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TITOLO TESI

HIV-1 infection: back and forth between virus and host.

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ABSTRACT

The main obstacles to HIV-1 eradication are linked to the viral ability to evade immune system and integrate into the host genome thus establishing a reservoir where virus is transcriptionally latent but able to replicate. The introduction of a combined antiretroviral therapy (ART) determined a major control of viral infection and spread in HIV-1 infected patients. Nevertheless, it cannot reach and eradicate the viral reservoirs. IFN action and RFs expression, dominant proteins that target multiple steps of the HIV-1 lifecycle, represent an early line of defence during HIV-1 infection. It is known that these proteins can interfere with viral replication by acting on different steps of viral cycle such as cellular entry and uncoating, reverse transcription, nuclear import, integration, budding and viral spread. Because of their interplay with viral replication, and in particular with reverse transcription and integration which are essential steps for the onset of latency, we would like to study the relationship between restriction factors and the viral amount in latently infected cells.

The first part of this project investigates the variations of the RFs expression levels in HIV-1 patients during the course of infection before and after ART administration by using Real Time qPCR. The immunological and virological (plasma HIV-RNA load and total HIV-DNA) parameters were analyzed on PBMCs from two cohorts of HIV-1 patients: 14 HIV ART naïve patients enrolled at diagnosis (T0) and followed at 4 (T1) and 8 (T2) months after ART administration; 14 HIV treated patients with undetectable viral load. We observed a restoration of immunological conditions in all patients during ART. HIV RNA load reached undetectable levels at 8 months (T2) as constantly observed in Group 1 during all follow-up period, while total HIV-DNA showed a decreased amount, but always detectable, after therapy in both groups of patients. Among the selected RFs (APOBEC3G, BST2, TRIM5α, MX2, SAMHD1, SERINC3/5, IFI16 and STING), APOBEC3G, MX2, SAMHD1, SERINC3 expression showed higher values in naïve patients and decreased levels after therapy (Group 1 at T2) and in TND patients. On the other hand, BST2, IFI16, SERINC5, STING and TRIM5α expression appeared to be reduced only after 8 months of antiviral treatment.

Plasma levels of HIV-RNA showed a positive correlation between several RFs (APOBEC3G, SAMHD1, MX2, BST2, SERINC3, IFI-16).

The second part of this study deals with the role of IFNs, in particular IFNα and IFNγ, and their role in the immune system disfunction that has been described during chronic inflammation associated to cancer, viral infection such as HIV-1, and autoimmune-disease. During the IFNs induction, RFs gene expression is induced as well as those of a second category of host proteins defined as Immune Check Point proteins (ICPs). ICPs are a group of inhibitory receptors expressed on the cellular surface of immune cells and trigger immunosuppressive signaling pathways leading to *T-cell exhaustion* an immune cells state of reduced effector function, sustained expression of immune checkpoint molecules (such as PD-1 and its ligand the PD-L1, TIGIT, LILRB2), poor recall responses. The major aim of this project is to assess the clinical meaning of ICPs expression in HIV-1 chronically infected patients to better characterized their involvement in immune system

disfunction. We performed immunofluorescences tests on Total CD4+, CD8+ T-lymphocytes, Myeloid cells, derived from PBMCs of healthy donors, and their respective different population subsets in order to analyze the expression of markers associated to cellular activation and exhaustion (PD1, PD-L1, TIGIT and LILRB2) after 24 hours of IFNs stimulation. Despite stimulated T-cells did not show any significant differences compared to unstimulated one, myeloid cells and its different subsets appeared more susceptible to IFN stimulation as indicated by a percentage increment of cells that are positive for PD-L1 and LILRB2 compered to control. Moreover, PD-L1 MFI analysis showed that under IFN stimulation, the expression pattern of PD-L1 on cellular surface is different between cells and the PD-L1 enhancement appeared mostly evident after IFN stimulation.

RFs play pivotal roles during the early phases of infection, in absence of antiviral treatment and therapy might drive downregulation of RFs. Moreover, the role of ICPs during immune activation is relevant in the determination of chronic infection as HIV-1. Deeper understanding of the molecular mechanisms of regulation by these factors are necessary to assess their utility as a therapeutic strategy. Acting on IFN pathway, for example by administration of JAK-STAT inhibitors, could lead toward new therapeutic strategies that associate to ART, could be represent new approaches in HIV-1 cure.

Introduction

The human immunodeficiency virus (HIV) type-1 is an RNA reverse transcribing virus belonging to the *Retroviridae* family, *Orthoretrovirinae* subfamily, genus *Lentivirus*. It was first described in the early 1980s when the first cases of acquired immunodeficiency syndrome (AIDS) were observed. Initially the infection was associated with men who have sex with men, then with drug users, people who received blood transfusions and finally the general population. The epidemic was rapidly spreading as one of the most devastating infectious diseases. The first clinical observations began in 1981 in the United States but only in 1983 that the HIV-1 was firstly isolated from patients with AIDS. Since its discovery, decades of intense research on the virus itself allowed its characterization, its interplay with the host, its pathogenesis and the development of approaches to test, treat and prevent HIV infection spread.

The genomic analysis permitted the identification of HIV origins, diversity and evolution as the result of cross species transmission of Simian-Immunodeficiency virus (SIV). Soon after the isolation of HIV-1, the identification of CD4 molecule on T-cells surface , as the main receptor for HIV provided evidence that the main target of this virus is the immune system, in particular immune cells that express on the cellular surface the CD4 receptor and one (or both) of the two coreceptors, the CCR5 and the CXCR4, which allowed the viral internalization into the host and defined the viral tropism.

The outcome of HIV infection in the last decades has been revolutionized by progress in the therapeutic strategies, which have transformed HIV infection from acute and fatal to a chronic disease: the introduction of a combined antiretroviral therapy (cART) has offered an increment of adult life expectancy and a prevention in the mother to child transmission of HIV. Despite the introduction of cART, HIV remains a persistent infection that it is still difficult to eradicate and it is associated to chronic inflammation and immune activation that lead to the immune system disfunction. The continuing evolution of drug resistance among circulating HIV variants leads to the global challenge of viral eradication with the development of new therapeutically strategies.

The latest data collected by UNAIDS revealed that 1.7 million of people were newly infected with HIV in 2018 and 37.9 million are people living with infection. 770 thousand are people who died of AIDS related illness. Despite the new UNAIDS statistics, the new HIV infections have been reduced by 40% since 1997 and the AIDS related deaths have been reduced by more 56% since the peak in 2004 (https://www.unaids.org/en).

The scientific progresses and the increasing access to therapeutic treatment are still going on and there is still lot of work to do to achieve total viral eradication.

Classification

Epidemiological end genetic studies performed soon after HIV isolation, led to the classification of HIV into two types, HIV-1 and HIV-2, both associated with AIDS. Type1 and type 2 are genetically different in the 30% of viral antigen expression. Moreover, while HIV-1 evolved to SIV isolated from Chimpanzee (SIVcpz) in the central African continent, the HIV-2 derived from an evolution of SIV isolated from *Sooty Mangabey* monkeys SIV infected (SIVsm) in the western African side where HIV-2 is largely represented between HIV infected population. Small percentages of HIV-2 infection are also registered in Portugal, France, Spain and South America. However, little is still known about HIV-2 infection. It is well characterized to be less virulent than HIV-1 with a longer asymptomatic stage and lower transmission rate (1).

According to the HIV sequence database (https://www.hiv.lanl.gov/) different HIV subtypes were genetically described. In particular, HIV-1 includes 4 groups named respectively M-N-O-P.

Group M is the "main" group of viruses in the HIV-1 global pandemic and the genetic clustering pattern of HIV-1 M group includes different subtypes and sub-subtypes that are labeled as A1-A2-A3-A4-A6-B-C-D-F1-F2-G-H-J and K, each one representing different lineage of HIV and geographically distributed. M group contains also multiple circulating recombinant forms (CRF), deal with recombinant HIV-1 genomes that have infected more than three persons who are not epidemiologically related.

Group N has only been identified in a few individuals in Cameroon and very few isolates have been identified and sequenced. N is sometime referred to different meanings as Not-M or Not-O as well as "new" group.
HIV-1 group O is the "outlier" group and it contains very diverse viruses even if it is still rarely found. It is originated in a transmission to human from gorilla. Subtypes within the HIV-1 O group are not yet defined, although the diversity of sequences within the HIV-1 O group is nearly as great as those in the HIV-1 M group.

Group P is a human isolate closely related to SIVgor.

The existence of a subtype L in HIV-1 classification has been further described even if this isolate is not typically included in reference sequence alignments used to classify HIV sequences. Despite that, it remains possible that others L-strains might be circulating (2).

HIV-2 is very distinct from HIV-1 and it includes group A-B-C-D-E-F and it appears that each group of HIV-2 represent at least one separate transmission event from SIVsm to human. HIV-2 recombinants are rare and in 2010 a HIV-2 CRF was described (3).

