Activator of transcription*) is a 14 KDa trans activator of proviral genome transcription while Rev is a phosphoprotein of 19 KDa involved in m-RNA maturation and transport and gets its name from *Regulator of Virion Expression*.

Viral Infectivity Factor, Vif, is about 23 KDa and plays a central role in the APOBEC cellular enzyme ubiquitination and degradation, directly disrupting its antiviral activity (Stanley et al., 2008).

Viral protein R, Vpr, is a structural virion protein, is 14 KDa in size involved in regulation of nuclear import of pre-integration complex (PIC). In addition, Vpr has an important role in apoptosis regulation and in G_2 cell cycle phase arrest (Bukrinsky and Adzhubei, 1999; Muthumani et al., 2006).

Virus Protein U, Vpu, is the distinguishing character of HIV-1, the protein is in fact not present in HIV-2 and SIV. Vpu is a multimeric integral membrane phosphoprotein of 81 amino acid (Wray et al., 1999). The protein is involved in increasing virus release and in CD4 degradation (Bour and Strebel, 2003).

Finally, the virulence factor Nef which, being the topic of this work, will be in depth treated during the following sections.

Human Immunodeficiency Virus (HIV)

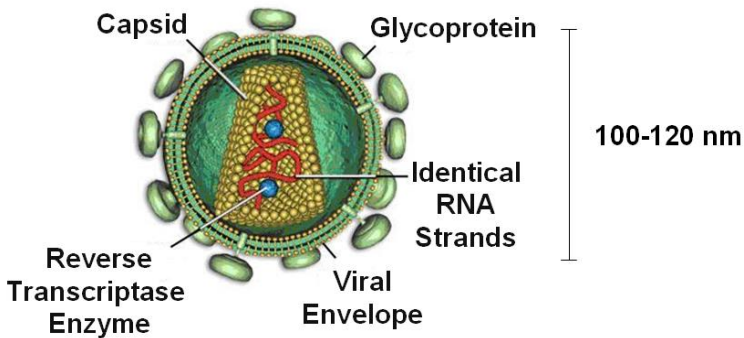**Figure 1.1. Human Immunodeficiency Virus (HIV): virion anatomy. (www.con-tatto.org).**
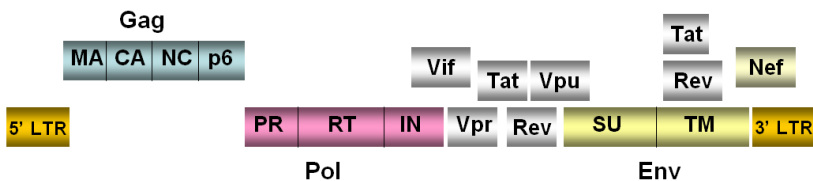
Genomic Organization of HIV-1

Figure 1.2. HIV-1 genomic organization. HIV-1 has three structural genes (gag, pol and env) codifying for structural proteins and enzymes. HIV-1 genome also contains six overlapping open reading frames codifying for the regulatory and accessory proteins Tat, Rev, Nef, Vif, Vpr and Vpu.

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1.1.2. HIV-1 life cycle.

HIV-1 life cycle, as that of the others retroviruses, consists in a multi stage process and each step is crucial for successful replication (Fig. 1.3).
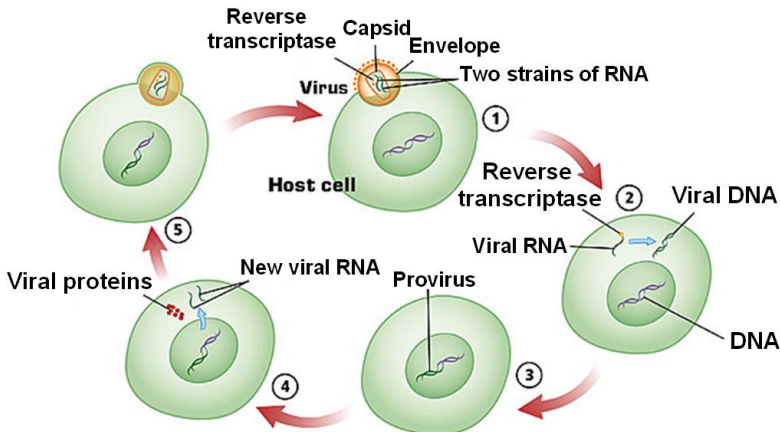
Figure 1.3. Schematic representation of HIV-1 life cycle. The figure shows the principal stages of the viral cycle: 1) host cell penetration; 2) uncoating and reverse transcription of HIV genome; 3) Integration of the retroviral DNA; 4) transcription of the proviral DNA; 5) Virus budding. (Modified from Manninen, 2001).

The first step of HIV-1 infection, is characterized by the interaction between the surface subunit gp120, of the trimeric viral envelope glycoprotein (Env) gp120/gp41, presents on the virion surface and the CD4+ receptor presents on suitable host cells (*i.e.* T helper lymphocytes, machrophages and dendritic cells) (Dalgleish et al., 1984). The binding of gp120 to CD4 receptor is necessary but not enough for the entry of HIV into the cells: for an efficient process also a co-receptor (*i.e.* CCR5 or CXCR4 chemokines receptors) is required (Edinger et al., 1998; Simmons et al., 1998). The binding of gp120 with the CD4 receptor, in fact, promotes its further binding to the co-receptor. This event leads to a conformational change in gp120 that results in viral fusion mediated by gp41 trans membrane subunit (Blumenthal et al., 2012). After membrane fusion, *penetration* and

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uncoating steps occur. During this process, who's molecular mechanisms have not yet been completely elucidated, viral capsid enters the cytoplasm and is digested (uncoating) inside the host cell releasing RNA genome and the three essential replication enzymes integrase, reverse transcriptase and protease to allow the formation of the reverse transcription complex (Arhel, 2010). Therefore, reverse transcriptase begins the reverse transcription of viral RNA. This enzyme presents two catalytic domains: an aminoterminal DNA polymerase active site and a carboxyterminal RNase H active site (Skalka and Goff, 1993; Tanese and Goff, 1988).

Reverse transcription is an extremely complex process comprising three main stages that are hereafter briefly schematized (an exhaustive description of the process lies outside the aim of this work): 1) Single stranded viral RNA is transcribed by DNA polymerase domain in a RNA/DNA double helix; 2) the RNA/DNA duplex is then processed by RNase H that degrades RNA strand; 3) the single stranded DNA is used as template by DNA polymerase to be converted in a full-length linear double stranded DNA bordered at each end by the long terminal repeat (LTR) containing all the genetic information for viral gene expression.

Once reverse transcription is concluded the integration of the viral DNA in the genome of the host cell takes place. Integration is crucial for retroviral life cycle and also accounts for the ability of viruses to persist in the infected cells as provirus and to permanently enter the germline (Goff, 2001).

The integration of retroviral DNA is mediated by the viral integrase protein. Integrase cleaves, at a highly conserved CA sequence, a dinucleotide from each 3' end of DNA creating two sticky ends with protruding 5' ends. Integrase then transfers the DNA into the cell nucleus and facilitates its integration into the host cell genome (Gallay et al., 1997). The mechanisms of integration consists in a Sn2-type reaction in which the 3' OH ends generated by integrase are used to attack the phosphodiester bonds of the host DNA (Fujiwara and Mizuuchi, 1988). Thus, the protruding 5' end of the proviral DNA is joined to the host DNA in a process that doesn't require the viral integrase.

The integration of the viral DNA signs the end of the early phase of HIV-1 replication cycle and the beginning of the late phase mediated mostly by host enzymes. The late phase is characterized by the synthesis of viral RNA and proteins and progeny virions assembly (Freed and Martin, 2001).

Transcription of proviral DNA into mRNA mediated by cellular Pol II polymerase starts at U3 part of 5' LTR sequences which are the major determinants regulating virus replication. This process is controlled and

regulated by the viral regulatory protein Tat that stimulates transcriptional elongation of the full length viral mRNA. Viral mRNA migrates from the nucleus to the cytoplasm in a process involving Rev regulatory viral protein (Karn and Staltzfus, 2012). Into the cytoplasm a portion of the transcripts that remain unspliced is utilized as the viral RNA genome and also serves as Gag and Gag Pol mRNAs. Another portion is partially spliced for Env, Vif, Vpr and Vpu mRNAs and completely spliced to generate Tat, Rev, and Nef (Freed and Martin, 2001). Some of these proteins are translated as protein precursors and then processed by the viral protease during and after virion assembly to produce viral mature proteins; this step is critical for the creation of an infective virus. After synthesis, viral proteins and RNA genome came together to form the capsid. This immature viral particle buds out the cell and acquires the envelope containing both host and viral proteins. After this process the virus matures, becomes infective and equipped to infect other cells.

1.1.3. HIV-1 tropism, pathogenesis and progression to acquired immunodeficiency syndrome (AIDS).

How emerges from the modalities of virus entry, HIV infection is confined mainly to the specific sub set of immune system cells that presents CD4 receptor on its surface, such as CD4+ T-helper lymphocytes, macrophages and dendritic cells, and carrying the co-receptors CCR5 or CXCR4. On the basis of the type of co-receptor utilized in *in vitro* fusion assays, a classification of HIV-1 isolates has been proposed (Baba et al., 1999; Donzella et al., 1998).
HIV-1 strains that use CCR5 receptor are predominantly macrophage- or M-tropic (able to replicate in macrophages), nonsyncytium-inducing (NSI) and are defined *slow/low* because are characterized by slow replication and low production of viral progeny, these isolates are called R5.
HIV-1 strains that use CXCR4 co-receptor (X4 viruses) present a T-cell line tropism (TCL-tropic), are syncytium-inducing (SI) and, on the basis of their characteristics of replication and particles production, are defined *rapid/high* and exhibit an enhanced cytopathicity. Although the *in vivo* situation is obviously more complex, on the basis of this simplified classification, it has been observed that HIV-1 R5 strains are predominant during the asymptomatic phase of infection (Zhu et al., 1993) while X4 viruses emerge mostly during the acute symptomatic phase (Koot et al., 1993). During the asymptomatic phase, in fact, the major part of the

depleting CD4+ T lymphocytes are memory T-cells expressing CCR5 that are located principally in the gastrointestinal tract and in lung; only a low percentage of these R5-target cells are usually present in the peripheral blood (5-10%), so that no relevant T-lymphocytes depletion is yet observable in this tissue at this time of disease (Mehandru et al., 2004). The emerging of X4 strains during the late acute phase is accompanied by the substantial depletion of peripheral blood CD4+ T-lymphocytes (to < 200 cells/μl) that represents the salient clinical manifestation of HIV disease. The major part of the CD4+ T-cells circulating in peripheral blood (80% to 95%), in fact, are naïve T-cells expressing CXCR4 receptor and suitable to X4 virus infection. This massive T-lymphocytes depletion is associated with a rapid progression to AIDS (Connor et al., 1993).

How mentioned above, T-lymphocytes are not the only sub set of immune cells to be infected by HIV. A pivotal role in HIV dissemination and persistence in target tissues (*i.e.* lung, gastrointestinal tract, bone marrow, central nervous system, lymph nodes), in fact, is ascribed to macrophages and dendritic cells.

Macrophages are involved in mucosal transmission of the virus and represent the main viral reservoir. Macrophages are the first cells to be infected and are involved in viral spreading because able to directly infect CD4+ T-cells through a transient adhesive cell to cell contact (Groot et al., 2008). Further, macrophages exhibit a host/pathogen interaction response that significantly differs from that observed in T-lymphocytes and consisting in a great resistance to the cytopathic effects of the virus. More, in macrophages, HIV is able to grow inside endocytic compartments designated as multivescicular bodies (MVBs) that confer protection. Altogether these features counter for the long-term persistence of productive infection mediated by these cells (Carter and Ehrlich, 2008).

Like to macrophages, dendritic cells (DC) are also infected by HIV and, being antigen presenting cells (APC), should be the first defence line during infection, but in the case of HIV-1 infection, DCs appear to promote viral spread. The interaction between DCs and HIV-1, in fact, differs from the mechanisms of infection observed in machrophages and CD4+ T-cells. In DCs the virus interaction is mediated mainly by a different kind of receptor known as DC-SIGN that binds and retains HIV on dendritic cell surface and mediates the infection of CD4+ T lymphocytes in a process called *trans-infection* (Geijtenbeek et al., 2000).

To resume, the specific contribution made by diverse and specific immune system HIV suitable cells on the intricate dynamics of HIV-infection, converges in the massive depletion of CD4+ T-lymphocytes. This event

constitutes a strong insult to the immune system and results in the wide plethora of clinical manifestations, normally do not present in a health immune system, typical of AIDS. These manifestations include: generalized lymphadenopathy; a wide variety of severe opportunistic infections mainly caused by *Pneumocystis carinii*, *Toxoplasma gondii* and cytomegalovirus; the development of unusual neoplasms such as Kaposi's sarcoms and non-Hodgking's lymphoma, due principally to oncogenic viruses co-infections (*i.e.* Epstein-Barr virus, papilloma virus, herpesvirus).

Although after the discover that HIV is the etiological agent of AIDS several successes have been reached to contain the pandemy, AIDS is still a great menace for public health and represents also a huge worldwide socio-economic problem. The ability of the virus to mutate and to escape the immune system accounts for the difficulty to find a resolutive therapy. So far antiretrovirals that interfere with the crucial steps of HIV cell cycle have given the best results, while no efficacious vaccines have been still developed. However, also the antiretroviral therapies present several limitations in terms of costs and side effects. Hence, the necessity to find new targets to prevent or block HIV disease. Nef protein represents a good candidate to this purpose and is already object of intense study from a large part of the scientific community.

1.2. Nef protein and its role in HIV pathogenesis.

1.2.1. HIV-1 Nef: an overview.

Nef is a primate lentiviruses (HIV-1, HIV-2 and SIV) accessory protein encoded by the *nef* gene that is localized at the 3' end of the viral genome (see figure 1.3).