Structure

Viral genome

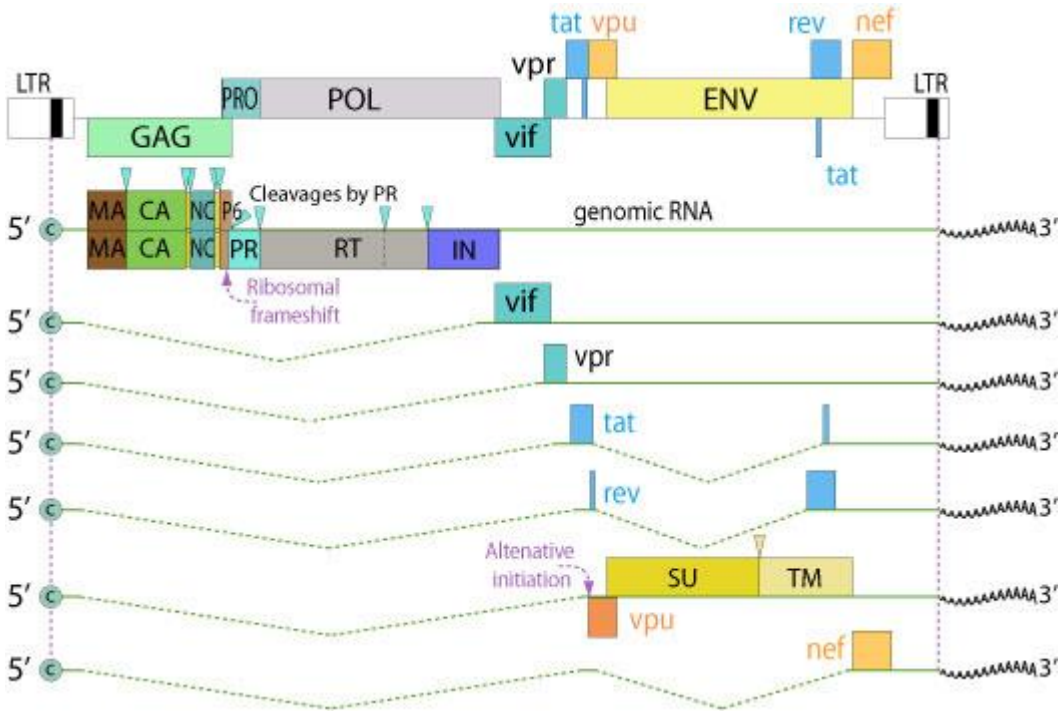


Figure 1: HIV-1 genome organization (https://viralzone.expasy.org/)

HIV genome is around 10 kb long and consists of two identical single-stranded RNA molecules that are enclosed within the core of the virus particle. The genome of the HIV provirus, also known as proviral DNA, is generated by the reverse transcription of the viral RNA genome into DNA, degradation of the RNA and integration of the double-stranded HIV DNA into the human genome. The DNA genome is flanked at both ends by LTR (long terminal repeat) sequences. The 5' LTR acts as the promotor region for transcription of viral genes and it encodes sequences for multiple transcription factors like NFkB, Sp1 and others. It further contains the Tat binding trans activating response (TAR) elements, relevant for the transcription of integrated HIV proviral DNA. In the direction 5' to 3' the reading frame of the *gag* gene follows, encoding the proteins of the outer core membrane, or matrix (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7) and a smaller, nucleic acid-stabilising protein (p6). The gag reading frame is followed by the *pol* reading frame coding for the protease (PR, p12), reverse transcriptase (RT, p51) with RNase H (p15) and integrase (IN, p32). Adjacent to the *pol* gene, there is the *env* reading frame that the two envelope glycoproteins [the gp120 (surface protein, SU) and the gp41 (transmembrane protein, TM)] are derived. In addition to the structural proteins, the HIV genome codes for several regulatory proteins: Tat (transactivator protein) and Rev (RNA splicing-regulator) are necessary for the initiation of HIV replication, while the other regulatory proteins Nef (negative regulating factor), Vif (viral infectivity factor), Vpr (virus protein r) and Vpu (virus protein unique) have an

impact on viral replication, virus budding and pathogenesis. HIV-2 codes for Vpx (virus protein x) instead of Vpu, which is partially responsible for the reduced pathogenicity of HIV-2 (Figure 1).

Particle structure

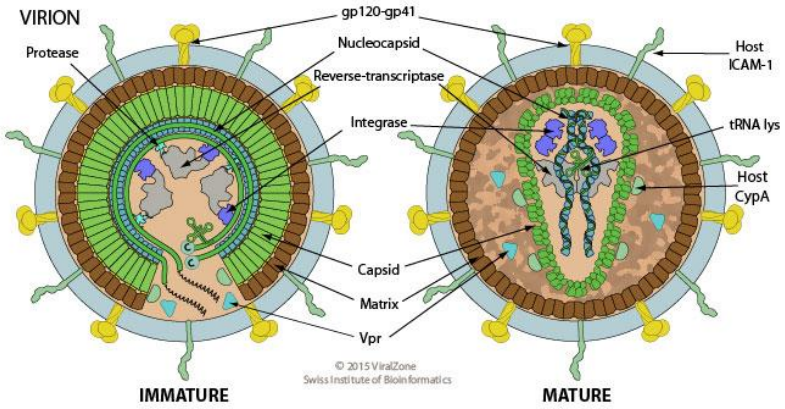


Figure 2: HIV-1 virion structure and organization (https://viralzone.expasy.org/).

The electronic microscopy was useful in order to characterize the viral structure of HIV-1. The virus appeared as enveloped, spherical to pleomorphic in shape, approximately 100 nm in diameter. Moving from the outsider to the inner part of the mature viral particle, the main elements that characterized its structure are the envelope, the matrix and the capsid forming the core shell that contains the viral core (Figure 2).

The envelope is a lipid bilayer deriving from the membrane of infected cell when immature viral particle are budding from the surface. The envelope contains 72 knobs, the trimers of Env proteins composed by gp120 and gp41, the surface and the transmembrane proteins, respectively. Other proteins are present and belong to the lipidic bilayer of infected cell.

The envelop itself covers the capsid membrane which is formed by the matrix protein (MA, p17) that sustains the viral structure. During the HIV-1 lifecycle, the p17 localizes at the level of host membrane thanks to its N-terminal portion where the presence of a myristic acid residue and the electrostatic interaction with the lipid elements of the bilayer.

The viral core is composed by the viral capsid protein (CA, p24). The capsid itself surrounds the nucleocapsid made of viral genome associated with other viral proteins, as the NC (p7 and p9), and different enzymes required during viral replication: two molecules of viral tRNA that triggers the viral reverse transcription, the protease, the integrase and polymerase reverse transcribing the two molecules of RNA into viral DNA.

Viral proteins

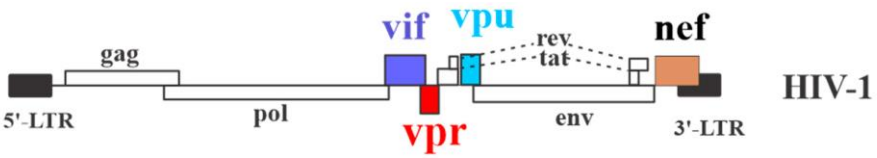


Figure 3: HIV-1 genome organization and gene coding viral proteins localization (4).

HIV-1 genome encodes for several proteins that can be grouped into three categories based on their relevant roles: structural, regulatory and accessory (Figure 3 and Table 1). While structural and regulatory proteins are essential for HIV replication irrespective of the cellular context, accessory genes encode for proteins that can be dispensable for HIV-1 spread in ex vivo cell line cultures as they, among other functions, mediate the interaction of infected cells with the host immune system. Gag Env and Pol code for the main structural proteins including viral enzymes required during replicative lifecycle. Tat (transactivator protein) and Rev (RNA-splicing regulator) are necessary for the initiation of HIV replication and they are considered the two main regulatory proteins of HIV-1. The main accessory proteins are Nef (negative regulating factor), Vif (viral infectivity factor), Vpr (virus protein r) and Vpu (virus protein unique) have an impact on viral replication, virus budding and pathogenesis. They also have distinct patterns of temporal expression (5). In the past decade it has become increasingly clear that the function of these non-enzymatic viral proteins is to modulate the cellular environment within infected cells to promote efficient viral replication, transmission and evasion from innate and acquired immunity as well as the counteraction of host cell barriers against retroviral replication (6–8).

Gene	Size*	Protein	Function
gag		Pr55Gag	precursor of the inner structural proteins
	p24	capsid protein (CA)	formation of conical capsid
	p17	matrix protein (MA)	myristilated protein, forming the inner membrane layer
	p7	nucleoprotein (NC)	formation of the nucleoprotein/RNA complex
	p6		involved in virus particle release
pol		Pr160GagPol	precursor of the viral enzymes
	p10	protease (PR)	proteolytic cleavage of Gag (Pr55) and Gag-Pol (Pr160GagPol) precursor protein; release of structural proteins and viral enzymes
	p51	reverse transcriptase (RT)	transcription of HIV RNA in proviral DNA
	p15 (66)	RNase H	degradation of viral RNA in the viral RNA/DNA replication complex
	p32	integrase (IN)	integration of proviral DNA into the host genome
env		PrGp160	precursor of the envelope proteins SU and TM, cleavage by cellular protease
	gp120	surface glycoprotein (SU)	attachment of virus to the target cell
	gp41	transmembrane protein (TM)	anchorage of gp120, fusion of viral and cell membrane
tat	p14	transactivator protein	activator of transcription of viral genes
rev	p19	RNA splicing regulator	regulates the export of non-spliced and partially spliced viral mRNA
nef	p27	negative regulating factor	myristilated protein, influence on HIV replication, enhancement of infectivity of viral particles, downregulation of CD4 on target cells and HLA cells on target
vif	p23	viral infectivity protein	critical for infectious virus production in vivo
vpr	p15	virus protein r	component of virus particles, interaction with p6, facilitates virus infectivity, effect on the cell cycle
vpu	p16	virus protein unique	efficient virus particle release, control of CD4 degradation, modulates intracellular trafficking
vpx	p15	virus protein x	interaction with p6 in virus particles, involved in early steps of virus replication of HIV-2, component of virus particles
tev	p26	tat/rev protein	Tat-Env-Rev fusion protein, regulates the activity of Tat and Rev in nucleus
*Numbers correspond to the size of the proteins (p) or glycoproteins (gp) in 1,000 Da.			

Table 1. Overview of HIV-1 proteins and their function (9).

Structural Proteins

Gag proteins derives from the proteolytic cleavage of a common precursor, the Pr55Gag polyprotein that during the final step of viral lifecycle move towards the inner part of cellular membrane where it is cleaved in the two structural protein Matrix, the p17 (MA), p24 (CA) and the nucleocapsid (NC) proteins p7 and p6. These events lead to important changes for the final steps of assembly and maturation of new viral particle. It was described that while MA proteins remain anchored at the inner layer of viral membrane, the CA proteins associate to form a shell surrounding the RNA/NC complex. Other two small peptides derive from the Pr55Gag proteolytic cleavage. Their roles in the viral biology is still unclear but it has been supposed that their presence in the Pr55Gag allow the proteolytic cut on the precursor polypeptide. They may also influence the correct assembly and morphogenesis in the viral particle.

p17 protein drives the targeting of Gag protein towards the membrane as well as the Env glycoprotein incorporation and the first earlier events of viral internalization in the host. P17 presents also a nuclear localization sequence (NLS) whose deletion impair the viral replicative ability and the pre-integration complex (PIC) carriage into the host nucleus.

p24 protein encloses the viral genomic RNA associated with the core proteins. It also plays a pivotal role in the viral assembly and maturation and during the first steps of viral lifecycle.

p7 proteins structure is characterized by the presence of CysX2-Cys-X4-His-X4-Cys (CCHC) that act as the zinc fingers domains which bind the DNA. As NC proteins it localizes in the core associated with viral RNA whom it drives the dimerization, reverse transcription and PIC stabilization.

Finally, but still not well described, there is the **p6** protein. Probably its role is associated with new viral particle budding and release as it was suggested the p6 truncation leads to an accumulation of virions at the level of cellular membrane.

Viral enzyme

Pol gene that lacks of the start codon, partially overlap Gag gene. The result is the synthesis of a 160 kDa polyprotein (Gag-Pol) derived from the fusion of Pol and Gag proteins (Pr160-Gag-Pol). Pol gene codes for the main viral enzymes: the protease (PR), the reverse transcriptase (RT) and the integrase (IN).

The PR plays a pivotal role in the assembly and maturation of new virions whom events must be coordinated in order to produce viral particles highly infectious and thus a productive infection. An overproduction of PR proteins may be deleterious for the onset of a productive infection because a big amount of truncated products result by the proteolytic activity of this viral protein with consequently impaired of infectivity.

The RT is fundamental for the onset of a reverse transcription reaction which allow the synthesis of proviral DNA starting from viral RNA. Indeed, RT acts as a polymerase RNA dependent. The molecular structure of this enzyme is represented by a heterodimer composed by two main subunits: the p51 (440-aminoacid residues) and the p66 subunit (560 aminoacid residues). p66 contains the main domain with RNase activity by which viral RNA of the complex RNA-DNA is degraded during the RT activity.

Once the pro-virus entries within the nucleus, the third viral enzyme, IN, allows the integration of reverse transcription product into the host genome. The viral integration is an essential step for the onset of viral reservoir, a pool of latently infected cells ready to produce new virus, and it involves a series of controlled and well-defined mechanisms which can be summarized in three steps: first the IN-exonuclease activity acts by cutting the double strand of HIV-DNA; subsequently a cut is also performed on the genome of the infected cell creating a site for the insertion of the viral genome. Finally, the IN-ligase activity allows the formation of covalent bonds at the ends of the proviral DNA allowing integration of the virus into the host genome (10,11). This integration seems to be strictly determined by the state of the chromatin rather than by specific sequences: open and transcriptionally active regions seem to be a preferential target for the integration of the provirus within the nucleus (12).

The envelope proteins

Env proteins are coded by Env gene and they are the only surface proteins that constitute the envelope. Env is a heavily glycosylated trimer of 160 kDa glycoprotein (gp160) that is pivotal for viral binding and entry into the host cell. Once after its synthesis, gp160 move towards the Golgi apparatus where it is cleaved by a host

protease in to the two mains glycoproteins, the gp120 and gp41 heterodimers that form the spikes which are present on virion surface and triggers the interaction between host and virus.

gp120 subunit is responsible for receptor binding and its structure contains two domains: the inner formed by five conserved regions (C1-C2-C3-C4-C5) and an external domain characterized by five variable loops (V1-V2-V3-V4-V5). Among the conserved regions, the C1 and C5 bind the gp41, while C2-C3-C4 forms a hydrophobic core within the molecule structure. Variable regions are so called referring to its relative genetic heterogeneity. Each of the variable regions is comprised of a loop structure formed by a disulfide bond at its base, with the exception of V5. The variable loops lies predominantly at the surface of gp120 and plays critical roles in immune evasion and coreceptor binding, particularly the V3 loop which binds the coreceptor triggering the fusion membranes process.

gp41 subunit is a membrane protein characterized by a cytoplasmic-transmembrane and an extracellular domain which drives the conformational changes relevant for the viral membrane fusion with cell membrane. The transmembrane portion anchors Env to host membrane while the N-terminal portion of gp41, thanks to a glycin-rich domain, triggers the fusion step between membranes. During the fusion mechanism, the central region of gp41 assumes an helicoidal structure, defined as TM-core, that it is not present in the native structure of this domain before viral binding and fusion.

Regulatory proteins

Tat protein is a small protein of 14kDa of 86 to 103 amino acids residues. Tat is encoded by two exons spliced together but while the first is conserved, the second exon is not as well conserved as the first one and it contains the arginine-glycine-aspartic acid sequence (RGD) that allows Tat interaction with surface proteins such as integrins. Tat is a protein with several domains. Among them, the hydrophobic core motif is pivotal for Tat transactivating activity through its binding to transactivation response RNA element (TAR) of new HIV genomic RNAs. By using other domains, Tat localizes to the nucleus, binding of Tat to TAR element, and the internalization of Tat protein into bystander cells by its interaction with surface proteins such as heparan sulfate proteoglycans (13).

During viral replication, after viral DNA integration and transcription, Tat controls HIV transcription. In particular once transcription starts, the first transcription events triggered by RNA Polymerase II leads to the production of short transcripts that are translocated to the cytoplasm where they are translated into Tat and Rev proteins. Newly translated Tat enters the nucleus to activate RNA Polymerase II and drives transcription elongation by binding to P-TEFb, a complex made up of CDK-9 and Cyclin T1. Thus, Tat proteins complexed with P-TEFb bind the TAR element and increase processivity of RNA Polymerase II. Tat can be released from infected cells and taken up by both uninfected and HIV-infected cells mimicking the transcriptional and cytotoxic effects of the protein. The current cART prevents the transcription and/or synthesis of Tat protein. Tat uptake has been shown to activate transcription factors like Sp1, NF-kB, modulate the expression of both HIV and host genes as well as different pro-inflammatory cytokines (like TNF-a, CCL2, IL-2, IL-6, and IL-

8), adhesion molecules and sometimes, pro- and anti-apoptotic factors upregulated by these transcription factors via Tat activities. Soluble Tat, in the absence of the virus, has been shown to cause: induction of apoptosis, release of neurotransmitters, oxidative stress, and inflammation (13).

Rev is a small regulatory protein of 16 kDa, with 116 aminoacids. As Tat, Rev plays a pivotal role during productive infection: it binds a specific sequence, the Rev Response Element (RRE), on neosynthesized viral transcripts and the RRE-Rev complex itself binds to Crm1 cellular factors to induce the transport of viral transcripts from the nucleus to the cytoplasm. Thanks to a specific arginine-rich region as NLS and a leucine-rich portion that act as a NES (nuclear export sequence) Rev acts as a shuttle for new viral transcripts: NLS associated with the importin-B and other factors, allow the re-internalization of Rev in the nucleus, while NES triggers its release by interacting with CRM1 protein.

Accessory proteins

Vpu is a small accessory protein of 16 kDa and it is a 77-86 amino-acids membrane associated protein. Vpu was initially identified as the product of an open reading frame (ORF), referred as the U ORF located between the first exon of the tat and env genes of HIV-1. Its expression is coordinated with the translation of Env.
As a membrane protein, Vpu is characterize by a short luminal N-Terminal domain, a single transmembrane (TM) spanning domain and a charged C-Terminal hydrophilic domain that extends into the cytoplasm. Vpu is largely expressed on intracellular membranes, which correspond to the ER, the trans Golgi as well as endosomal compartments. This small accessory protein exerts two relevant roles during HIV-1 replication. First, Vpu induces a rapid degradation of newly synthesized CD4 receptor molecules in the ER via the ubiquitin-proteasome system. In addition to its effect on CD4 catabolism, Vpu promotes the release of progeny virions from HIV-1- infected human cells by counteracting Tetherin, a host restriction factor that strongly inhibits the release of virions from the host cell surface (14). It is well established that Vpu, which is expressed late during the virus life cycle, acts on newly synthesized CD4 molecules in the ER (15). Besides its role in CD4 ubiquitination and dislocation across the ER membrane so that receptor molecules can be accessible to the cytosolic proteasome, there are further evidence that Vpu retains CD4 in the ER (16). It was initially found that Vpu was targeting CD4 molecules that were retained in the ER through formation of a complex with Env. In a recent study the role of HIV-1 infection in loss of peroxisomes in macrophages and brain tissue has been described. It has been shown that Vpu is necessary and sufficient for the induction of microRNAs that target peroxisome biogenesis factors. The ability of Vpu to downregulate peroxisome formation depends on the Wnt/_-catenin pathway. Thus, in addition to revealing a novel mechanism by which HIV-1 uses intracellular signaling pathways to target antiviral signaling platforms (peroxisomes), we have uncovered a previously unknown link between the Wnt/_-catenin pathway and peroxisome homeostasis (17).