HIV-1 Nef is a 27-35 Kda regulatory myristoyled phosphoprotein and despite to the originally belief that confined Nef functions to downregulation of virus replication (Nef, in fact, derived its name from *negative factor*), is now clear that the protein has not negative effects on virus replication but is strongly involved in maintenance of high-viral load *in vivo* and plays a crucial role in progression to AIDS (Hanna et al., 1998; Kestler et al., 1991). The central role of Nef in pathogenicity *in vivo* is supported by studies on animal models and seropositive patients that showed that *nef* defective viruses lead to an attenuated clinical phenotype with a reduced viral load. Are reported, in fact, a number of long term non-progressor (LTNP) individuals whose viruses presented marked depletion in the *nef* gene (Kirchhoff et al., 1995). Further, it has been demonstrated that *nef* transgenic mice develop an AIDS-like disease (Hanna et al., 1998). Nef, actually, is considered an important virulence factor that, lacking of any enzymatic activity, fulfils its functions working as a molecular adaptor. It has been reported, in fact, that Nef interacts and interferes with the activity of more than 30 intracellular partners (Fackler and Baur, 2002) mostly involved in membrane receptors trafficking (Doms and Trono, 2000) and in signal transduction pathways (Geyer et al., 2001).

Well ascribed Nef functions *in vitro* include downregulation of diverse cell-surface molecules (such as CD4, MHC-I, MHC-II, CD3 receptor complex, CD28) (Garcia and Miller, 1991; Schwarz et al., 1996), increase of virus infectivity (Miller et al., 1994), regulation of apoptosis (Fackler and Baur, 2002) and modulation of cell signalling such as T-cell activation pathways (Sawai et al., 1994). These functions will be analyzed in detail in the following sections after a preliminary description of the Nef structure.

1.2.2. HIV-1 Nef structure and the "Nef cycle".

Nef is a small protein of about 200 amino acids that exists in diverse allelic forms varying slightly in length (Percario et al., 2011), however in this work we will refer mainly to SF2 Nef allele that is about 27 Kda in size in its full length myristoylated form or 25 Kda in the trunked form that is translated from a second start codon. Nef is expressed early and abundantly during the early stages of the viral cycle and is post-translational modified by phosphorilation and by N-terminus myristoylation that is critical for its functions.

NMR spectroscopy and X-ray crystallography have been used to determine the three-dimensional structure of Nef protein (Grzesck et al., 1997; Arold et al., 1997). The structure of whole Nef protein has been determined trough the overlap of the single fragmented components separately analyzed, because the full-length structure is quite difficult to obtain due to problems in its crystallization. Nef presents a specific cleavage site for a viral protease (between Trp 57 and Leu 58 residues) that has been a good tool for the determination of its structure because splits the protein in its two principal domains: the N-terminal anchor domain that is globally unfolded and structurally flexible and the C-terminal core domain that is the only part of the protein to present a stable tertiary fold (respectively 2-61 and 62-210 residues), both involved in cellular signalling and trafficking (Geyer et al., 1999; Breuer et al., 2006; Geyer and Peterlin, 2001); further a flexible loop, also important for Nef functions, of about 30 amino acids, projects out of the core domain (Fig. 1.4).
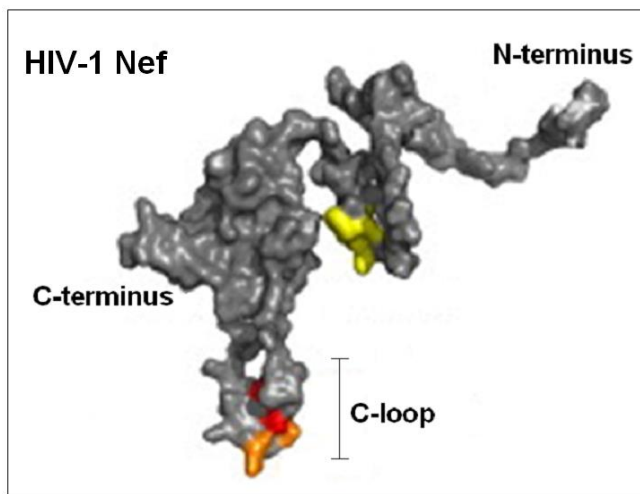
Figure 1.4. HIV Nef structure. The principal Nef domain are shown: the unstructured N-terminus anchor domain, the well conserved and folded C-terminus core domain and the C-loop that projects out of the core domain. (Modified from Götz et al., 2012).

The core domain has been studied both alone and in association with SH3 domains of Nef interaction partner proteins. It is a highly conserved region characterized by the presence of a PxxP motif that, in association with the SH3 (*Src homology 3*) domain, assumes a left-handed polyproline type II helix. Over PxxP motif, is also present an α-β motif in which a central anti-parallel β sheet of four strands is flanked N-terminally by two long anti-parallel α-helices and C-terminally by two short α-helices (Arold et al., 1997; Lee et al., 1996). Diverse binding sites are present in the core domain. PxxP motif is one of the most important, allowing the binding of a great part of Nef molecular partners. Further, core domain plays a role in the Nef oligomerization that has been observed both *in vitro* and *in vivo*, even if its significance has still to be clarified (Arold et al., 2000; Arold and Baur, 2001).

The structure of the anchor domain has been characterized both in the presence and in the absence of N-terminus myristoylation. The anchor domain is a genetically varied unstructured region of about 60 amino acids.

Globally, in the not myristoylated anchor domain secondary folded elements have not been found except for a short two-turn α-helix (H2) between Arg35 and Gly41 and for another helical secondary structure element (H1) in the arginine-rich region (Arg17 to Arg22). The myristoylation, that involves the N-terminal glycine residue, confers stability to this secondary elements and renders it more defined (Geyer et al., 1999).

How mentioned above the anchor domain is a moderately conserved region and the only sequence highly conserved is the motif MGxxx(S/T) that is the *consensus* sequence for N-myristoyl-transferase target proteins (Resh, 1999; Geyer et al., 2001) and this accounts for the crucial role that myristoylation plays in the Nef functions specially in its signalling activity (see below in the chapter). Later in this chapter, will be described detailed examples relative to the importance of myristoylation in Nef biology. However, N-terminus myristoylation plays a role in the cellular localization of Nef that is determinant for Nef molecular interactions and signalling. How mentioned above, in fact, Nef is a molecular adaptor that alters cellular pathways via multiple protein-protein interactions and diverse Nef interaction motifs have been already identified (table 1.1). Indeed, Nef presents both cytoplasmic and membrane localization and the wide plethora of Nef-mediated effects are the results of the different set of effectors that interacts with Nef depending on its cellular localization and on the particular phase of the viral life cycle. In this regard it has been speculated on the existence of a Nef cycle that consents a time/space-dependent exposure of different Nef motifs (Arold and Baur, 2001). The great plasticity of Nef, in fact, is certainly due to its peculiar flexible structure that consents drastic conformational adjustments that are basic for Nef multiple interactions, and these conformational changes could occur during the hypothetic Nef cycle. The Nef cycle is a speculative model that follows the path of Nef immediately after its translation until its bind to cellular membrane. According to this model, after protein translation, Nef assumes a "closed conformation" in which presumably are shown just some of its interaction sites. After the bind with the membrane, mediated by myristic acids, Nef adopts a semi-open form that binds first with signalling molecules downstream the T-cell receptor (TCR). These interactions lead Nef to assume the open signalling conformation involved in the interaction with endocytotic machinery and in Nef trafficking and signalling (see next paragraph) (Fig. 1.5).
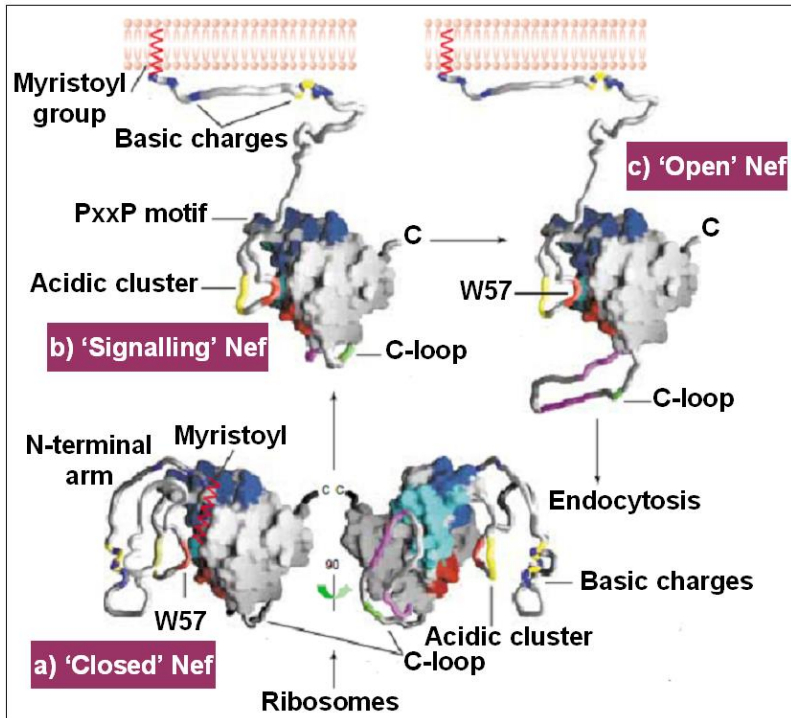
Figure 1.5. The Nef Cycle. On the basis of this speculative model, Nef assumes different conformations depending on its cellular localization and on the viral cycle stages. a) the closed conformation that Nef could adopt immediately after translation, in this form Nef hides most of its interaction sites; b) after the bond with membrane, Nef adopts a semi-open signalling conformation mainly involved in the interaction with T-cell receptor signalling molecules; finally, Nef assumes an open conformation and exposes the C-loop. This conformation could be involved in the interaction with endocytic machinery. (Modified from Arold and Baur, 2001).

Name	Nef residues implicated in binding
Signalling proteins	
Src family tyrosine kinases (*i.e.* Hck,Lyn, Fyn,Src,Lck)	SH3-binding site (*i.e.* P72, P75, R77, K82, D86, F90,W113,I114, T117, Y120);N-terminal residues for binding to Lck SH2 (Arold, 1997)
Protein kinase C	Not clearly identified (Smith et al., 1996)
p21-associated kinase 1/2	SH3-binding site and P69, Q73, L76, R106, D108, L112, P122, D123 (Manninen et al., 1998)
Mitogen-activated protein kinase Erk-1	Not identified (Greenway et al., 1996)
Ser/Thr protein kinase Raf1	D174, D175, E179 (Hodge et al., 1998)
TCRζ	Core-domain residues (Xu et al., 1999)
Vav	SH3-binding site (Fackler et al., 1999)
LAT	Association assumed (Hanna et al., 1998)
CD4	A57, W56, L59, G95, L97, L100, I101, R106, I109, L110 (Grzesiek et al., 1996)
MHC-1	Residues of helix H1, Pro-rich region and E62EEE65 in HIV-1 Nef (Greenberg et al., 1998)
Proteins implicated in trafficking	
Adapter protein complexes	E160, L164, L165, D174–E179 (Craig et al., 1998)
β-COP	E155, E156 (Piguet et al., 1999)
Vacuolar ATPase	D174, D175 (Lu et al., 1998)
Thioesterase	D108, L112, P122, D123 (Cohen et al., 2000)
PACS-1	E62EEE65 (Piguet et al., 2000)
Others	
Nef (oligomerization)	R105, D108, I109, L112, Y115, F116, P122, D123 (Arold et al., 2000)
Cellular membrane	G2 (carrying the myristoyl group), K4, K7, R17, R19, R21, R22 (Welcker et al., 1998)

Table 1.1. The table shows a list, although non exhaustive, of proteins that have been found associated with Nef.Abbreviations: β-COP, β subunit of COPI coatomers; LAT, linker for activation of T cells; MHC-1, major histocompatiblity complex 1; PACS-1, phosphofurin acidic cluster sorting protein 1; SH2/3, Src-homology domain 2/3; TCRζ, ζ chain of the T-cell receptor; vATPase, vacuolar ATPase. (Modified from Arold and Baur, 2001).

1.2.3.   HIV-1 Nef functions.

How mentioned above, Nef is an important virulence factor able to interfere with the normal functions of the cells showing a wide range of phenotypes. In this paragraph we will consider what is known about the main Nef functions, even if their molecular mechanisms are still only partially solved.

Negative modulation of membrane receptors.

Nef interferes with the endocytic machinery decreasing, in the infected cells, the expression of certain membrane receptors such as CD4 and histocompatibility complex class I (MHC I) antigens.
Downregulation of CD4 is a common phenomenon, utilized also by other retroviruses, to avoid superinfection (Michel et al., 2005) and to enhance the release of viral progeny (Ross et al., 1999). Further, this could represent a strategy to control signalling events in the infected cells.
Nef promotes CD4 endocytosis recruiting clathrin molecules to the membrane, in a process that involves the Nef binding to CD4 and to the clathrin adaptor AP-2. Furthermore, it has been reported that the interaction between Nef and β-COP protein is responsible to direct CD4 molecules to the endosomal pathways leading to its degradation (Benichou et al., 1994; Landi et al., 2011).
MHC-I downregulation is a defense mechanisms involved in HIV immuno-evasion that, rendering infected cells less visible to circulating CD8^{+} cytotoxic T lymphocytes (CTL), confers resistance of infected cells to CTL killing (Adnan et al., 2006).
Despite to the Nef-CD4 interaction that happens when the receptor is already present on the cell surface, the Nef binding to MHC-I takes place early in the secretory compartment. Particularly, Nef with its acidic and polyproline domains interacts with the cytoplasmic tail of MHC-I to form a complex able to recruit and bind AP-1 (Roeth et al., 2004). The formation of this complex creates an alternative route for MHC-I that, instead to be directed to the cell surface, is transported to lysosomes to be degraded, in a process that also in this case involves the interaction with the protein β-COP (Schaefer et al., 2008).

Nef-mediated cell signalling deviation and activation of T-cell receptor (TCR).

In T-cells Nef activates TCR signalling pathways miming what activated by its exogenous stimulators, and thus is able to manipulate T-cell activation by interacting with several proteins downstream the TCR, which include PI3K, Vav or DOCK2-ELMO, small GTPases, Pak2 and PKC (Roeth et al., 2006).
It has been reported that Nef exerts modulatory opposite effects on TCR signalling in infected T-cells depending on its cellular localization. It has been observed, in fact, that Nef promotes T-cell activation when is myristoyled and thus anchored to the membrane while exerts an inhibitory effect on T-cell activation in its non-myristoyled cytosolic form (Baur et al., 1994).
On the basis of the current models, Nef mediates the control of T-cell activation both in a TCR-dependent and independent way. Nef can bind directly the ζ chain of TCR promoting, in concert with other signalling proteins (such as Lck, LAT and Vav), its phosphorilation and activation or can influence the process through the indirect activation of Inositol 1,4,5-trisphosphate receptor type 1 (Manninen and Saksela, 2002).
However it has been hypothesized that the strategy adopted by Nef to lead T-cell activation is in decreasing the threshold of T-cell activation instead to promote the activation of the resting cells (Schrager and Marsh, 1999).
To conclude, activation of T-cells is a necessary event for integrated proviral genome transcription (Zack et al., 1990) and is evident the role of Nef in the regulation of this process.