Nef is a small protein of 27 kDa acts as protein adaptor without enzymatic activity to hijack central host cell transport and signal transduction pathways and to optimize virus spread in the infected host. An important aspect of these activities is the re-routing of transmembrane receptors (such as the HIV entry receptor CD4 or

major histocompatibility complex (MHC)-I molecules) as well as peripheral membrane proteins (such as Src family kinases) from the plasma membrane to intracellular membrane compartments . In addition, Nef also impairs host cell actin dynamics and motility, drives the release of extracellular vesicles, and has been implicated in the regulation of cell survival. Nef elevates the infectivity of virus particles, an activity that provided an intuitive link to the observation that the viral protein elevates viral titers in infected host by several orders of magnitude. The effect of Nef on virion infectivity was detected in single round infection assays in which wild-type HIV-1 was two-to-tenfold more infectious than the HIV-1Dnef counterpart. This effect required the presence of Nef in the producer but not the target cell or the virion itself and was due to an effect on the infectivity of HIV-1 particles and not the amount of particles released from the producer cells. Which step in the viral life cycle is facilitated by Nef remained somewhat controversial: most studies detected a positive effect of Nef on early post entry events without enhancement of fusion, other studies reported a mild effect of Nef on the fusion of HIV-1 particles with target cells. A caveat for the relevance of the Nef effect on infectivity was that it was relatively mild (two-to-tenfold fold depending on the producer cell used). This changed when Pizzato and Göttlinger described a clone of Jurkat E6.1 cell that, when used for virus production, revealed an up to 100-fold enhancement of virion infectivity by Nef. In this system, the effect of Nef on virion infectivity depended on the GTPase dynamin 2, clathrin and the AP-2 adaptor complex, indicating that Nef may affect virion infectivity by modulating host cell endocytic trafficking (6).

Vpr is a 14 kDa protein that is necessary for optimal replication in macrophages where it enhances infection and increase viral burden in tissue where macrophages reside, but it also facilitates viral replication in CD4+ T-cells. Vpr is packaged into the virion in large quantity by interacting with the P6 region of viral Gag precursor (18,19), localizes to the nucleus (20) and it was observed that it may enhance early viral replication events and its role continues into late stages in the HIV replication cycle (21). Vpr is known to play multiple roles at different stages of HIV-1 viral life cycle such as arresting the cell cycle at the G2/M phase, increasing the activity by regulating apoptosis (22) by hijacking E3 ubiquitin ligases. Thus, Vpr targets multiple cellular proteins to proteasomal degradation and in consequence causes global remodeling of the cellular proteome (23). In this context, recently was further described that Vpr blocks autophagy earlier after HIV-1 entry. In infected CD4+ T cells, autophagy is an anti-HIV -1 process and its block allows HIV-1 replication. Vpr coming from the viruses decreases autophagy triggered during viral entry. Interestingly, Vpr decreases the expression of three ATGs, LC3, Beclin-1 and BNIP3, at their transcriptional level. Furthermore, Vpr induces the degradation of the transcription factor FOXO3a (21).

Vif is a small accessory protein of 23 kDa and its role is strictly associated to the host restriction factors APOBEC3 (A3). Indeed, Vif role is relevant in the production of mature virions highly infectious as well as it promotes degradation of the antiviral A3 proteins through the host ubiquitin proteasome pathway to enable viral immune evasion. It was infact observed that in the absence of Vif, A3 family members are encapsidated into HIV virions and inhibit viral replication primarily by deamination of cytidines to uridines in the viral complementary DNA during reverse transcription (24,25). The resulting hypermutation renders the viral infection nonproductive. It was further observed that the correct recruitment of A3 by Vif required the Vif

interaction with the specific cellular cofactor core binding factor (CBF)-β (26). Recently, proteomic analysis have been obtained in order to perform a comprehensive time course analysis of viral and cellular proteins during HIV infection and clusters of proteins have been identified according to their patterns of temporal expression regulated by HIV, including candidate resistance/restriction factors and HIV accessory protein targets. It was observed that other proteins as A3 would be Vif targets and demonstrated Vif-dependent remodelling of the cellular phosphoproteome during HIV infection (5).

Hiv-1 replication cycle

HIV-1 life cycle consists of several steps that occur sequentially and require the presence and the activity of both viral and cellular factors to be completed. Similarly to other viruses, HIV-1 replication can be divided into two phases: an early phase, including entry, uncoating, reverse transcription and integration into host genome, and a late phase, which includes the events leading to viral particle production, namely transcription, and production and release of new virions (27–29) (Figure 4).

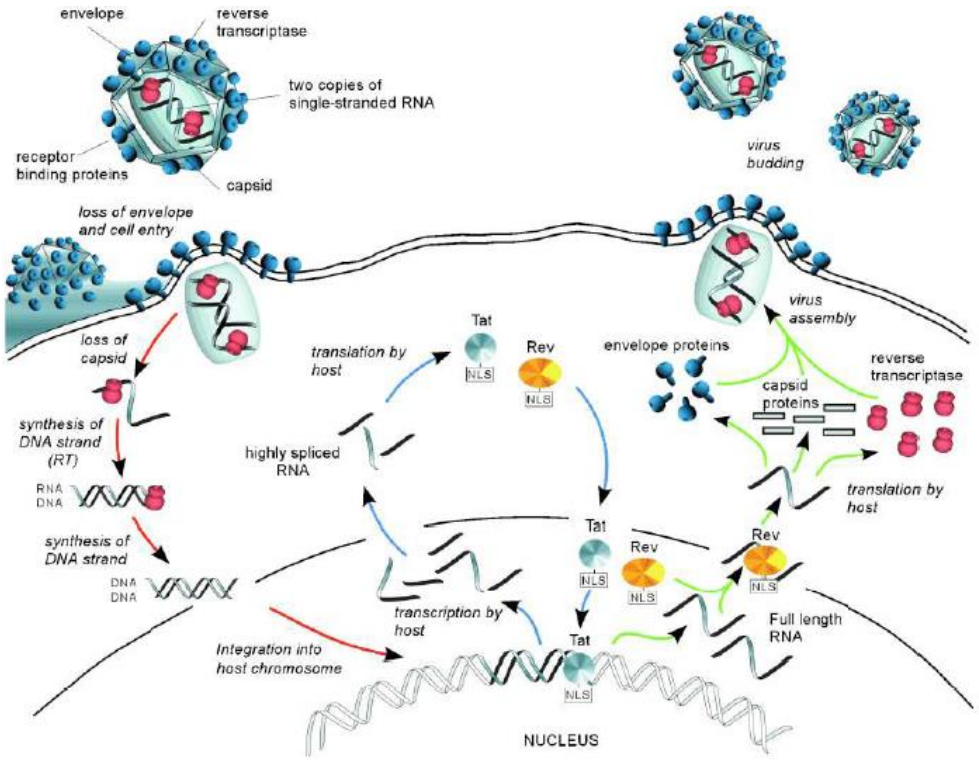


Figure 4 : Diagram showing the replication cycle of HIV-1 (30)

Early phase

Entry and uncoating

HIV-1 entry in the host cell is a complex mechanism that starts with the contact between the viral receptor Env and cellular membrane to culminates with the release of the virus nucleocapsid into the cytoplasm. The first

contact between the viral and the cellular membranes is mediated by electrostatic forces, resulting from the interaction between Env and various cell attachment molecules such as the negatively charged heparin sulphate proteoglicans and the α4β7 integrins (31–33). The fusion between viral and cellular membranes occurs in two steps: initially, the gp120 molecule binds to the cellular receptor CD4 inducing a conformational change that exposes the domains responsible for the interaction with the co-receptor molecule. Subsequently, the engagement of the co-receptor exposes the gp41 fusion peptide, which is finally responsible for the apposition and fusion of the membranes.

In addition to cell-free virus infection, cell-to-cell transfer of virions represent an alternative mechanism for viral spread. Cell-to-cell transmission requires close proximity between the cells harboring the virus and the uninfected target cell. Physical connection between donor and target cells occurs via the formation of an organized membrane structure, referred as virological synapse, as well as via the establishment of membrane protrusions, or via generation of cell syncytia. This route of entry is more rapid and efficient than fusion of cell-free virions, because it avoids the early steps of attachment, and, importantly, it allows evasion of humoral immune response (34). Although cell-to-cell spread of HIV-1 was initially described in CD4+ T cells, infected macrophages have been also shown to efficiently transmit virions to T cells via the same mechanism (35). Furthermore, trans-infection of T cells has been observed following the contact with dendritic cells, which are able to capture the virions and transfer them to target cells through cell-to-cell contact, despite being resistant to HIV-1 infection (35,36).

Irrespectively of the route, the entry process results in the release of the viral capsid into the cytoplasm. An efficient infection requires the correct dissociation of the capsid protein (CA) from the HIV-1 core (37). While initial observations suggested that uncoating was a passive process taking place immediately after entry (38,39), it is now known that the organized lattice made by CA plays a pivotal role in the trafficking of the particles to the nucleus. Several reports have shown that CA is essential for reverse transcription as well as for integration of viral genome, supporting the notion that disassembly of the capsid does not occur immediately after membrane fusion (40,41). Currently, there are two models that describe the uncoating stage: according to the first, the intact capsid docks at the nuclear pore where reverse transcribed genome is translocated inside the nucleus (40); according to the second, the capsid loses integrity during the trafficking from the cellular membrane to the nuclear pore and only some CA molecules that are associated with the viral ribonucleoprotein are retained. The cytoplasmic uncoating is supported by dependence of the process on microtubule stability (42–44) and by recent observations, made using live-cell imaging approach in combination with fluorescently tagged markers, showing that uncoating takes place in the cytoplasm in the first thirty minutes after entry (45).