Regulation of apoptosis.

Nef plays a central role in the regulation of apoptosis during the course of HIV-1 infection, protecting infected cells and inducing apoptosis mainly in bystander non-infected T-cells (Finkel et al., 1995). The relevance of this function is documented by the strong correlation existing between apoptosis and the massive CD4+ T-cell depletion typical of AIDS.
Nef, as already said, activates T-cell and it has been reported by several authors that the activation of T-cell is a central requisite that renders T-cells highly susceptible to apoptosis in a process known as *activation-induced cell death* (Alimonti et al., 2003).
The induction of apoptosis in bystander T-cells can occur both in a direct way in which is involved the Nef interaction with the CXCR4 receptor

(James et al., 2004; Homann et al., 2009), and in indirect way in which Nef increases the expression of FasL on infected cells (Xu et al., 1999). On the other hand, Nef protects infected cells by apoptosis expounding an inhibitory effect on pro-apoptotic proteins such as ASK1, Bad or p53 (Greenway et al., 2002).

1.2.4.   Nef and macrophages.

Several studies have highlighted on the important role that Nef exerts in macrophages that are used as an extremely valid *in vitro* model to better clarify the role of this protein in the HIV-1 infection context.

In macrophages HIV-1 induces the production and release of chemotactic factors able to activate and recruit T-cells to the infection site, rendering them more susceptible to virus attack. Studies carried out by infecting macrophages with HIV-1 M-tropic strains both containing *nef* gene or *nef* deleted have demonstrated that Nef is responsible for the chemokines production observed (Swingler et al., 1999). Indeed, the endogenous expression of HIV-1 Nef in human monocyte-macrophages has been reported to induce production and the release in the macrophages supernatants of inflammatory proteins such as MIP-1α and MIP-1β and other soluble factors (Swingler et al., 1999 Alessandrini et al., 2000). Further, Nef-mediated activation of the *Signal Transducer and Activator of Transcription* STAT-1 has been demonstrated in monocyte-derived macrophages (MDMs) infected with a Δ*env* HIV-1 strain. Interestingly, the STAT-1 activation doesn't occur in MDMs upon infection with a Δ*env*/Δ*nef* HIV-1 strain (Federico et al., 2001). Further studies, conducted by using an adenovirus-based vector to express Nef in macrophages, suggest that Nef is able to hijack the cellular signal transduction pathways intersecting the CD40/CD40L signalling pathway (Swingler et al., 2003).

Recently, on the basis of the fact that extracellular Nef has been found in the serum of infected patients at a significant range of concentration (from 0.5 to 10 ng/ml) (Fujii et al., 1996), the interest has been also focused in exploring the capability of macrophages to internalize Nef, also evaluating its effect on cell signalling (Olivetta et al., 2003).

Particularly, *in vitro* studies carried out in the laboratory where I have performed my Ph.D., have demonstrated that Nef is efficiently internalized by primary human non-infected MDMs and leads to effects very similar to those observed in cells endogenously expressing Nef.

Here Nef mediates the cycloheximide-independent activation of the transcriptional activator NF-kB, the activation of specific MAPKs (*i.e.* ERK1/2, p38, and JNK) and of IRF-3 that is the main regulator of interferon β gene. These events lead to the synthesis and release of several cytokines and chemokines (*i.e.* IL-1β, IL-6, TNFα, MIP-1α, MIP-1β and IFN-β). These factors are in turn able to activate STAT-1, STAT-2 and STAT-3 in autocrine and paracrine manner (Fig. 1.6).
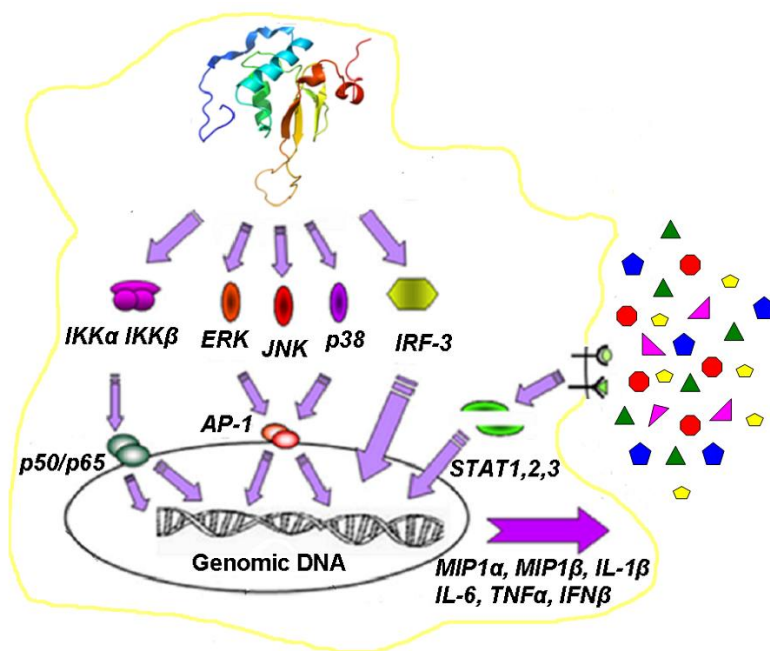

Figure 1.6. HIV-1 Nef mediated signalling in primary human non-infected monocytes derived macrophages (MDMs). Nef is efficiently internalized by MDMs, mediating the activation of NF-kB, specific MAPKs and IRF-3. These events lead to the synthesis and release of several cytokines and chemokines (i.e. IL-1β, IL-6, TNFα, MIP-1α, MIP-1β and IFN-β) able to activate the *Signal Transducers and Activators of Transcription* STAT1, STAT2 and STAT3 in an autocrine and paracrine manner. (Mangino et al, 2007).

These studies has been realized by using a myristoyled recombinant Nef (rNef) and a set of Nef mutants (Olivetta et al., 2003; Mangino et al., 2007).

1.2.5. The Nef anchor domain and its acidic cluster motif as potential targets to block Nef functions.

After the long series of Nef functions earlier described, it is not surprising ask about how to justify the intricate biology of Nef referring to its small size. Clearly, to answer this question we have to dwell in the Nef structure again. Particularly, in this paragraph we will focus on the N-Terminal anchor domain of Nef (also called Nef N-terminal arm), a protein region whose functions range over both structural and signalling tasks. This flexible unstructured region provides a large interaction surface that, in addiction to interact directly with the molecular effectors, seems involved in the conveying of binding molecules to the core domain interaction motifs or to other Nef binding site. Further, the anchor domain could behave as a regulative allosteric region involved in the exposure of specific Nef motifs through the induction of conformational changes (Baugh et al., 2008). It has been also assumed that the anchor domain could act as a spacer to consent, for example, a good juxtaposition between Nef and the membrane target receptors.

More over, the presence of a specific cleavage site for the viral protease between the two principal Nef domains suggests for this site a plausible protease-mediated regulative function (Freund et al., 1994) eliciting also the possibility that these two domains could work independently too, hence the importance of an in parallel modular study of the protein, that is object of this work.

As a matter of fact, the importance of the N-terminal anchor domain is documented also by the presence in this region of the most important sites subjected to post-translational modifications, such as myristoylation and phosphorylation sites, presumably responsible for the allosteric regulations mentioned previously.

Probing in detail the anchor domain it is possible to detect its two principal interaction motifs: the region including the first 22 amino acid residues (required for the membrane binding, mostly positively charged and containing the N term G fundamental for the myristic acid attachment) (Welcker et al., 1998), and the acidic cluster motif (AC).

The Nef acidic cluster is located at the border between the anchor domain and the core domain but is considered as a part of the anchor domain. It

encompasses, in Nef SF2 allele, the four glutamate residues from position 64 to 69 (A_{64}QEEEE$_{69}$) (Fig. 1.7).
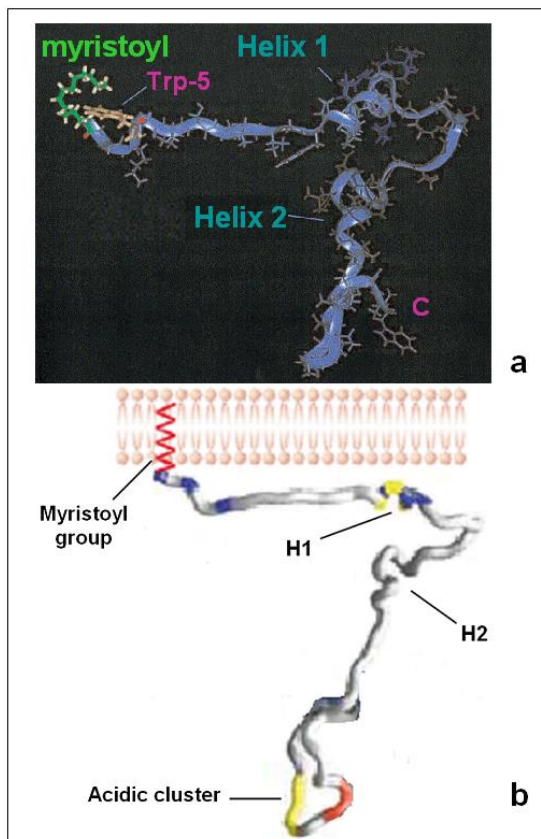

Figure 1.7. Structure of HIV-1 Nef anchor domain. Panel a shows the two principal secondary folded elements: helix 1 and helix 2. Panel b shows the localization of the acidic cluster domain (a: modified from Geyer et al., 1999; b: modified from Arold and Baur, 2001).

One of the first and highly important functions ascribed to the Nef acidic cluster is its involvement in the down-regulation of MHC-I mediated by the

AC binding with PACS-1 and PACS-2. This has been confirmed in studies carried out with Nef acidic cluster mutants, in which the substitution of the four glutamates in four alanines results in the loss of the Nef ability to down-regulates MHC-I (Greenberg et al., 1998). A large number of similar studies on SF2 Nef acidic cluster mutants has been carried out to define the AC role also for other functions of Nef (Baugh et al., 2008).

Recent studies, carried out by my co-workers, validate the involvement of the acidic cluster on the Nef signalling effects observed in macrophages that have been described in the paragraph 1.2.4.

Particularly, it has been demonstrated that the synthesis and the release of inflammatory factors and IFNβ after treatment of MDMs with myristoyled rNef depends on the integrity of the acidic cluster region (Mangino et al., 2011). Particularly, on the basis of previous results indicating that Nef exerts its signalling functions intersecting the CD40 path (Swingler et al., 2003), it has been proposed a direct interaction between the acidic cluster and members of the Tumor Necrosis Factor Receptor-Associated Factor (TRAFs) adaptors family. This interaction is supported by modelling analyses performed using crystallographic data obtained on both TRAF2/4-1BB (Ye et al., 1999) and TRAF6/RANK (Ye et al., 2002) complexes indicating plausible the acidic cluster as a putative binding motif for TRAF2 and TRAF6 *consensus* binding sequences (Fig. 1.8).

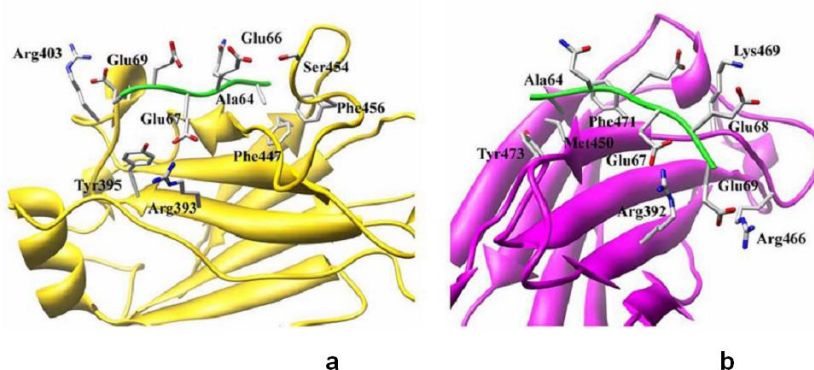### *HIV-1 Nef acidic cluster and TRAFs putative interaction*

Figure 1.8. Schematic representation of the modeled complexes formed by Nef acidic cluster with TRAFs. Panel a) putative interaction between Nef acidic cluster and TRAF2; panel b) putative interaction between Nef acidic cluster and TRAF6 (Mangino et al., 2011).

However, to really evaluate the effective involvement of TRAF in Nef mediated signalling and to confirm putative TRAFs-Nef acidic cluster interactions silencing studies and pull-down experiments have been carried out. Silencing experiments have been performed on human monocytic cell line THP-1 that presents a response to Nef very similar to that observed in MDMs. Three days post transfection of THP-1 with TRAF-2 or TRAF-6 specific siRNA pools, cells have been treated with rNef and the activation of STAT-1 and STAT-2 has been evaluated. As result, no STAT activation has been observed in transfected cells, suggesting that both TRAF-2 and TRAF-6 are involved in Nef mediated signalling.

On the other hand, pull-down experiments confirm the physical interaction between Nef and TRAF-2 even if the same result has not been reported for TRAF-6 (Mangino et al., 2011).

To resume, it has been demonstrated that the acidic cluster is responsible for the Nef signalling effects on macrophages and that in this process the acidic cluster plays a central role. Further, in this function is involved the direct interaction between the acidic cluster and TRAF-2.

Overall, considered the relevance of N-terminal anchor domain in Nef functions and consequently in HIV-1 pathogenesis, finding molecules able to block the main interaction sites of Nef could be of great therapeutic importance.

Starting from the results previously shown, the focus of my Ph.D. work has been directed on the search of Nef acidic cluster binding peptides through phage display methodology. This study has been carried out with two principal purposes: first, better elucidate the molecular mechanisms underlying the Nef interactions with its molecular partners and specifically the Nef/TRAF interactions dynamics and secondly, to find a potential Nef binding inhibitor. A peptide able to compete with TRAF in the acidic cluster binding, in fact, could represent a potential tool to control the Nef effects on the signalling and thus could represent a potential drug to contrast HIV-1 infection.

Phage display of random peptides, that consents to construct libraries with enormous molecular diversity and to select for molecules with predetermined properties, has been used in order to identify acidic cluster peptide ligands.

Further, this project has been also centred on the more generic study of Nef anchor domain and on its effects on cell signalling after internalization in macrophages, as it will be described in the "aim of the work".

1.3. Phage display technology.

Phage display is a powerful laboratory technique largely used in a wide array of application such as the study of protein-protein, protein-peptide, protein-nucleic acids interactions, the individuation of newly enzymatic catalytic sites or activities and providing also the starting point for *in vitro* evolution studies. This methodology, introduced for the first time in 1985 (Smith, 1985), has had a great impact on several biologic fields such as immunology, cell biology, protein engineering, physiology and pharmacology.
Phage display uses bacteriophage as vehicle of peptides, and providing the coupling between phenotype and genotype, consents selection, recover and identification of the interest molecules from highly broad peptide libraries. Phage display, in fact, is centred on the ability of filamentous phage to display capsid protein-fused foreign peptide on their surface.

1.3.1. Biology of filamentous bacteriophage.

Filamentous phage have a single-stranded DNA genome which is encased in a long cylinder approximately 6 nm wide by 900 to 2000 nm in length. The entire genome of these phage consists of 11 genes (Fig. 1.9). Two of these genes, X and XI, overlap and are in-frame with the larger genes II and I (Rapoza and Webster, 1995; Model and Russel, 1988). The arrangement of the genes on DNA is based on their functions in the life cycle of the bacteriophage. Two genes (gII and gX) encode proteins required for DNA replication while a third one (gV) encodes for a protein necessary both at the assembly and DNA level; a group of three genes (gI, gIV and gXI) is involved in the phage assembly process at membrane level, while a last group encodes the capsid proteins. In addition to the regions which encode proteins, is the "Intergenic Region" which contains the sites of origin for the synthesis of the (+) strand (phage DNA) or (-) strand as well as a hairpin region which is the site of initiation for the assembly of the phage particles (packaging signal). A phage expresses about 2700 copies of the major coat protein (pVIII, 50 aa long), and 3 to 5 copies of the minor coat protein (pIII, a 406 aa long) (Russel, 1991).
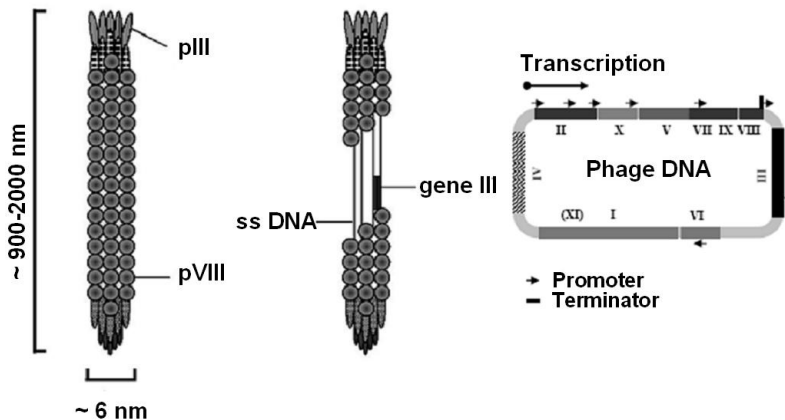
Figure 1.9. Schematic representation of filamentous bacteriophage particle and phage genome organization. The single stranded DNA is encased inside the cylindrical coat. pVIII is the major coat protein presents approximately in 2700 copies per particle, while the minor capsid protein pIII is present at the tip of the phage. The phage genome of 6407 nucleotides contains 11 genes.

The best characterized filamentous phage are M13, fl and fd. These phage infect a variety of gram negative bacteria such as *Escherichia coli* that contain the F conjugative plasmid. Because of their dependence on the F plasmid for the infection these phage are known as Ff phage. The infection starts when one end of the phage interacts with the tip of the F pilus whose gene is located in the F conjugative plasmid. Afterwards, the capsid proteins integrate into membrane and the phage circular single stranded DNA is delivered in the cytoplasm. Here, the complementary strand synthesis, operated by bacterial enzymes, and its conversion in a double stranded super coiled replicative form molecule (RF) occurs. RF provides the template for transcription and translation of the phage proteins. Now, phage proteins together with bacterial enzymes lead the synthesis both of further RF and of new phage DNA molecules. New phage particles are produced when the phage specific single-stranded DNA binding protein pV, reaching a limit concentration, forms complexes with the newly synthesized phage single-stranded DNA. pV-DNA complexes are not converted to RF but assembled to form new phage particles (Fig. 1.10). The assembly of phage occurs at the bacterial envelope and continues until the end of DNA, this

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fact renders Ff phage good cloning vectors because able to contain foreign DNA of big size (range from few to thousand nucleotides). Phage infection doesn't alter significantly the host duplication and results in the production of about 1000 phage particles during the first generation and 100-200 phage particles in the next generations (Model and Russel, 1988).
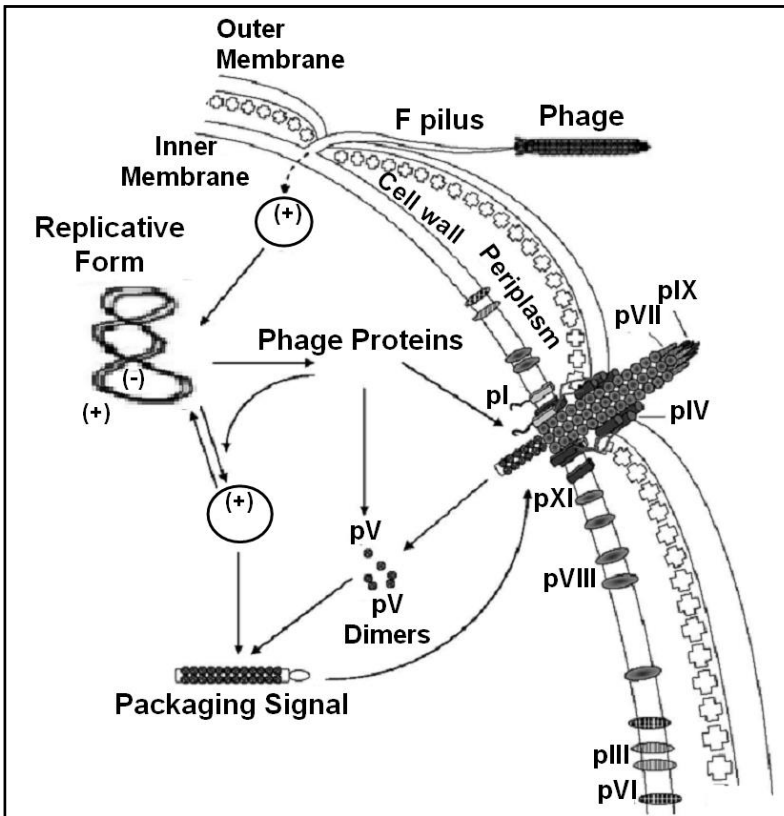

Figure 1.10. Bacteriophage life cycle. After interaction between protein pIII and a tip of the F pilus the phage DNA is translocated inside the cytoplasm. The phage DNA is converted in its replicative form (RF). pV protein phage bound phage ss DNA and direct it to the membrane. Here assembling of new phage particles occurs followed by particles extrusion from the cell.

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1.3.2. Phagemid vector construction and phage display principles.

Phagemid is a particular kind of cloning vector specifically designed for the application of phage display. It consists of a double stranded circular DNA molecule containing the replication origin and the packaging signal (PS) of the filamentous phage together with the origin of replication and gene expression system of the chosen plasmid. Inside the phagemid is possible the cloning of the insert of interest. The phagemid is usually engineered to contain near to the cloning site a sequence codifying for a phage capsid protein in order to obtain after plasmid expression a chimeric protein consisting in the capsid protein fused with the protein of interest. *E. coli* cells are thus transformed with this construct. The infection of the transformed cells with a filamentous helper phage actives the viral origin of replication of the phagemid, further helper phage supplies the proteins for the assembly. As result, the newly synthesized phagemid single stranded DNA carrying the insert of interest is assembled to form new phage-like particles. These recombinant phage carry on their surface the chosen capsid protein (it is usually used the minor capsid protein PIII) fused with the peptide encoded by the foreign DNA, so that phenotype and genotype are linked and, after selection cycles, it is possible to identify the peptide that presents desired properties.

1.3.3. Phage display of random peptides.

How mentioned above, one of the features that render phage display a powerful methodology is the possibility to obtain a peptide with desired properties starting from enormously variegate peptide libraries. The starting point to carry out phage display of random peptides is to design and produce synthetic oligonucleotides fixed in length but with unspecified codons so as to have a highly diverse mixture of random nucleotide sequences (about 10^9 to 10^{12} different sequences). These sequences can be cloned in phagemid vectors as fusion to M13 genes codifying for capsid protein such as PIII to obtain a phagemid library. After infection of the bacterial cells transformed with the phagemid library the viral particles produced represent a phage library of random peptides in which each phage theoretically displays on its surface a different random peptide.

One of the most important application of phage display of random peptide is the selection of random peptides whose bind with a molecule target, and this can be used, as in the case of the project to which we refer here, to

select for a binding peptide that can exerts inhibitory activities on the target molecule.

The selection cycles are performed by using a form of affinity selection known as "bio-panning" (Parmely and Smith, 1988).

The selection through bio-panning consists in the immobilization of the molecule that is object of study on a solid surface and in the incubation of the target molecule with the phage library to start the selection cycles. During the selection cycles phage that display specific binding affinity for the target can be selected, recovered and submitted to further more stringent selection cycles. The process goes on until the finding of the peptide that best resemble that with the ideal features (Fig. 1.11).
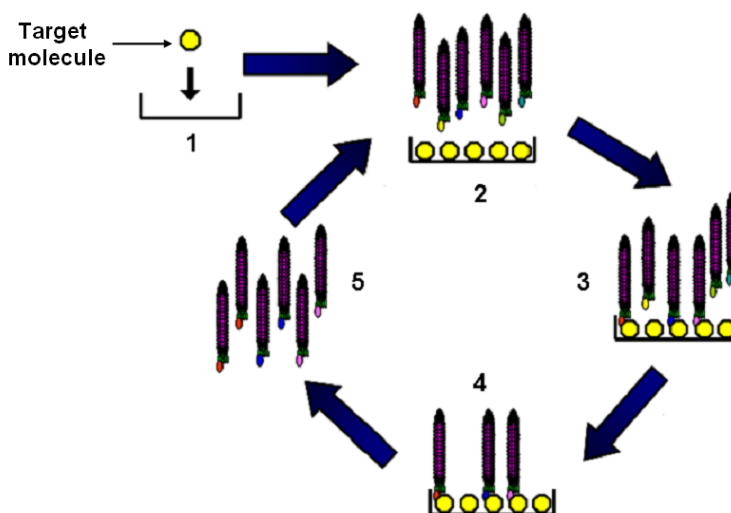

Figure 1.11. Schematic representation of bio-panning cycle. 1: the target molecule is immobilized in the plate; 2: incubation with phage library; 3: selection of the phage that displays peptides with binding affinity for the target; 4: to remove unbound phage several washes are performed; 5: bound phage are eluted, and amplified by infection of susceptible *E. coli* cells. After phage precipitation the enriched phage library is used to repeat the cycle.

2. AIM OF THE WORK

Since, in 1983, HIV was declared the causative agent of AIDS scientists around the world have not yet been able to find a resolutive therapy for this disease that still remains a very significant public health and socio-economic emergency.

This doctoral project falls within the scope of contributing to the elucidation of the action mechanisms of HIV-1 Nef that currently represents one of the most interesting targets for the development of new therapeutic strategies, and further to search for inhibitors of Nef functions to be tested as drugs for HIV disease.

In detail, the study has been focused on the N-terminal anchor domain of Nef that, as already reported in chapter 1, is a region of the protein exceptionally flexible in terms of structure and of molecular interactions and absolves a key role in the Nef activity. Thus, this project aims at studying the role of Nef anchor domain in Nef activities, and at the investigation of the molecular basis underlying the Nef/TRAF interaction evaluating the role of the Nef acidic cluster in this process in order to clarify the enigmatic functions of Nef. Moreover, the project is aimed at searching for inhibitors of Nef functions.

To reach these goals it has been decided to produce and purify a target region of Nef designed as N-Term76-Nef, encompassing the Nef N-terminal anchor domain and the acidic cluster region, to be utilized both to elucidate the role of Nef anchor domain in Nef signalling and as target to phage display experiments.

Using phage display approach peptides will be selected, from a phage random peptide library, that display binding affinity for Nef. Finally, once found a N-Term76-Nef binding peptide it will be used to verify its potential inhibitory effects. The attention will be also focused on the responses of cell culture systems, such as primary human non infected MDMs and human monocytic cell lines to N-Term76-Nef treatment alone or in combination with the wild type protein.

3. RESULTS AND DISCUSSION

3.1. Expression and purification of HIV-1 Nef protein N-terminal anchor domain.

Here we want to acquaint the reader with the experimental procedures that have been set up for obtaining the production and the isolation of the N-terminus anchor domain of Nef protein.

The molecular characterization of the Nef anchor domain, the elucidation of the molecular mechanisms underlying its involvement in the biology of HIV-1 and the search for inhibitors of its functions to be tested as drugs for HIV disease are the goals of this project, and the first step towards the realization of these intentions has been the production of large amounts of this Nef target region.

The protocol here proposed consists of three main successive phases: the amplification of the region of the *nef* gene codifying for the first 76 amino acid residues here called *N-Term76-nef*, the expression of this *nef* region, and the purification of the expressed protein.

3.1.1. *N-Term76-nef* amplification and plasmids construction.

The entire *nef* gene (SF2 allele) was initially cloned inside pCDNA3 plasmid (Mangino et al., 2011). Starting from this construct, the amplification of the *N-Term76-nef* has been carried out by polymerase chain reaction (PCR). The trunked region of the *nef* gene includes the anchor domain (aa 2-61) and a further region that extends over the viral protease cleavage site (between Trp 57 and Leu 58), encompassing the Nef acidic cluster ($A^{64}QEEEE^{69}$) that is considered as an extension of the anchor domain (see chapter 1).