Reverse transcription

Following entry, the establishment of a productive infection requires the conversion of the single stranded (ss) RNA genome into a double-stranded (ds) DNA. This reaction occurs within the reverse transcription complex (RTC) and is catalysed by the viral reverse transcriptase (RT). RT is a heterodimer composed by two subunits:

p51 and p66, containing the two catalytic domains responsible for the polymerase and the RNase H activity of the enzyme.

Reverse transcription proceeds through three main steps: synthesis of the (-) strand cDNA, digestion of the RNA template, and synthesis of the (+) strand DNA. Synthesis of the (-) cDNA starts from a tRNA associated to the viral RNA, which acts as a primer for RT, and proceeds towards the 5' end. The ends of the ssRNA genome are characterized by the presence of a specific motif made of a repetitive region (R), which is identical for the 5' and the 3' end, and a unique region, which differs between the 5' end (U5) and the 3' end (U3). By annealing to the R sequence at the opposite end of the template RNA, the newly synthetized cDNA is transferred from the 5' end to the 3' end, where synthesis of (-) strand is resumed. Once (-) strand DNA is completed, the RNAse H domain of RT removes the RNA template from the hybrid duplex. The RNA molecule is not degraded in correspondence of two purine rich tracts of the genome (Polypurine tracts, PPTs), which are resistant to RNAse H activity. This PPTs serve as primers for the synthesis of the (+) strand RNA and are removed subsequently. Following the degradation of the tRNA primer at the 3' end of the (-) strand DNA, a second strand transfer occurs which transfers the incomplete (+) DNA from the 3' to the 5' of the DNA template, allowing to complete the synthesis of the ds molecule (46–48).

Because of the two strand transfers, the motifs flanking the DNA produced by reverse transcription are longer than the ones of the RNA template, each one containing the U5, R, and U3 tracts. These regions, called long terminal repeats (LTRs), include the elements that will be required for viral transcription once the viral DNA is integrated, such as the viral promoter and polyadenylation tract (49).

Reverse transcription is crucial for the spread of the disease as it is responsible for sequence variation, one the key mechanisms that allow the virus to escape to immune response and antiretroviral therapy. HIV-1 variability mainly results from the error-prone nature of reverse transcription. Indeed, RT lacks proofreading activity, and thus dsDNA generated by reverse transcription is highly mutated compared to the RNA template (46,50). Furthermore, since the strand transfers can occur also between two different RNA templates, the process can generate recombinant ds molecules (51–54).

The late steps of the reverse transcription process are characterized by the transitions of the RTC into the preintegration complex (PIC) (46), the nucleoprotein complex which is responsible for the translocation of the dsDNA viral genome to the nucleus and for its integration. RTC already includes integrase (IN), the viral enzyme that catalyses integration of the viral DNA into the host genome. Interestingly, it is has been recently demonstrated that the presence of IN within the RTC is required to complete reverse transcription. Tekeste *et al* showed that mutations affecting the RT-binding site of IN prevent HIV-1 replication by blocking the early stages of reverse transcription, indicating that the RT-IN interaction is functionally relevant for RT activity (55).

Integration

The hallmark of retrovirus replication is the integration reaction, which consists in the insertion of the viral DNA into the host genome. The first step of this process is the translocation into the nucleus of the PIC, the

ribonucleoprotein complex containing the newly generated viral dsDNA (56,57) bound to a variety of viral and cellular factors, which are responsible as whole for viral genome stabilization and translocation, as well as for the integration reaction (58,59).

Because of their size, PICs cannot cross the nuclear membrane by diffusion, instead they are actively driven through the nuclear pores via the interaction with multiple components of the nuclear pore complex (NPC) (64,68).Several authors have identified the binding site of factors required for nuclear import in a pocket formed by the juxtaposition of the N-term and C-term domains of CA (62,67,68).

Integration starts within the PIC while the complex is translocated from the cytoplasm to the nucleoplasm. A tetramer of four IN molecules binds the viral DNA and removes several nucleotides from the 3' of each end of the strands. In order for the reaction to proceed further, the 3' ends must be processed to generate CA_{OH} overhangs, which serve as nucleophiles for the subsequent steps of the reaction. Within the nucleus, the CA_{OH} overhangs invade the target host DNA producing a hybrid duplex molecule. Integration is then completed through the action of cellular repair enzymes, which fill the single strand gaps at the 5' ends of the viral DNA to the phosphates of the target genome, generating the HIV-1 provirus (69,70). Linear DNA is the only substrate allowing proper formation of the provirus; however, integration can generate defective byproducts such as the circular forms 1-LTR and 2-LTR. 2-LTR circles are generated by the activity of the cellular repair system, whereas 1-LTR circles can result from defective reverse transcription, auto-integration from the rearrangement of circular forms, or homologous recombination between 2-LTR circles.

Unintegrated circular forms of HIV-1 are present prominently in the nucleus and can be considered as markers of the active transport of the PIC into the nucleus (61,71,72).

Late phase

Transcription and translation

Once the genetic material of the virus is integrated, the viral cycle proceeds to the late phase, which includes transcription and translation of the viral proteins, and assembly and release of new virions. Transcription from the provirus depends on the concerted action of viral and cellular factors and co-factors which regulate the activity of the 5' LTR, the viral promoter. Sufficient levels of activating transcription factors and the presence of a permissive chromatin landscape are both critical for transcription initiation (73). Even in presence of an environment favoring transcription initiation, initially only short messengers are produced, because of the rapid dissociation of the RNA polymerase from the DNA template (74). Production of the full length viral messengers occurs only after successful translation of the viral transactivating factor, the regulatory protein Tat. Tat binds to a stem loop on the nascent transcripts and recruits a group of cellular factors ad cofactors, known as the Super Elongation Complex that enhances RNA pol II processivity allowing completion of transcription. This mechanism allows the production of unspliced (9 kb) and partially spliced (5 kb) RNAs, which encode the polyprotein precursors and are packaged in the newly generated capsids as genomic RNA.

Large intron-containing transcripts such as the ones generated by HIV-1 transcription are not translocated to the cytoplasm by the cellular systems, which instead tend to process them further leading to their degradation. To overcome this problem and export unspliced and partially spliced RNAs, HIV-1 encodes an additional regulatory protein, Rev. Rev binds a cis-acting structure present on viral transcripts, the Rev Response Element (RRE) and guides their translocation to the cytoplasm through its nuclear export sequence. In the cytoplasm, the unspliced mRNAs are translated to produce the polyproteins Gag and Gag-Pol, which eventually will give rise to the structural and enzymatic components of the virion, whereas translation of the partially spliced mRNAs will result in the production of the virus envelope. The viral enzymes RT and IN are packaged as domains within the Gag-Pol polyprotein, which is generated when translating ribosomes shift into the −1 reading frame at a site near the 3′ end of the gag open reading frame, and then go on to translate the pol gene. While high-level HIV-1 transcription results in productive infection, the virus may also exist in a latent state, in which the provirus is present in a transcriptional inactive state. After integration, viral transcription depends on the balance between the cellular mechanisms that regulate gene expression. Position of integration has been considered a major player in the establishment and maintenance of HIV-1 latency. Nevertheless the diverse nature of the mechanism regulating gene expression has prevented the identification of a unique chromatin signature for latent integrants (75,76). Indeed, several authors have shown that silent proviruses are mapped in inactive regions, such as centromeres and heterochromatic regions, while productive integration events generally tend to occur in transcriptionally active regions of the genome (77–82). On the other hand, integration into highly transcriptionally active regions has also been associated with latency through a mechanism referred as transcriptional collision, by which ongoing RNA pol II activity on the host gene prevents the assembly of the transcriptional machinery at the 5' LTR thus blocking production of viral messengers (83–85).
Binding sites for a number of transcription factors have been found along the 5' LTR (86), including NF-κB, AP-1, and Sp-1 which have been shown to be crucial for viral replication (87,88). Segregation of these transcriptional regulators in the cytoplasm frequently occurs in metabolically inactive cell populations such as CD4+ T cells (89), which represent the most clinically relevant population harbouring latent proviruses (90,91). Likely, the lack of transcriptional activators in the nucleus of these cells contributes to shift the balance towards latency at the 5' LTR (92–94).

Virus assembly, budding and maturation

Assembly, budding and maturation are the last three phases of HIV-1 life cycle that lead to the release of infectious viral progeny.

During assembly, the components of the viral particle, including genomic viral RNA, Gag and Gag-Pol polyproteins as well as Env trimers, are packaged at the plasma membrane. Assembly constitutes a highly coordinated process through which Gag, Gag-Pol, Env, and viral RNA all come together on a concentrated location on the plasma membrane in order to form the new infectious particles. Assembly may take place in specialized membrane domains, known as detergent resistant domains (DRMs) (95–97). Recruitment of viral

components at these sites is mediated by the myristylated N-terminal domain of Gag and Gag-Pol polyproteins which binds the phosphatidyl inositol (4,5) bisphosphate (PIP2). Viral genomic RNA associates to Gag in the cytoplasm, via the interaction between the NC domain of Gag polyprotein and the RNA encapsidation signal sequence (ψ)(98–100). Besides binding to NC, viral RNA interacts also with a highly basic region of the MA domain, determining an increase in the affinity of Gag for lipid rafts (101). This latter interaction is lost at plasma membrane, where the conformational changes induced by binding of PIP2 and Gag multimerization displace the RNA from the MA region, allowing a tighten interaction between the myristic acid and the membrane phospholipids (102,103).

Contrary to the other components of the virion, Env is assembled in trimers into the plasma membrane in a Gag-independent fashion. Env transcripts are translated as a 160-kDa membrane glycoprotein precursor (gp160) by ribosomes associated to the ER, resulting in the incorporation of the protein in the membranes of the secretory pathway. While travelling from the ER to the membrane, gp160 undergoes several post-translational modifications including cleavage into its two functional domains and assembly in trimeric complexes. The Env trimers on the plasma membrane are subsequently internalized and directed to the endosomal recycling compartment, where Rab11-FIP1C and Rab14 subsequently direct their outward movement to the particle budding site.