In detail, both the amplifications of *N-Term76-nefWT* (wild type $A^{64}QEEEE^{69}$) and of its mutant in the acidic cluster region *N-Term76-nef4EA* ($A^{64}QAAAA^{69}$) have been performed in order to use this mutant as negative control during the experiments.

The design of three synthetic oligonucleotides (primers) has been based on the sequence of the specific *nef* gene region to amplify and on the type of plasmid utilized as cloning vector (how explained later, two different vectors have been used). The so designed primers are the follows: Nef-PshAI-Forward: 5' ATG GGT GGC AAG TGG TCA 3'; Nef-NotI-Forward: 5' ATA AGA ATG CGG CCG CAA TGG GCA AGT GGT CA

3'; Nef-BamHI-Reverse: 5' AAC GAA TGG ATC CTA TAA AGG TAC CTG AGG TGT 3'.

The PCR reactions led to the amplification of four PCR products, deriving from the use of the two different forward primers and the two different DNA templates (*nef WT* and *nef 4EA* genes) (Fig. 3.1).
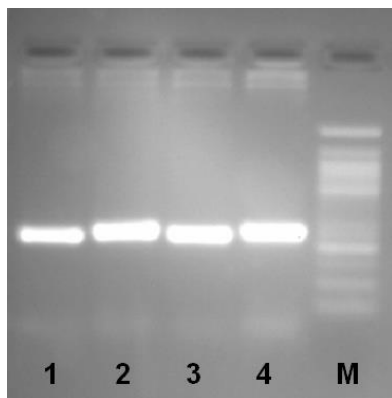

Figure 3.1. Amplification by PCR of *N-Term76-nefWT* and *4EA* gene region. Lane: 1 and 2 *N-Term76-nefWT* PCR products, lane 3 and 4 *N-Term76-nef4EA* PCR products. Lane M: 50 bp DNA Ladder.

How mentioned above, two different plasmids have been used for the cloning of the amplified PCR products in order to have the possibility to use different methodologies of purification on the basis of the experimental requirements. Both *N-Term76-nefWT* and *N-Term76-nef4EA* PCR products have been cloned inside pET-14b (previously digested with PshAI and NotI restriction enzymes) or inside pET-42-CM (previously digested with PshAI and BamHI) expression vectors (Fig. 3.2).
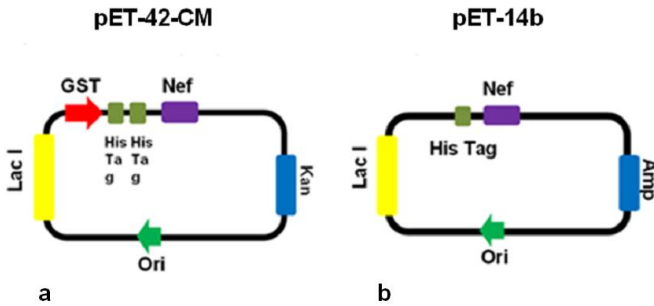
Figure 3.2. Schematic representation of the plasmids used as expression vectors. Panel a: pET-42-CM carrying two different tags: GST and two hexa histidine-tag (His-Tag) upstream *N-Term76-nef.* Panel b: pET-14b carrying only a His-Tag upstream the cloning site.

pET-14b plasmid carries an N-terminal His-Tag sequence followed by a thrombin site and three cloning sites. On the other side, pET-42-CM vector, that has been modified from the original one (pET-42b) by adding two His-tag sequences upstream the cloning site and the not translating DMY sequence, that is used to check the enzymatic double digestion in the plasmid, consents a high expression level of the inserted sequences fused to the 220 aa GST (glutathione S-transferase) Tag protein (Fig. 3.3). As results, two different constructs have been realized which are genetically equipped for the expression of Nef anchor domain flanked by two different tags (His sequences and GST protein).
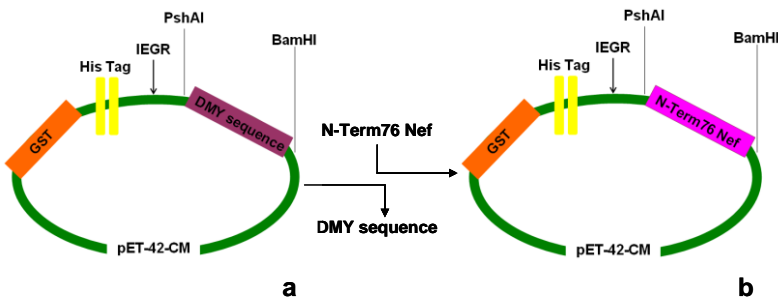

Figure 3.3. Schematic representation of pET-42-CM vector. After digestion with PshAI and BamHI the DMY sequence (a) is removed and N-Term76-Nef gene sequence is cloned inside the plasmid (b).

3.1.2. Expression of the recombinant N-Term76-Nef.

The ligation reactions of the PCR products with the appropriate expression vectors have resulted in four different plasmid constructs (pET-14b-*N-Term76-nefWT*, pET-14b-*N-Term76-nef4EA*, pET-42-CM-*N-Term76-nefWT*, pET-42-CM-*N-Term76-nef4EA*) that have been first used to transform *E. coli* DH5α strain. After transformation, PCR colony and plasmid extraction have been performed and, after sequencing, the plasmid DNA of the positive clones has been used to transform BL21 expression competent strains.

In order to obtain the expression of the recombinant proteins, *E. coli* BL21DE3 cells have been first used. However, because of the poor yield of expressed protein obtained using this strain, it has been soon replaced with its derivative *E. coli* Rosetta strain in order to improve protein expression. Nef protein, in fact, is normally expressed in eukaryotic cells, and *E. coli* Rosetta strain has been designed to enhance the expression of eukaryotic proteins whose genes contain codons rarely used in *E. coli.*

The expression of the proteins has been obtained adding IPTG 0.1 mM in the BL21 Rosetta growth medium. The expression of the cloned genes, in fact, is under the control of the *lac* promoter activated by IPTG. In order to avoid the accumulation of the expressed proteins inside the inclusion bodies, the induction temperature has been maintained at 25 °C. Thereafter a part of the IPTG induced bacterial culture has been subjected to a preliminary check to verify the successful protein expression, and to evaluate the cellular localization of the protein after expression. During this procedure the bacterial pellet, resuspended in appropriate buffer, has been subjected to five cycles of freezing in liquid nitrogen followed by rapid thawing in hot water. The resulting viscous solution has been sonicated and the two resulting fractions (*i.e.* the cytosolic soluble fraction and the fraction containing inclusion bodies including membranes) have been separated trough centrifugation. These fractions have been tested by SDS-PAGE and western blot analysis. As results the protein was successfully expressed and almost completely localized in the soluble fractions (cytosol) while only a minor part was localized in the inclusion bodies (Fig. 3.4).
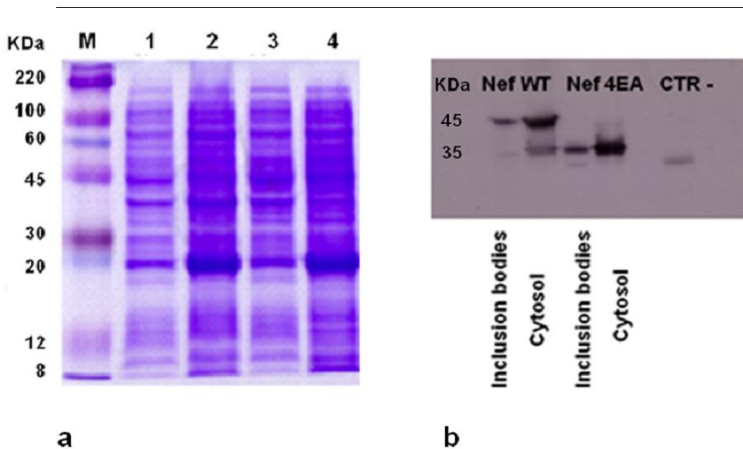
Figure 3.4. Localization of the recombinant expressed proteins after cell extraction. The figure shows the GST-N-Term76-Nef recombinant proteins after extraction procedure: the recombinant proteins are located almost exclusively on the soluble fractions (cytosol). Similar results have been obtained for His-N-Term76-Nef WT and 4EA in pET-14b (data not shown). Panel a: 12% SDS-PAGE. Lane 1 and 3 N-Term76-Nef WT and 4EA (respectively) inclusion bodies fractions. Lane 2 and 4 N-Term76-Nef WT and 4EA (respectively) soluble fractions (cytosol). Panel b: corresponding Western Blot performed by using a primary antibody directed against HIV-1 Nef. The last lane represents the negative control (fractions derived from cellular lysate of non inducted BL21 cells). How it is possible to observe in panel 2, migration profile differs from Nef WT to 4EA because of the acidic cluster mutation (for further details see Baugh et al., 2008).

On the basis of these results it has been carried out the procedure for the extraction of the proteins from the soluble fractions.

To resume, as result of the different plasmids utilized to transform the bacterial competent cells it has been obtained the expression of both N-Term76-Nef WT and 4EA that carries the GST tag followed by two his tags (GST-N-Term76-NefWT and GST-N-Term76-Nef4EA) and the expression of the WT and 4EA recombinant proteins carrying only one series of 6 histidines as tag (His-N-Term76-NefWT and His-N-Term76-Nef4EA in pET-14b) (Fig. 3.5).
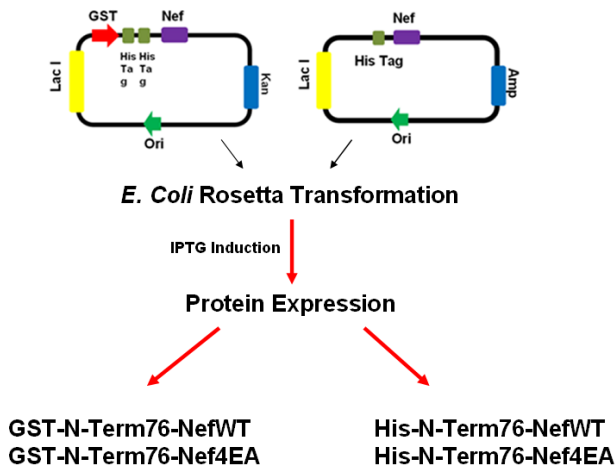
Figure 3.5. Expression of recombinant proteins. After single transformations of BL21 Rosetta strain with pET-42-CM-N-Term76-NefWT and 4EA and with pET-14b-N-Term76-Nef WT and 4EA, proteins expression has been induced by IPTG. As result, two different tagged proteins (GST-N-Term76-Nef and His-N-Term76-Nef) have been obtained both for the wild type and for its mutant on the acidic cluster region (4EA).

These recombinant proteins have been mechanically extracted from cells trough cell lysis with alumina that is a less aggressive procedure than the treatment with liquid nitrogen and heat described above, and doesn't affect the overall structure and functionality of the extracted proteins.

3.1.3 N-Term76-Nef purification procedures.

For the aims of the project an extremely high level of Nef anchor domain purity is required, and the choice to produce two differently labelled N-Term76-Nef proteins resides also in the necessity to individuate the best purification method.

The His tag allows to use as purification methods the immobilized metal ion affinity chromatography (IMAC) because of the great binding affinity to poly-histidine exhibited by several metal ions such as nickel and cobalt. On the other hand GST tag allows the purification of the protein through glutathione affinity chromatography. The two procedures are based on the same principle of purification however may lead to different results in terms of purity of the sample, and can be more or less advantageous depending on the case.

His-N-Term76-NefWT and 4EA have been purified by IMAC. Although these recombinant proteins present the advantage to have a small tag that doesn't require its removal at the end of the purification process, their purification by IMAC has been resulted in a great presence of contaminants in the eluted fractions due to the poor specificity of IMAC. In contrast, the purification of GST-N-Term76-NefWT and 4EA fusion proteins by glutathione affinity chromatography, that is more selective than IMAC, has allowed the recovery of fusion proteins with less contaminants. Also if previous works have described that the size of GST tag could affect the overall structure of the full length Nef protein (Finzi et al., 2003), the Nef anchor domain is an unstructured region with no global tertiary fold (Geyer et al., 1999) so that, in this case, GST tag would not negatively interfere.

Thus, GST-N-Term76-NefWT and 4EA fusion proteins have been used and purified by in batch glutathione affinity chromatography. For our purposes, such methodology presents several advantages respect to the in column purification such as the possibility to treat a great volume of bacterial lysate without any risk to saturate and/or occlude the column. In this methodology the stationary phase (glutathione-sepharose matrix) is not packed in the column but suspended in the mobile phase (the bacterial lysate, containing the GST-fusion proteins, suspended in the equilibration buffer) and the entire purification procedure takes place inside a beaker.

The affinity binding has been achieved shaking the above described mixture for an opportune incubation time (see material and methods section). The separation of the mobile phase (flow through) from the solid phase (which is now linked to GST-fusion proteins) has been obtained by centrifugation, as well as washing and elution steps. In detail, washes have been repeated

until the value of OD_{280} resulted near to zero, and this value corresponds to the absence of contaminants in the flow through.

Before proceeding to GST tag cleavage, that has been carried out on the GST-N-Term76-Nef still bound to the stationary phase, a preliminary check to evaluate the purification trend has been carried out. After binding GST-N-Term76-Nef has been eluted from the stationary phase. The elution procedure has been performed by incubating the glutathione-sepharose matrix with a buffer (elution buffer) containing an excess of free glutathione molecules that competes with GST-fusion proteins for the binding to the matrix.

As it is shown in figure 3.6, the binding of GST-N-Term76-Nef to the stationary phase has been successful and the matrix has been able to retain the target protein almost completely (Fig. 3.6, lane 2). Further, after elution, almost all of the GST-N-Term76-Nef initially linked to the matrix has been recovered (Fig. 3.6, lane 3,4,5).
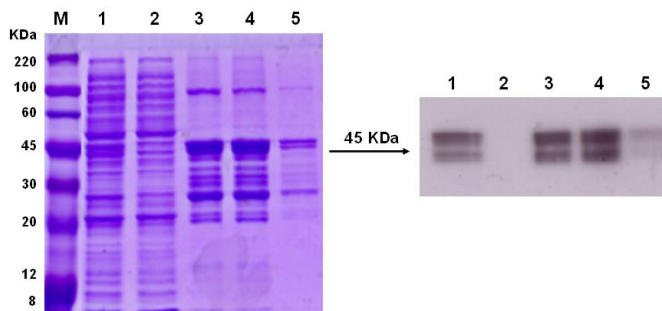

Figure 3.6. Glutathione-sepharose affinity chromatography preliminary check. SDS-PAGE (panel a) and Western Blot (panel b) assays to evaluate the purification trend have been carried out. What follows refers to both panel a and b, lane 1: cellular lysate before the in batch chromatography that reveals the presence of GST-N-Term76-Nef (MW about 45 KDa). Lane 2 cellular lysate after incubation with the gluthatione-sepharose matrix where it is possible to observe the capacity of the matrix to retain almost all the target protein, as documented by the absence of the proteins in the supernatant. Lanes 3, 4 and 5: three sequential elution steps, in which the GST tagged N-Term76-Nef is completely recovered from the stationary phase.

After this preliminary check, the purification and the cleavage of the fusion proteins have been carried out.

The first step of the whole procedure is very similar to that carried out for the check and consists on the immobilization of GST-N-Term76-Nef to the stationary phase, followed by a series of washes to remove the contaminants (as described above). The second step, that contrary to the check procedure doesn't foresees the elution step, consists in the cleavage of the fusion proteins from their GST tag still bound to the glutathione-sepharose matrix. The cleavage has been achieved using the site-specific protease Xa (coagulation activated factor X) that specifically cuts the amino acid sequence IEGR presents in the expressed GST-N-Term76-Nef fusion proteins. The obtained N-Term76-Nef WT and 4EA (obviously separately purified) have been recovered under non-denaturing conditions by centrifugation (Fig. 3.7) (for further details see materials and methods section).
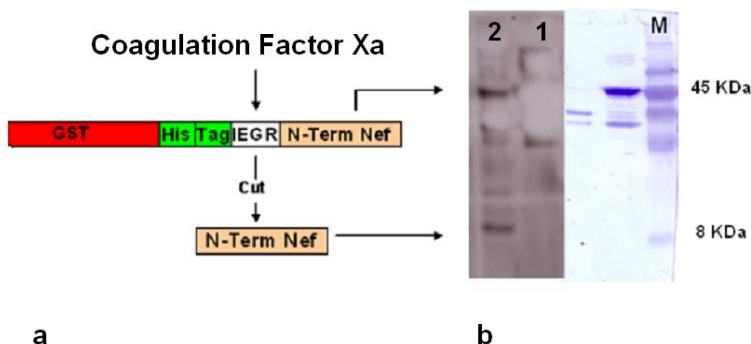

Figure 3.7. GST-N-Term76-Nef digestion by Coagulation Factor Xa. Panel a: schematic representation of the recombinant GST fusion protein and factor Xa cleavage at the IEGR sequence. Panel b: Western Blot and SDS-PAGE of the purified eluted fractions. Lane 1: purified GST-N-Term 76 eluted before the Xa cleavage. Lane 2: N-Term76-Nef (after the cut). Western Blot has been performed using a primary antibody directed against both GST and HIV-1 Nef. In lane 1 are shown the ghost bands derived from GST-fusion protein. In lane 2 the ghost band, that migrates lower than 45 KDa, is due to the GST without N-Term76-Nef which is represented by the band at 8 KDa.

It is notable that the in batch chromatography presents several advantages as well during the cleavage step. In fact, it is possible to use less reaction volume with respect to the in column cleavage allowing the use of a less amount of proteolytic enzyme. Further, the reaction can be carried out longer and at a more suitable temperature. Altogether these conditions have been resulted in a good protein recovery. After the cleavage, the elution step has been performed in order to elute the GST. The entire procedure is shown in figure 3.8.
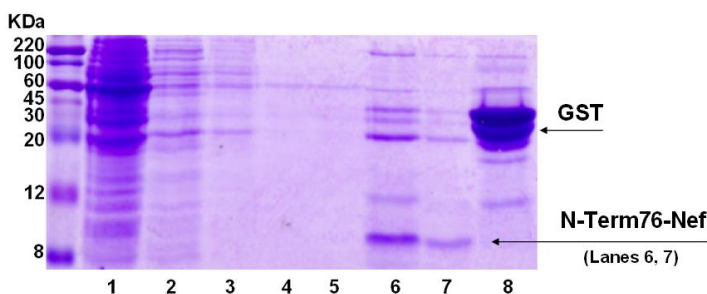

Figure 3.8. SDS-Page of in batch glutathione-sepharose affinity chromatography, in batch factor Xa cleavage and GST elution. Lane 1: cellular lysate before incubation with the stationary phase. Lanes 2-5: washes carried out to eliminate unbounded proteins (flow through). Lane 6 and 7: in batch factor Xa digestion that shows the cleaved N-Term76-Nef of about 8 KDa. Lane 8: GST elution; notably the GST alone migrates at an apparent molecular weight of about 30 KDa that is lower than that observed with the GST-N-Term76-Nef that is about 45 KDa.

However, glutathione-sepharose affinity chromatography alone has not been enough to grant a high purity level of the N-Term76-Nef. In order to eliminate from the eluate the Xa factor and the rests of contaminants a second step of purification has been necessary. This has been carried out by using size exclusion chromatography (*i.e.* gel filtration) that allowed the isolation of the small sized N-Term76-Nef (~8 KDa) from the residual bacterial contaminants that are all of a greater size, and has been resulted in the recovery of a highly pure target protein.

The target protein has been eluted at 0.25 ml/min flow rate after 70 minutes corresponding at 17 ml of volume. This value reflects the theoretical recover time of the protein on the basis of the molecular size, the flow rate and the column parameters, and matches with the presence of a 17 ml pick on the FPLC graphic (Fig. 3.9).
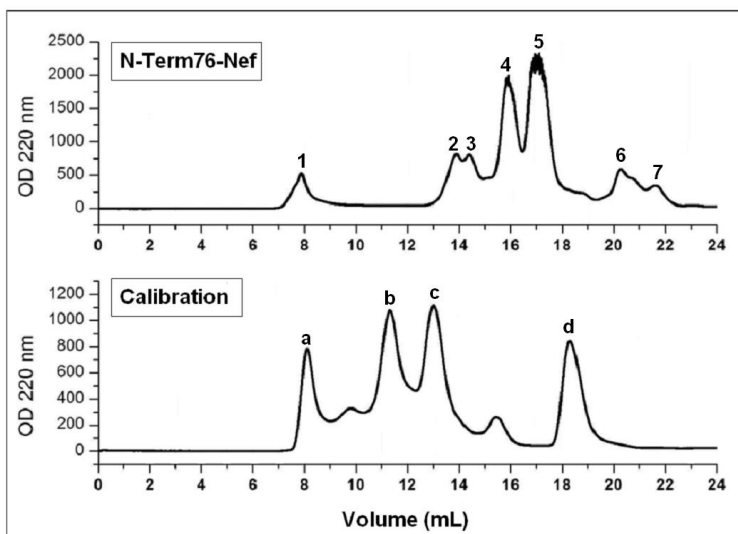
Figure 3.9. Gel filtration chromatography FPLC graphic. The figure shows the trend of size exclusion chromatography with the picks corresponding to the different eluted molecules. On the top is the gel filtration profile of the sample derived from the glutathione-sepharose affinity chromatography containing the cleaved N-Term76-Nef domain and its corresponding pick eluted at a volume of 17 ml (pick 5). On the bottom is shown the calibration curve obtained by the gel filtration of a heterogeneous solution containing compounds with known molecular weight: a: Ferritin, 450 KDa; b: Immunoglobulin (IgG), 150 KDa; c: Bovine Serum Albumin (BSA), 69 KDa; d: Cytochrome C, 12.4 KDa.

Further, the totality of the fractioned picks have been separately recovered and analyzed by SDS-PAGE to evaluate the presence and localization of N-Term76-Nef. To confirm the previous data, the Nef positive fraction coincides with the 17 ml pick 5 (Fig. 3.10). Further, SDS-PAGE also shows the high level of purity of the N-Term76-Nef obtained after gel filtration, as confirmed by mass experiments (data not shown).
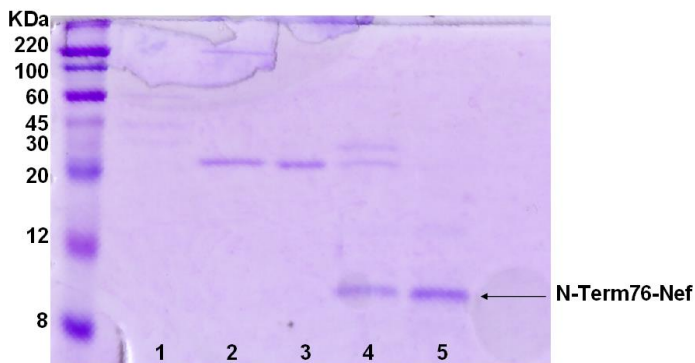
Figure 3.10. Gel filtration SDS-PAGE. The fractions corresponding to each pick from gel filtration have been separately analyzed by SDS-PAGE. The numeration of the lanes corresponds to the picks from 1 to 5. Lane 5 that corresponds to pick five (Fig. 3.10) shows the presence of N-Term76-Nef. Fraction 4 (lane 4) also contains Nef due to the partial overlap of picks 4 and 5 (see Fig. 3.10). However, due to its major purity only fraction 5 has been considered for the next experiments.

The concentration of the recovered N-Term76-Nef proteins has been estimated reading the absorbance of the sample at 280 nm and at 260 nm to correct for nucleic acid contaminations: Concentration (mg/ml) = (1.55 x A_{280}) - 0.76 x A_{260}). As result, starting from four liter of bacterial culture, we have obtained a total yield of 0,8 mg of purified N-Term76-Nef.

3.2. HIV-1 Nef anchor domain: its involvement in cell signalling and strategies to block its activities.

Could HIV-1 Nef anchor domain by itself has an independent role in HIV-1 infection context? Based on this assumption, is it possible to speculate on a so far unexplored new mechanism of action of Nef protein? Further, what is the real significance of the Nef acidic cluster region? All these questions, arising from new experimental findings, will be discussed in the following sections in which will be also described possible strategies aimed at blocking the functions of Nef.

3.2.1. Treatment of MDMs cells with exogenous recombinant HIV-1 Nef full-length protein: state-of-the-art.

As already explained in chapter 1, recent studies have demonstrated that Nef protein full-length is efficiently internalized by human non infected monocyte-derived macrophages (MDMs) and hijacks cellular signalling pathways. In particular it has been observed a rapid activation of NF-κB, MAPKs and IRF-3 that leads to the production of a set of chemo-cytokines ultimately resulting in the activation, by tyrosine phosphorylation, of certain STATs family members such as STAT1, STAT2, and STAT3 (Mangino et al., 2007). Nef seems, in fact, to intersect the CD40 pathway miming the events downstream its cascade signalling (Swingler et al., 2003).

Further studies, similarly carried out by treating MDMs with exogenous recombinant Nef full-length mutants (*i.e.* G2A lacking the myristoylation site and particularly 4EA carrying Glu to Ala substitution within the Nef acidic cluster $A^{60}QEEEE^{65}$), highlighted on the importance of the integrity of the acidic cluster region for Nef signalling activity that, as shown in figure 3.11, is required for STATs activation. Particularly, a TRAFs involvement in Nef mediated signalling has been reported, and Mangino et al. (2011) have demonstrated as possible the interaction between Nef and TRAF2 and it is highly probable that the consensus binding sites on Nef encompasses the AQEEEE acidic cluster (see chapter 1).
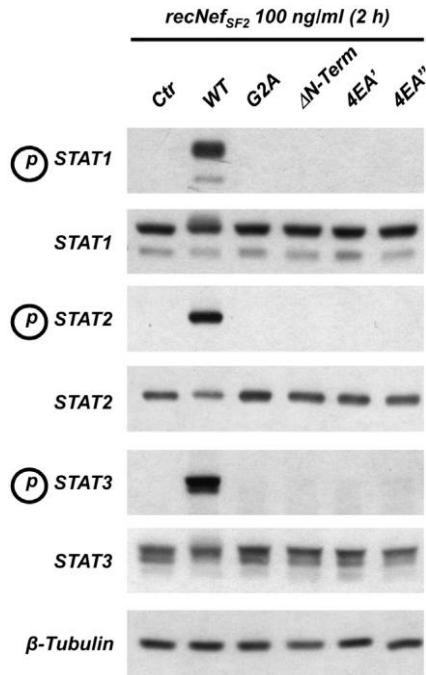
Figure 3.11. Tyrosine phosphorylation of STAT1, STAT2 and STAT3 requires the Nef Acidic Cluster. MDMs from four independent healthy donors have been treated with 100 ng/ml of myr+ wt as well as with DN-Term, G2A or myr+ 4EA recNef. Western blot assays have been used to evaluated STATs phosphorylation and steady-state expression levels of STAT1, STAT2 and STAT3. β-tubulin steady-state expression level has been used as a loading control. (Modified from Mangino et al., 2011).

3.2.2. Treatment of THP-1 cells with HIV-1 recombinant N-Term76-Nef.

Preliminary experiments have been done using human cell cultures in order to verify if the trunked N-Term76-NefWT protein could affect cell signalling as the Nef WT full length protein does.

Experimental procedures: cells differentiation methodologies.