Progression to virion budding starts with the arrangement of Gag in spherically shaped immature particles underneath the regions of the plasma membrane harbouring Env trimers. Subsequently, the cellular endosomal sorting complexes required for transport (ESCRT) machinery promotes the membrane remodelling required for to fission and release of newly synthetized virions.

Budding virions are released in an immature form and require proteolysis of their components to achieve infectivity. Cleavage of the polyproteins in their mature constituents is mediated by the protease domain of Pol, which initially cleaves itself out from the Gag-Pol precursor through an autocatalytic reaction. Activation of the protease domain requires its dimerization and depends on the correct stoichiometry of Gag-Pol precursor, being induced by the increasing concentration of Pol within the developing virion but inhibited by its overexpression (104). Sequential proteolysis of Gag then releases the single structural components of the virion and triggers a number of conformational changes resulting in the condensation of the viral genome and reorganization of the capsid in a conical shaped particles, ultimately converting the virion into a particle that can enter and replicate in a new host cell.

Pathogenesis

The pathogenesis of HIV-1 infection is the result of a highly dynamic interplay between virus life cycle, host cellular environment and the immune responses via the cell-mediated and immune-mediated reactions (28,105,106).

The evolution of HIV-1 infection is characterized in almost 95% of cases by a marked reduction of T-cells thus leading to death. Infection can be divided in two main phases: primary acute infection and late infection.

Transmission occurs generally across mucosal surfaces or by direct inoculation and primary HIV-1 infection starts when the host is infected for the first time and culminate in the seroconversion, when non neutralizing antibodies are produced. This first phase is characterized by a viremic peak associated with a relevant decline in CD4+ T-cells and there are evidences that HIV-specific cytotoxic CD8+ T-lymphocytes limit the viraemia (107–109). Viral replication leads to loss of CD4+ T-cells, which could be due either to increased cell death, or to reduced production, or both. The increased turnover of both CD4+ and CD8+ T cells in HIV-1 infected subjects compared to controls supports the killing of virally infected cells by HIV-specific CTL as a leading hypothesis for CD4+ T-cell decline in HIV infection. However, the direct relationship between plasma viral load and rate of CD4 decline suggests that viral replication also contributes, directly or indirectly, to CD4 loss. The balance between viral replication and the antibody response pinpoints the late stage of HIV-1 infection where an increased rate of CD4 loss and expansion or broadening of the viral tropism (by a switch in co-receptor usage from CCR5 to CXCR4) occur. Thus, the absence of an efficient antiretroviral therapy leads the HIV-1 infected individuals to death by the development of a series of opportunistic infections that immune system is unable to control and eradicate because deeply compromised.

In the following graph (Figure 5) is depicted the trend of plasmatic viraemia and T-cells count during the cours of infection.

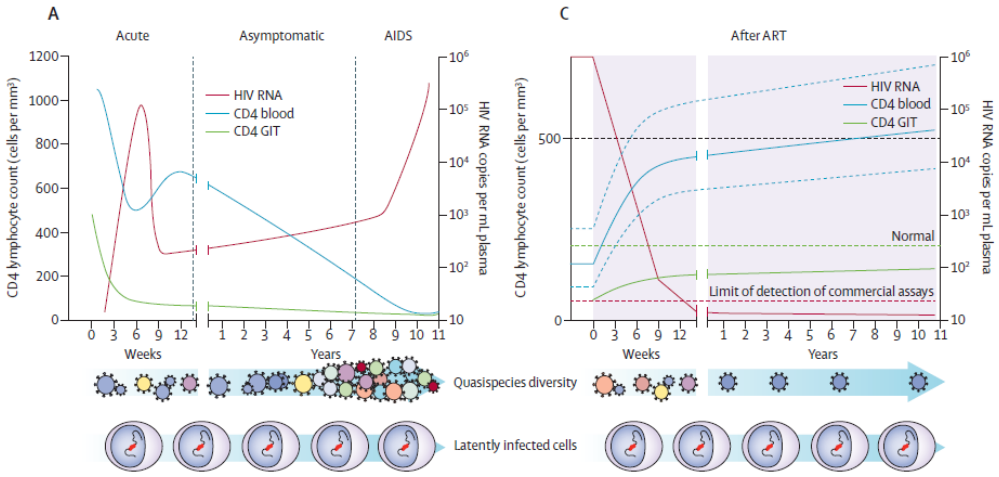


Figure 5*:* Natural history of untreated HIV infection and changes after antiretroviral therapy (105)**.**

When a virion infects its target cell, unrestrained viral replication occurs during the first two weeks and new virions disseminate in all tissues and organs. During this phase, called *window period*, viraemia is still not detectable, immune response occurs, and the infected subject appears asymptomatic.

The acute phase arises between the second and the fourth weeks of primary infection and it occurs when the plasmatic viraemia significantly increases (around 10^7 copies of viral RNA/ml) and the number of infected CD4+ T-lymphocytes grow in blood and lymphnodes. Symptoms like fever could occurs and the onset of

immune response appears during the peak of viraemia. At this time the immune activation leads to the production of antibodies against viral proteins and the cytotoxic CD8+ T-lymphocytes response against antigens expressed in infected cells. Probably the viraemia peak depend on the combination of different factors: the absence of an early immune response and the existence of an higher number of CD4+ T-lymphocytes activated after pathogen recognition resulting, however, deleterious for infected individuals because activated T-cells are the main target during HIV-1 pathogenesis.

The acute phase is followed by an intense reduction of viraemia thanks to both immune system, which partially controls the infection, and to T-lymphocytes reduction, as the main target of HIV-1. As far as concern the CD4+ T cells, there is a rapid reduction of this T-cells subtype that ends when the viraemia reaches its peak. After that, there is a partial CD4+ T-cells recovery.

Chronic infection, also defined as clinical latency that lasts from one to 20 years, starts with the mild recovery of CD4+ T-lymphocytes followed by a slow and constant decline associated to the increased plasmatic viraemia. During this stage of infection, patients are still able to prevent other opportunistic infection as well as the immune system is still able to block or limit the onset of infections by other pathogens.

Neutralizing antibodies production starts after three months from the infection onset. However, one of the hallmarks of HIV-1 is its ability to evade immune system and to be a fast-evolving virus. Thus, antibodies are not able to protect the host in the long term. (105,110).

When the CD4+ T cells count drastically reduces below the 200 cells/µl, infected individuals enter in the final phases of HIV-1 infection where the reduced cell-mediated immunity leads to a wide range of opportunistic infections and cancers. This coincides with the onset of AIDS, acquired immunodeficiency syndromes (111).

It has been hypothesized and demonstrated that the first target infected by HIV-1 is a memory CD4+T lymphocytes that express the CCR5 coreceptor. These cells are the main viral target because the most abundant, especially in the mucosal lamina propria. Viral replication in mucosa is crucial for the infection transmission and spread towards other body districts. In the mucosal district where virus firstly replicate and disseminate, the majority part of CD4+ T cells are memory subtypes which expresses high levels of CCR5 coreceptor than the CXCR4, mostly expressed by the naïve lymphocyte subpopulation. It has been further described that CCR5 coreceptor expression appeared increased during HIV-1 infection and immune system activation meanwhile the CXCR4 decreases. Moreover, CD4+T cells that do not express markers of activation, are able to support high levels of viral replication in the GALT (Gut Associated Lymphoid tissue).

CD4+ T lymphocytes are not the only viral target. The CD8+ T-cells, Natural Killer (NK) and Dendritic Cells (DCs) have been described as other cellular targets as well as the main cells that play a pivotal role in the viral dissemination through the “immunologic synapsis”, a strategy of infection spread by which new viral particles infect new cells without budding from cellular surface. Monocytes infection has been also described. In particular, the infected monocytes migrate to body district and differentiate into macrophages. This strategy,

well known as the “trojan horse” is a way by which HIV-1 reach the nervous system and onsets a reservoir. It has been described the existence of HIV-1 different macrophages-tropic strains in the nervous system able to infect microglia and the perivascular macrophages, the trojan horse (112,113).

Diagnosis

Several tests have been developed for the diagnosis and monitoring of HIV-1 infection. In particular the Italian guidelines (http://www.salute.gov.it/portale/hiv/dettaglioContenutiHIV) adopt two diagnostic strategies to detect virus in plasma samples. The first one, defined as first level tests or tests of screening, are based on the immune-assay technology by which viral antigens and antibodies are detected. Currently, the test of fourth generation are used in several laboratories qualified in the HIV-1 diagnosis. The combination of antigens and antibodies in the same test has the advantage of detecting the infection even when no antibodies have been produced, as occurs during the window period, when there is still no antibody response, or in some cases of immunosuppressive patients. A positive or uncertain result must be confirmed by a second test, usually western blot followed by an additional test based on molecular assay such as the Real Time RT-qPCR, as mentioned in the Italian guidelines. Moreover, a further test could be included to verify the time of infection. This test, defined as avidity test, allowed to distinguish between recent infection (less than 6 months after the estimated time of infection) and previous infection (more than 6 months after the estimated time of infection) with the aim to start promptly the antiretroviral therapy, reduce the plasmatic viraemia and prevent the risk of viral spread and infection in healthy population. Meanwhile, sequencing analysis are further performed on viral strain isolated from blood sample of the infected individual to identify the existence of genetic variants on HIV-1 genome that lead to the development of drug resistance at the antiretroviral therapy.

Immunological diagnosis is based instead on the lymphocytes counts. In particular, the amount of CD4+ T-cells is considered a marker of prognosis associated to the clinical progression of HIV-1 infection (a too low CD4+ T-lymphocytes percentage is associated to a faster disease progression) and to therapy efficacy. The CD4+ T-cells recovery after the antiretroviral therapy beginning has been associated to their nadir value, aging and coinfections (114–116).

Another relevant parameter followed during the follow up of HIV-1 infected patients under antiretroviral therapy administration, is the CD4+/CD8+ ratio because considered as an index of immune system restoring. Before therapy administration, patients are characterized by an inverse ratio that only in few cases of infected individuals appeared to be restored (117,118) .

Based on immunological and virologic parameters, two different categories of patients have been described as individuals with an atypical pattern of infection: the *elite controllers (ECs)* and the *post-treatment controllers (PTCs)*. These patients are described as individuals able to control the viral infection although the viral and host mechanisms involved are still unclear. *ECs* are able to maintain viral replication and viraemia under undetectable levels for prolonged period in the absence of antiretroviral therapy (119). They are 1% of infected

population and are characterized by an higher number of CD4+ T lymphocytes and a slower progress of HIV infection. Viraemia is under detection limit with available tests validated for the diagnostic routine, but it appears detectable by using a cell associate RNA and proviral-DNA assays. Despite the huge variability between patients, genetic and immunologic parameters have been described to well characterize this group of patients. Among the genetic variants, the existence of HLA protective alleles, such as the HLA-B*57, HLA-B*27 and HLA-B*5801, has been analyzed in elite controllers. The immunologic determinants are represented by the presence of polyfunctional CD8+ T-cells with a superior HIV-1 inhibition (120). On the contrary, PTCs differ from ECs firstly because the HIV-1 infection has been diagnosed when the acute phase was still detectable and, furthermore, these patients assumed the antiretroviral therapy for a prolonged period (around 4 years). Once therapy has been stopped, these infected individuals can maintain the plasmatic viremia under the detectable limit of detection with commercially available test for a long period before a viral rebound. These patients have high level of viraemia and the onset of symptoms associated to acute infection allow in the early diagnosis. Protective HLA- class I alleles variants are not determinant in PTCs, except for the HLA -B*35 variant associated to a faster disease progression. CD8 T-lymphocytes ability to successfully inhibit HIV-1 replication is smaller than ECs. Moreover, the expression of a selected group of immunoglobulin-like receptors, called KIR, on NK-cells surface, appears to limit the viral replication. Among them, the KIR3DL1 expression has been associated to a slower disease progression and IFNγ production by NK-cells is higher in this group of patients thus conferring a major control on virus (121).

Antiretroviral therapy

The introduction of an efficient antiretroviral therapy (ART) can effectively control viral replication and spread maintaining the viraemia under undetectable levels (⩽20 copies/ml), further allowing the control of infection spread towards healthy population (https://www.unaids.org/en/topic/prevention). However, ART can not reach the viral reservoir and latently infected cells persist over the time. So, latency represents one of the major obstacle to infection eradication and the main goal for research activities is the modeling of new molecules of strategies that, in addition to current therapy, can purge viral reservoir and eradicate HIV-1 infection. Thanks to ART, viral infection shifts from an acute to a chronic one and life expectancy increased for HIV-1 infected individuals, improving the immune system abilities, reducing chronic inflammation and reducing the risk of AIDS evolution (122). However, it is well known that ART interruption leads to a rebound of viraemia within few weeks. Some patients following regularly ART can enter in a state of ART resistance (https://hivdb.stanford.edu/DR/) caused by the ability of this virus to be genetically fast evolving, a strategy by which it prevents not only the immune system control, but also the therapeutic properties of ART to block some steps viral lifecycle. ART presents some side effects, too. Comorbidity associated to HIV-1 infection and treatment are largely described and they are one of the main research topics. For example, cardiovascular and neurological complications as well as kidneys, bones, liver disease and the onset of diabetes mellitus are extensively described (123,124).

ART includes different drugs that could be grouped into six classes on the basis of what step of the replication lifecycle or viral target they interfere with. The therapeutic regimen provides a cocktail of three drugs, two of them belonging to the same class while the third is part of a different group. Italian guidelines (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2696_allegato.pdf) and Temprano studies (125) revealed that ART beginning is important both in patients in acute infection and ECs in order to prompty reduce the disease progression and the impact on the size of the HIV reservoir in infected patients. Moreover, a recent study confirmed that an early beginning of ART administration guarantees a rapid clearance of that pool of cells that are the early targets during acute infection (126).

Nucleoside RT inhibitors (NRTIs) are molecules of drug that work by targeting the viral RT enzyme. Once the RT converts the viral RNA into DNA, the NRTIs disrupt the construction of a new piece of proviral DNA, thereby stopping the reverse transcription process and halting HIV replication. This class of molecules is sometimes referred to as the 'backbone' of a first-line HIV treatment combination. The ability of these molecules to block the reverse transcription activity, is associated to their molecular structure mimicking a nucleotide or nucleoside. Thus, they act as nucleoside/nucleotide analogs that interfere with the natural substrate at the active site of viral enzyme. Among the several drugs belonging to this group, Tenofovir Disoproxil Fumarate (TDF) has been largely described as one of the first drug administered after the first diagnosis of acute infection because of its high genetic barrier. However, association between TDF assumption and toxicity leading to kidneys disfunction with Fanconi syndrome outbreak and bone tissue compliances have been observed (127). Good clinical practices have recently approved the use of Tenofovir alafenamide (TAF), instead of TDF, that maintain a high genetic barrier as TDF but shows reduced side effects on kidney and bones thanks to its major tissue penetration compared to TDF. Abacavir (ABC) side effects are associated to a severe allergic reaction and it has been observed its correlation with the existence of HLA-B*5701 allele. It means that before ABC administration, patients must be tested for HLA-B*5701 allele. Moreover, a further association between ABC and cardiovascular risk increment have been analyzed, especially in patients with other factors risk. Another NRTIs is the zidovudine (AZT) whose severe side effects led to its interruption in the clinical practice, except in Sub-Saharan area and other regions of African continent where AZT is included in the second lines of therapeutic protocols. Together with AZT, stavudine is another NRTIs whose administration have been limited because of its severe hepatic toxicity with the onset of liver steatosis, lactic acidosis and lipoatrophy. Moreover, while AZT assumption is further associate to anemia, stavudine affects mostly brain wit the development of neuropathies (128).

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) also target the viral reverse transcriptase, but in a different way compared to NRTIs. They interfere with the reverse transcriptase enzyme by binding a site that is different to that of active site. The binding leads to several conformation changes of enzyme structure altering its functionality and block the reverse transcription process. Generally, this is a class of powerful and safe drugs, except for efavirenez that presents high toxicity at the nervous system level with an increment of depression and suicide. Nevirapine, on the contrary, causes liver failure in patients characterized by a high

number of CD4+ T-cells. Finally, rilpivirina is the safest between the NNRTIs even if it is the less powerful. For this reason its administration is recommended in patients with viraemia under 100000 RNA copies/ml and CD4+4 T-lymphocytes count more than 200 cell/µl (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2696_allegato.pdf).

Protease inhibitors (PIs) block the activity of the protease enzyme, which HIV uses to break up large polyproteins into the smaller pieces required for assembly of new viral particles. While HIV can still replicate in the presence of protease inhibitors, the resulting virions are immature and unable to infect new cells. This group of drugs are administered in combination with NRTIs and with inhibitors of hepatic metabolism (ritonavir o cobicistat, defined as *booster*) because PIs drugs required a step at the liver level to be metabolized. The main side effects described in patients treated with PIs are mild gastrointestinal symptoms, dyslipidemia and cardiovascular disturbs as well as the iperbilirubin in concomitance with atazanavir use (128).

Integrase inhibitors (INSTIs) target the viral enzyme integrase which is essential for viral replication and integration of viral DNA into the host genome, an essential step of viral lifecycle for the onset of latency. Integrase inhibitors stop the virus from inserting itself into the DNA of human cells. This class of drugs is well tolerated and safe, with a high genetic barrier. Raltegravir (RTG) and Dolutagravir (DTG) are the most used while Elvitegravir, because of its hepatic metabolism, required to be administered with a booster of cobicistat (COBI) to prevent hepatic clearance. This therapeutic combination increases the serum creatinine that required an ongoing monitoring of patients with kidney disfunction (129).

Entry inhibitors stop HIV from entering human cells. There are two types: CCR5 inhibitors and fusion inhibitors. Maraviroc, a CCR5 inhibitors, prevents the CCR5- tropic strains to enter by binding to it. Thus, before administration, a sequencing analysis is required for the identification of virus-tropism and the exclusion of a CXCR4 tropism. This is the reason why this class of drugs are very rarely used for first-line treatment. Enfuvirtide on the contrary, binds directly to the virus to prevent the fusion of the HIV-1 envelope protein with the CD4 cell. As a fusion inhibitor, it is used only for people who have no other treatment options. Moreover it requires more than one administration per day by injection (128). Finally, a new drug in experimental clinical phase 3 is the Fostemsavir. This drug inhibits the viral entry by blocking the conformational changes of gp120, an essential step for the onset of a new viral lifecycle in the host. Preliminary results revealed a good safety and efficacy for both virus tropism in patients with repeated events of therapeutic failure and with limited or poor therapeutic options (130). However, it has been observed that polymorphisms on gp120 sequence, most frequent in non-M group, could lead to the onset of drug resistance (131).