THP-1 cell line (human leukemic monocytes) has been used as macrophage-like cell model. THP-1 cells have been treated with phorbol myristate acetate (PMA) to induce macrophage differentiation. The concentration of PMA initially used was 200 nM, however we have recently modified the differentiation protocol since we observed stable differentiation also treating cells with 10 nM PMA. This allowed a major cells viability and avoids aberrant PMA-induced gene expression resulting in more physiological conditions. Park et al. (2007), in fact, reported that higher PMA concentrations might upregulate the expression of some genes in differentiated macrophages, which could superimpose the increase of gene expression induced by other stimuli. Moreover, we have also set up another protocol to completely eliminate the PMA contribution. It is known, in fact, that PMA induces a massive production of M-CSF (machrophage colony-stimulating factor) by THP-1 cells, and M-CSF itself induces monocyte-machrophage differentiation. Thus, we have in parallel employed a diverse differentiation procedure by using a THP-1 conditioned medium (CM1). THP-1 cells have been first treated with 150 nM PMA and, after differentiation, the supernatant has been removed and cells have been repeatedly washed with PBS in order to eliminate any residue of PMA. Fresh RPMI medium has been added to the differentiated cells and has been maintained in cell contact for five days. The supernatant has been recovered and filtered and the so obtained conditioned medium (CM1) has been used to efficiently differentiate new THP-1 cells. HPLC analysis of samples derived from CM1 confirm the presence of M-CSF in the medium that is otherwise absent in another conditioned medium derived from cells initially differentiated with 10 nM PMA. Further, the supernatant (CM2) derived from cells differentiated with the conditioned medium (CM1) doesn't induce THP-1 differentiation (see materials and methods chapter).
To conclude PMA 10 nM and MC1 have been used to induce THP-1 macrophage differentiation. The experiments have been performed in parallel by using these two different methodologies, and the results obtained

in terms of cellular response to Nef stimuli in both the cases have been found comparable (see below). However, its important to remark that M-CSF is a cytokine that *in vivo* directs hematopoietic stem cells toward macrophages differentiation (Stanley et al., 1997). Therefore, its use for THP-1 differentiation could lead to a more physiological *in vitro* model.

N-Term76-Nef induces STATs activation in THP-1 cells.

In the paragraph 3.1 we have described the purification procedures that have been performed to obtain the N-Term76-Nef recombinant protein that is shown in figure 3.12.
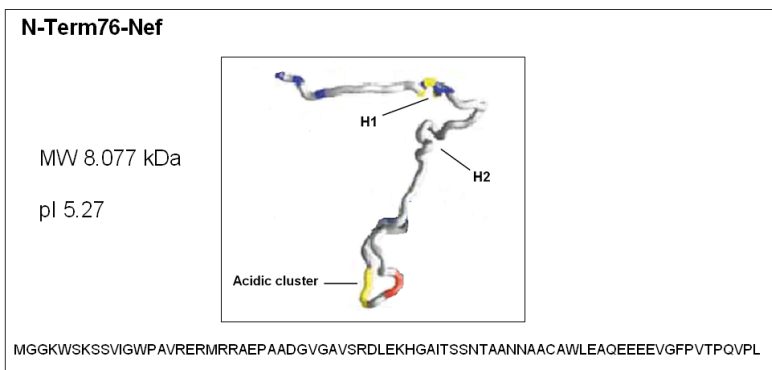

Figure 3.12. N-Term76-Nef. The figure shows the N-Terminal anchor domain followed by the acidic cluster region. The N-Term76-Nef is 8 kDa in size and its 76 aa sequence is shown on the bottom of the figure.

The recombinant purified N-Term76-Nef has a molecular weight of about 8 KDa, it is not myristoyled and has been used to treat THP-1 cells.

THP-1 cells have been first induced to differentiate into macrophages using 10 nM PMA or 50% CM1 at 37 °C as described in meterials and methods section. The treatment with N-Term76-Nef has been performed five days after induction. Cells have been treated with N-Term76-Nef at two different final concentrations. The first concentration has been chosen to be equimolar to Nef full-length that is of 100 ng/ml (4 nM). Based on the molecular weight of N-Term76-Nef this concentration has been fixed at 29,6 ng/ml (4 nM). Furthermore, differently from the Nef full-length, N-Term76-Nef is not myristoyled, therefore we have also treated cells with N-Term76-Nef at a concentration 10 times higher, 40 nM, to possibly improve the rate of its activity. Indeed we know from prevoious data that the myristoylated Nef full length protein works about 10-fold better than the non myristoylated full length protein (Mangino et al 2007) that is possibly myristoylated by the cells as demonstered by the lack of activity of the G2A mutant protein. Two different controls have been carried out: a negative control (untreated, differentiated THP-1), and a positive control in which cells have been treated with 100 EU/ml of bacterial lipopolysaccharide (LPS). LPS interacting with Toll-Like receptor 4 (TLR4) leads to NF-kB, p38, JNK and IRF-3 functional activation, inflammatory cytokines production and STATs activation.

N-Term76-Nef has been tested to evaluate LPS contamination. Although the samples resulted endotoxin free, they have been incubated with low amount of Polymyxin B (PMX B) that is well known to inhibits LPS activity to avoid possible signalling activity of undetectable amount of contamination (see Matherials and Methods).

Thus, THP-1 cells have been treated with N-Term76-Nef for 4 hours, cellular lysis has been carried out and the lysates have been analyzed by western blot assays to evaluate STATs tyrosine phosphorylation (see material and methods) (Fig. 3.13).
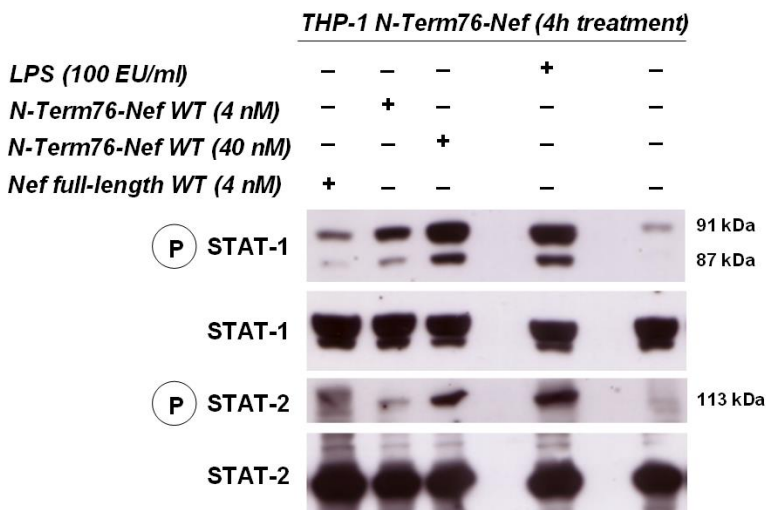
Figure 3.13. Treatment of THP-1 macrophages with N-Term76-Nef WT. Western blot assay reveals the N-Term76-NefWT-induced tyrosine phosphorylation of STAT-1 and STAT-2 and the steady-state expression level of STAT-1 and -2. LPS 100 EU/ml has been used as positive control.

The results show that N-Term76-Nef WT is able to activate STAT-1 and -2 as Nef full-length WT does. After 4 hours of treatment, the activation of STAT-1 and STAT-2 is induced by both the tested concentration of N-Term76-Nef (4 and 40 nM) and increases in a dose-dependent manner.

Nef has been described to form homodimers and oligodimers both *in vitro* and within HIV host cells (Poe and Smithgall, 2009), therefore we have also investigated Nef-Nef interactions in order to evaluate possible competitive, additive or synergic effects between N-Term76-Nef and Nef full-length protein. To this purpose, THP-1 macrophages have been co-treated with N-Term76-Nef and Nef full-length WT. Particularly, N-Term76-Nef and Nef full-length have been mixed and then used to treat cells. Nef full-length has been used at 100 ng/ml (4 nM), while the concentration of N-Term76-Nef has been for the first condition 29.6 ng/ml (4 nM) and for the second condition 296 ng/ml (40 nM), also in this case samples have been pre-

treated with PMX B before addition to the cell cultures. After 4 hours of treatment, cells have been tested to evaluate STATs activation (Fig 3.14).
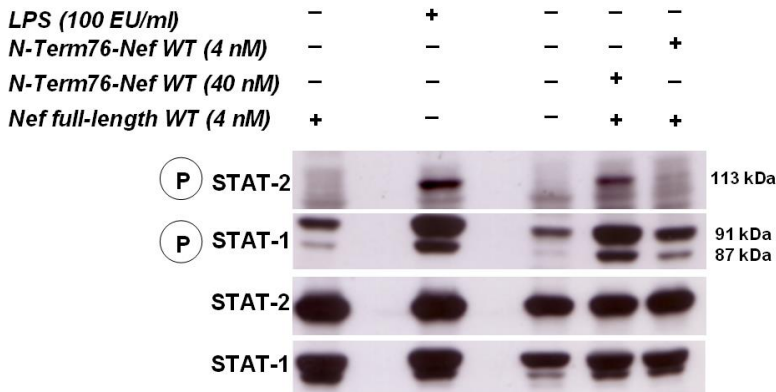
Figure 3.14. THP-1 macrophages co-treatment with N-Term76-Nef WT and Nef full-length WT. Western blot assays have been performed to evaluate STAT-1 and STAT-2 tyrosine phosphorylation after co-treatment with full-length and N-Term76 WT Nef proteins.

As results, N-term76-Nef WT and Nef full-length co-treatment lead to a cellular response very similar to that observed when cells are treated with N-Term76-Nef or with Nef full-length separately, suggesting that, in these experimental conditions the presence of both N-Term76-Nef and Nef full-length doesn't affect the signalling.

3.2.3. Assuming a novel mechanism of action of Nef: a possible role of HIV-1 protease in regulating Nef activity.

The finding that N-Term76-Nef alone is able to induce the same response mediated by Nef full-length on macrophage cellular systems certainly clarify the importance of the anchor domain in Nef-mediated signalling, eliciting the evidence that the core domain is not required for this process. Importantly, this suggests the possibility that the two principal domains of Nef could work independently too. It is known, in fact, that Nef contains a cleavage site for the viral protease between Trp 57 and Leu 58 amino acid residues. *In vitro* studies have been reported that after protease cleavage Nef protein is fragmented in its two principal domains. This cleavage site is well conserved and it has been already hypothesized a possible biological regulatory significance (Freund et al., 1994; Schorr et al., 1996).

Here we want to speculate on the possibility that the great array of phenotypes that emerge after Nef exposure both in *in vitro* cellular systems and in a HIV infection context could be something more than the result of Nef space-temporal dependent multiple interactions, extending the Nef cycle hypothesis (see paragraph 1.2.2.) to the prospect that Nef protein activity could be actively and elegantly regulated also by the viral protease cleavage, and thus directed to exert different functions depending on the viral cycle stages. Particularly Nef could affect distinct cellular pathways according to its full-length or fragmented form as well as could be regulated just after the cleavage. In this last case, according to the Nef cycle, Nef could hide the cleavage site in its cytosolic closed conformation to expose it when, once bound to the membrane, adopts the open signalling conformation.

Another question is on the significance of the acidic cluster region that, as already mentioned, is required for the Nef full-length mediated signalling observed in macrophages and possible involved in TRAFs interaction. The approaches to answer this question will be in part discussed in conclusion and perspective section and in the third part of this chapter in which we will discusses the strategies that we have been set up to find binding peptide of Nef interaction sites/cellular partners (*i.e.* acidic cluster/TRAFs).

3.3. Searching for Nef peptide inhibitors: Phage display experiments.

This third part of the chapter will describe the phage display of random peptides that has been used as tool to find peptides exhibiting binding affinity for the principal interaction sites of N-Term76-Nef WT with particular reference to the Nef acidic cluster. This study attempts both to better characterize the interaction sites of Nef and to find binding Nef functions inhibitors.

3.3.1. Design and production of synthetic oligonucleotide random library.

The first step for the construction of random peptide libraries consists in the design of synthetic degenerate oligonucleotides that will be then cloned upstream gIII (M13 gene codifying for pIII minor capsid protein) in a phagemid to obtain libraries of peptides expressed as N-terminal fusions to pIII.
We decided to produce a 50 amino acid long random peptide library starting from the design of a 150 bp long DNA library. The DNA library has a codon scheme NNK, where N is an equimolar mixture of all four bases and K is either G or T. This scheme uses 32 codons to encode all 20 amino acids and 1 stop codon (TAG), yielding an acceptably low frequency of stop codons when used to encode short polypeptides. The random nucleotides, encoding 50 amino acids, are flanked by fixed residues that are necessary for annealing, cloning and recovery.

3.3.2. Construction of phagemid vector and phagemid library.

The phagemid pIII-DMY-His-tag has been used for the cloning of the random DNA library constructed as described above.
pIII-DMY-His-tag is a derivative of pHEN phagemid and differs for the presence of a His-tag sequence, of the DMY sequences and factor Xa cleavage site sequence (Xa). The DMY sequences (738 bp long), containing 3 stop codons in the 3 different reading frames, allows a visual control during the cloning step and is helpful to reduce the production of non-recombinant fusion proteins in case of phagemid self-ligation (Fig. 3.15).
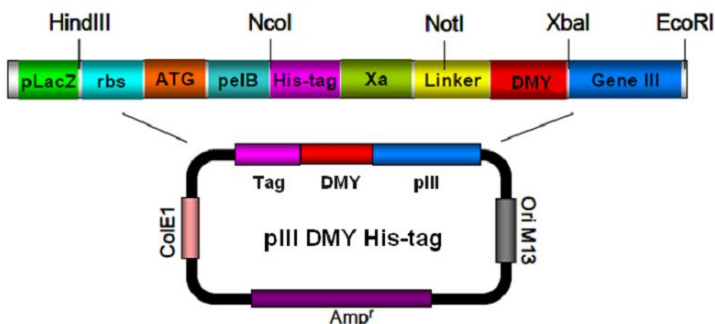
Figure 3.15. pIII-DMY-His tag phagemid. The pIII-DMY-His tag phagemid has been used for the cloning of the oligonucleotides random library. The two cloning sites NotI and XbaI surround DMY sequence. After digestion and ligation steps, DMY sequence is replaced by the foreign DNA insert. Gene III is located downstream the cloning sites to allow the expression of random libraries of peptides fuse to pIII phage protein.

The 150 bp oligonucleotide random library, produced as described in paragraph 3.3.1, has been amplified by PCR using F-AMPLI-LIB-NotI and R-AMPLI-LIB-XbaI-BamHI primers (see material and methods section).

After PCR reaction the cloning step has been carried out. PCR products and pIII-DMY-His-tag phagemid have been digested with NotI and XbaI restriction enzymes and the reaction of ligation has been performed. In order to obtain the library, the phagemids have been used to transform *E.coli* cells (XL1Blue MRF' strain). Cells have been transformed by electroporation in order to obtain a high efficiency of transformation and thus to obtain a largely representative phagemid library. As result, it has been obtained a phagemid library with a complexity of about 10^4 different random sequences per milliliter of the recovered phage solution. From phagemid library, 10 sequences of the phagemid library have been analyzed to verify their correctness and randomness. The majority of the sequences, about 80%, were found to be correct, the remaining part of the sequences consisted of either not in-frame sequences or vectors with no random sequences.

3.3.3. Production of phage library.

For the production of phage libraries the 3+3 monovalent phage system has been used which allows to obtain phage particles containing the phagemid and a single random peptide fused to the minor capsid protein pIII. This model provides only one pIII fusion protein on the possible five allowing the infectiveness of the phage (Fig. 3.16).

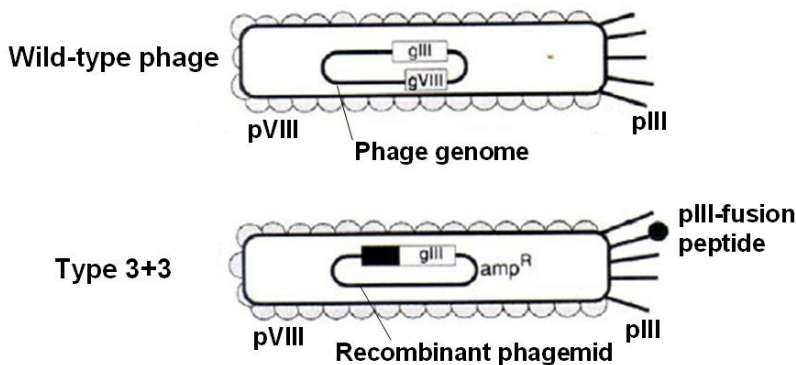## M13 wilde-type and recombinant viral particles

Figure 3.16. Phage display of random peptides. The figure on the top shows the M13 phage WT containing the phage genome. Are indicated the pIII and pVIII proteins codified by gIII and gVIII respectively. The figure in the bottom shows the recombinant phage according to type 3+3 system. Here the phage genome is replaced by the phagemid containing the random foreign DNA insert (black box). It is evident the link between genotype and phenotype, the latter documented by the display of the expressed random peptide (black sphere) fused to pIII protein on the phage surface.

In order to produce recombinant viral particles carrying the recombinant phagemid instead of M13 genome and thus expressing the fusion peptides, XL1Blue MRF' cells, previously transformed with the phagemid library, have been infected with M13K07 helper phage that is a derivative of M13 phage. The recombinant phagemid contains the origin of replication of the M13 phage and the packaging signal (PS) in addition to the origin of replication of the original plasmid. The infection with M13K07 helper

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phage is necessary to activate the origin of replication of the phagemid and thus to lead the synthesis of the single stranded phagemid DNA. Further the helper phage provides the proteins necessary to the assembly of phagemid DNA into phage-like particles. Moreover, in order to induce the expression of recombinant fusion random peptides, IPTG has been added to the grow medium of the infected cells because the expression of the fusion gene (random seguence/gIII) is under the control of pLac gene.

The infection with the helper phage can lead to the production of four different final products of which only one is the recombinant that presents both the phagemid genome and the corresponding random peptide fused to pIII on the phage surface (link between genotype and phenotype) (Fig 3.17). After infection, in fact, inside the cell is present also the M13K07 helper phage genome, however, the presence of a phagemid with a wild-type M13 origin leads to a preferential packaging of the phagemid DNA, in addition we have improved the experimental protocol with strategies to promote the production of recombinant phage. As result of the infection, we have obtained a phage library with a recombinant/wild type phage production ratio of about 10000:1.
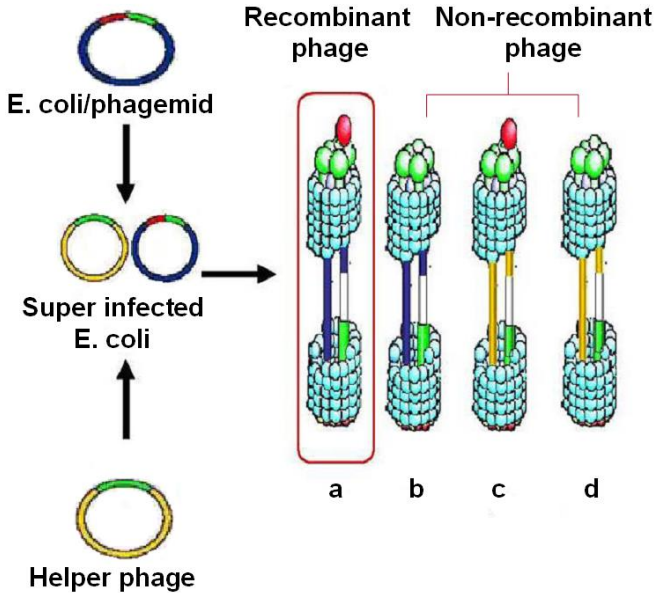
Figure 3.17. The four possible final products after M13K07 helper phage infection of transformed _E. coli_ XL1Blue MRF' cells (phagemid library). a) The recombinant phage presents both the phagemid and the fusion protein (link between genotype and phenotype); b) the phage presents only the phagemid while the pIII proteins are all non-recombinant; c) the phage contains the phage genome and the recombinant fusion protein on its surface (there is no link between genotype and phenotype); d) is refigured the wild-type phage.

To resume, a 50 amino acid long random peptide phage library has been produced starting from a phagemid library in bacteria with a complexity of 10^4 CFU/ml and we have estimated the final phage titre to be about 2.6 x 10^{10} CFU/ml. A schematic representation of the phage library production procedure is shown in figure 3.18.
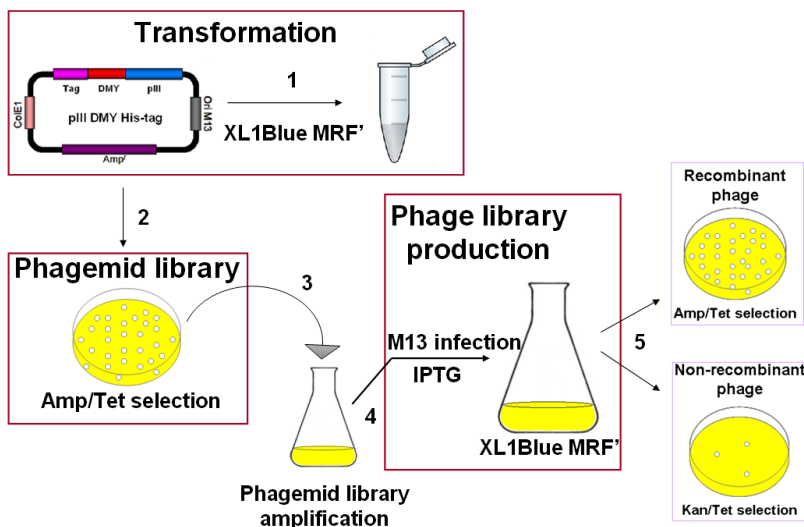
Figure 3.18. Schematic representation of phage library production procedure. 1) XL1Blue MRF' have been transformed with the phagemid library cloned inside a pIII-DMY-His tag phagemid; 2) the transformed cells have been plated in a selective medium to select the transformed clones and to estimate the complexity of the phagemid library (XL1Blue MRF' strain carries tetracycline resistance and the phagemid carries ampicillin resistance); 3) the phagemid library has been regenerated and amplified; 4) to produce the phage library, the regenerated cells have been infected with M13K07 helper phage. One hour after infection IPTG has been added into the grow media. Cells have been incubated over night in presence of ampicillin, tetracycline and kanamycin antibiotics (the helper phage carries kanamycin resistence); 5) after phage precipitation and recovery, new *E. coli* cells are infected and plated on selective media containing Amp/Tet or Kan/Tet to select recombinant phage and non-recombinant phage respectively and to determine the phage library titre.

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3.3.4. Phage display of random peptides: selection cycles to find N-Term76-Nef binding peptides.

The phage library has been used to perform selection cycles, through bio-panning affinity methodology, in order to select for peptides capable of binding N-Term76-Nef WT. The process involves several steps of selection before to find the peptide that best resemble the desired properties, i.e. a peptide displaying high binding affinity for N-Term76-Nef WT.

The first step has been to immobilize the target molecule (N-Term76-Nef) in a suitable solid support. To better mimic the cellular localization of Nef (hence its membrane binding) and to allow the exposure of Nef interaction sites (particularly the acidic cluster region) to the random peptide library, N-Term76-Nef has been bound to the solid support through its N-terminus. To perform it, it has been utilized as support a functionalized 96 wells plate that specifically and covalently immobilizes amino surfaces. The plate is, in fact, functionalized with an electrophilic group that reacts with a good nucleophile such as primary amine. Further, the protocol avoids the bind between the plate and the others weak nucleophiles present in the target molecule (such as tyrosine hydroxyl and histidine imidazole groups) so that N-Term76-Nef has been immobilized exclusively through its N-terminus end.

To start phage display selection each plate well has been first incubated O.N. with 10 μg/ml of N-Term76-Nef in a final volume of 100 μl/well. After washing, the first selection cycle has been carried out. The phage library has been diluted to have a phage titre of about 10^8 CFU/ml. The same dilution of the helper phage solution has been used as control to verify the rate of unspecific interactions. The 96-wells plate previously treated with N-Term76-Nef and the untreated plate have been incubated with phage library or helper phage alone. The first selection cycle has been performed under mild conditions (*i.e.* number of washes, incubation time, temperature as described in Materials and Methods). This consents to control the experimental conditions cycle by cycle and to avoid the premature loss of representative phage, however the subsequent cycles of selection have been gradually more stringent. After incubation and washes, the second step has been the recovery of the bounded phage. Thus precipitation and regeneration of the recovered phage has been carried out. This process leads to the generation of a new random peptide library that has been named sub-library 1. The titre of the regenerated sub-library 1 has been estimated to be 1.4 x 10^{10} CFU/ml, while the non-recombinant phage that have been selected during this first selection cycle are in the range of about 10^5

CFU/ml. The experiments carried out with the helper phage alone, instead, have been resulted in a null phage recovery suggesting that unspecific bounds between phage and plate do not occur.

As previously reported, the number of non-recombinant that have been selected during this first experiment is very low with respect to the recombinant phage but still too high for the experimental purposes, however it is the result of the poorly stringent conditions that have been adopted. Thus the sub-library 1 has been utilized to perform the second selection cycle in more stringent conditions. We have continued the selection cycles to generate a series of sub-libraries until the obtainment of a final sub-library showing a recombinant/non-recombinant ratio completely shifted to the recombinants. The final sub-library should contain the peptides with the expected properties. XL1Blue MRF' cells have been infected with the final sub-library and, after plating, a statistically significant number of clones has been subjected to plasmid extraction and sequence analysis. In this way, thanks to the linkage between genotype and phenotype provided by phage display methodology, it is possible to go back to the sequence of selected peptides, starting from the random DNA cloned inside the phagemid. This last step, that is currently in process, will consist in the individuation of those peptide sequences that could represent good candidates for the binding to N-Term76-Nef, that will be characterized through modelling analysis and pull-down experiments. The outlooks of this work will be described in the "conclusion and perspectives" chapter.

4. CONCLUSIONS AND PERSPECTIVES

This Ph.D. project has been inserted in the broad field of HIV research.
The HIV-1 Nef virulence factor has been the object of the work.
The focus has been the N-Terminal anchor domain of Nef and the research has been based on the study of its interaction sites with particular attention to the acidic cluster region, of its involvement in cell signalling and on the search for potential peptide inhibitors of specific Nef functions to open novel perspectives in the finding of new HIV drugs.
During the first part of the work an efficient protocol for the production and the purification of the N-Term76-Nef has been set up. The purification protocol that we have here reported has been optimized in order to ensure a good result in terms of yield and purity of the final product ensuring the minimal time and material consuming. This is of particular interest for a modular study of the protein which is required to solve the intricate biology of Nef that overlaps with a large number of phenotypes. There are, in fact, increasing evidences that the N-terminal Nef anchor domain absolves a key role in the Nef activity and this is coherent with the presence in this region of various interaction and phosphorylation sites. Further, this unstructured region could act as a spacer to convey the interaction sites of Nef domains to the target molecules.
Therefore the purified N-Term76-Nef, encompassing the N-terminal anchor domain and the acidic cluster region, has been used both to evaluate the effects that exerts on macrophages-like cells (THP-1), and as target to perform phage display of random peptide experiments in order to characterize the Nef interactome and to find N-Term76-Nef inhibitor peptides.
In particular, during the second part of this Ph.D. project, it has been demonstrated that the N-Term76-Nef region alone is able to affect the macrophages cell signalling as the Nef full-length protein does. This finding highlights on the importance of the Nef anchor domain in the Nef functions and opens new intriguing perspectives regarding the so far enigmatic biology of Nef. Basing on these results and on the presence of a cleavage site for the viral protease between the two principal domain of Nef (the anchor domain and the core domain), we hypothesise that the Nef protein functions can be also regulated by the viral protease cleavage so that to consider a new mechanism of action for Nef, in which the anchor domain and the core domain can also have an independent role depending on the viral cycle stages. The future outlooks will be to further investigate on this possibility that by a side would justify the wild array of phenotypes that

emerge after Nef exposure and on the other side could open new outlets in the HIV research. The first step toward the realization of this goal will be the setting of experimental procedures first to evaluate the response of MDMs to both Nef anchor domain and Nef core domain independently and second to evaluate the role of HIV-1 protease in the regulation of Nef activities. Furthermore, we want also to verify if the acidic cluster region is involved in the protease cleavage too, because Nef mutant in the acidic cluster region doesn't show signalling activity.

The third part of the project has been focused on the search of N-term anchor domain inhibitor peptides. This has been performed by phage display of random peptide experiments. The sub-library deriving from the last selection cycle has been screened for the finding of those phage displaying the best binding affinity for Nef. In detail, sequencing analysis of the phage genome have been carried out to go back to the peptide sequences from DNA. The subsequent step, currently under process, is to individuate the most suitable peptide sequences through modelling analysis and pull-down experiments. Once found the peptide that exhibit the best binding affinity for N-Term76-Nef, the final step will be to test the selected binding peptide in cell culture systems (such as MDMs and THP-1). In detail, cells will be treated with Nef in presence of the potential inhibitor in order to verify if this treatment affects the Nef signalling activity by preventing the interactions between the main interaction sites of Nef and its molecular partners and, in particular, the interaction between the Nef acidic cluster and TRAFs proteins.

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