HIV-1 and inflammation

The IFN response

IFNs are cytokines that constitute one of the first-line of innate defense mechanisms against pathogens. Three main classes of IFNs have been described: type I, type II and type III, based on their structural homology, chromosomal location, and interaction with their various receptor chains (132). Type I IFNs are encoded by numerous genes that cluster on chromosome 9 (133), and include IFN-α (which comprises 13 human subtypes), IFN-β, IFN-ε, IFN-κ and IFN-ω. All type I IFNs bind to the same receptor, an heterodimeric complex consisting of two subunits: IFN-α/β receptor chain (IFNA R)1 and IFNA R2 (134). IFN I signalling induces a state of refractoriness to infections through the activation of multiple mechanisms of protection, such as the upregulation of proinflammatory genes and the activation of adaptive immunity (50,105,135–137) (Figure 6).

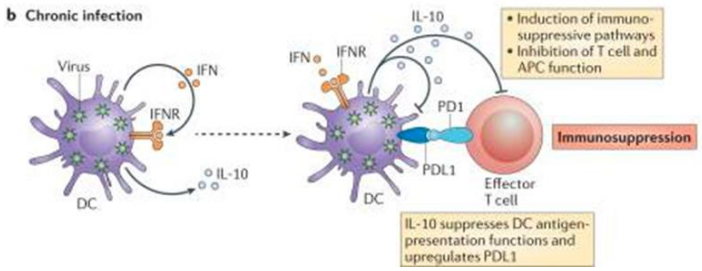


Figure 6: Persistent type I IFN exposure in chronic infection induces immunosuppressive pathways.

Binding of type I IFNs to their receptor triggers the JAK-STAT signalling pathway, eventually leading to the transcription of IFN stimulated genes (ISGs). ISGs include RFs as well as others mediators of innate and adaptive immune response, which act as the final effectors of IFN I activity (50,105,134,136). Notably, among the three IFN groups, type I IFNs, and in particular IFN-α, are the most involved in the antiviral defense against HIV-1 (50,105,135–137).

IFNγ, encoded by a group of genes localized on chromosome 12, is the only member of the Type II IFN group and is produce by T cells, NK-cell, monocytes, macrophages, and dendritic cells (138). IFNγ has immunoregulatory and inflammatory properties and it is known to enhance type I IFN production (139). Finally, type III IFN family comprises IFN λ1, λ2 and λ3 (also known as IL29, IL28A, and IL28B, respectively), whose genes are located on chromosomes 19. They all bind to IFNλR1 (or IL-28RA) and IL-10R2 receptors (140). Type III IFNs share a similar activity with type I IFNs (141); however their action appears to be restricted to the epithelial-mucosal surfaces (142).

Generally, the activation of IFN response is triggered by detection of pathogen-associated molecular patterns (PAMPs), structurally conserved motifs of proteins and nucleic acids derived from invading organisms. PAMPs expression is restricted to pathogens, allowing the cells to distinguish between self and non-self antigens, thus avoiding unspecific activation of IFN response. PAMPs are recognized by specific cellular

molecules, known as pattern recognition receptors (PRR). PAMP-PRR binding induces a downstream cascade culminating in transcription of IFNs, which, once secreted, reprogram the host cellular landscape via autocrine and paracrine signaling. Furthermore, IFN signaling starts a positive feedback loop that potentiates secretion of type I and type III IFNs, as well as of proinflammatory cytokines and chemokines that recruit and activate innate immune cells, including macrophages, NK cells and dendritic cells (143). In the case of HIV-1 infection, PAMPs are mainly constituted by the viral nucleic acids, which are recognized by the toll-like receptors (TLRs) and the retinoic acid inducible gene 1 (RIG-1)-like receptors, or by dsDNA cytosolic sensors such as the interferon inducible protein 16 (IFI16) and the cyclic GMP-AMP synthase (cGAS) (144).

TLRs are a group of PAMPs belonging to the endosomal compartment that are able to recognize a variety of microbial antigens, such as ssRNA, and to sense the presence of RNA-DNA heteroduplexes and dsDNA outside the nucleus. TLR7, which is activated by the binding to ssRNA, has been shown to trigger IFN response in dendritic cells after HIV-1 exposure (145). TLR9, which responds to the presence of unmethylated CpG DNA, has been also linked to IFN response activation following HIV-1 infection, although the mechanisms by which viral DNA would reach the endosomal compartment to induce activation is still debated (146,147) . While TLR mediated recognition of HIV-1 PAMPs is restricted to endosomes, other sensors are involved in the detection of viral components that have reached the cytoplasm, such as the RIG-I-like receptor and the melanoma differentiation-associated gene 5 (MDA5). Although both proteins are ubiquitously expressed, only RIG-I-like receptor has been implicated in the detection of cytosolic HIV-1 RNA. Specificity of RIG-I-like receptor for viral RNAs results from its ability to recognize secondary and tertiary RNA conformations, such as the stem loops formed by the A-U rich sequence of the HIV-1 untranslated region (148).

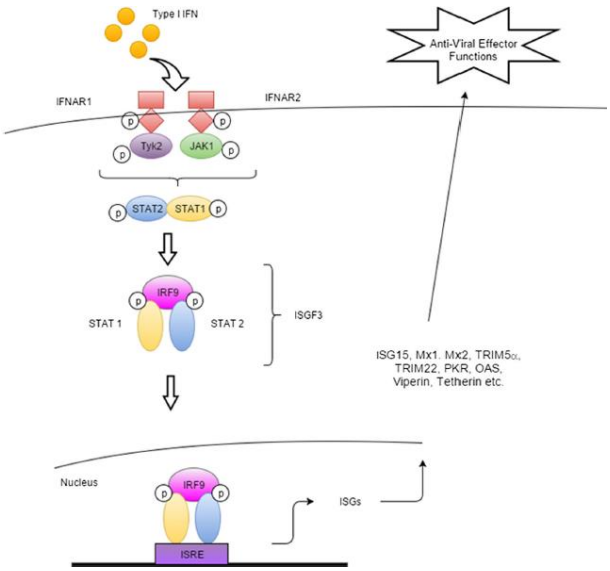


Figure 7: IFNs pathway activation (134)

Presence of both single and double strand DNA in the cytosol of infected cells is also a strong activator of IFN signaling (149–152). The host protein cGAS can bind dsDNA through its amino terminal domain, generating

a dinucleotide product, the cyclic GMP-AMP (c-GMP), that acts as a second messenger in the activation of innate immune response (153). In addition, IFI16 senses ssDNA, which is present in the cytoplasm as an intermediate product of reverse transcription (150). Engagement of DNA sensors in the cytoplasm triggers a downstream transduction cascade involving the adaptor molecule stimulator of interferon genes (STING) (154). Interaction between STING and TBK1 mediates the activation of the interferon regulatory transcription factor 3 (IRF3) and its translocation to the nucleus where it promotes the transcription of type I IFN and ISG genes (155,156).

Restriction factors

Restriction factors target all phases of HIV-1 life cycle

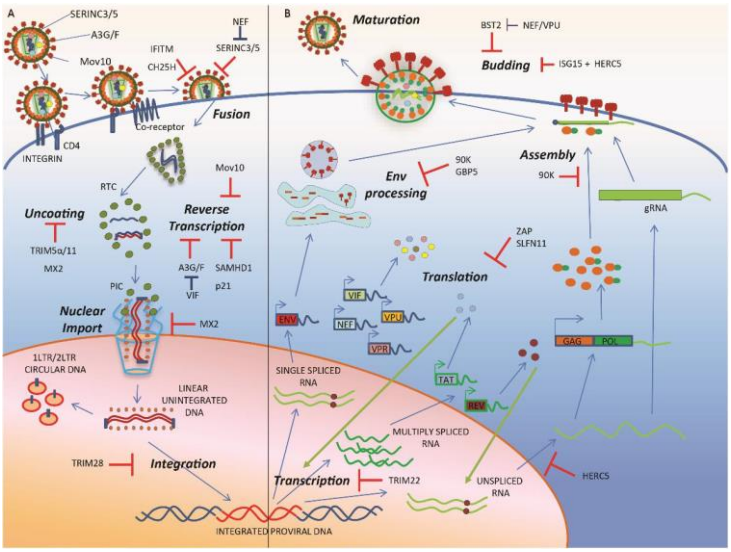


Figure 8: Overview of the impact of RFs on HIV-1 replication.

RFs preventing entry and uncoating: SERINC3/5 and TRIM5α

SERINC3 and SERIC5: The serine incorporator (SERINC) protein family is a class of transmembrane carrier proteins that play a pivotal role in lipid metabolism, regulating the incorporation of the polar amino acid serine in cell membranes, thus facilitating the biosynthesis of phosphatidylserine and sphingolipids (157). Among the five members of the SERINC family, SERINC3 (SER3) and SERINC5 (SER5) have both been identified as host restriction factors that are able to reduce HIV-1 infectivity of Nef-defective strains (158,159). The HIV-1 accessory protein Nef stimulates viral replication via different mechanisms, including downregulation of CD4 receptor and class II MHC, modulation of cell activation status and cytokine networks, and enhancement of virion infectivity. The latter effect depends on the presence of Nef in virus producing cells, suggesting that Nef could counteract the activity of some cellular factor (160). Using complementary approaches, two independent groups have identified SER3 and SER5 as the restriction factors targeted by Nef. Performing immunoprecipitation experiments comparing virion composition after infection with WT or Nef defective HIV-1 strains, Umami *et al.* found that SER3, and to lesser extent SER5, could be consistently identified in virions produced in absence of Nef (158). On the other hand, a transcriptomic analysis of cells showing different susceptibility to Nef+ strains performed by Rosa *et al.*, identified SER5 as the gene whose expression better correlated with the requirement of Nef for HIV entry (159). Consistent with these observations, knockdown of SER3 and SER5 in T cell lines increased virus infectivity (158).

Although the discovery of these two restriction factors is strictly linked to Nef antagonism and their ability to restrict HIV-1 infection in vivo is still unclear, some recent insights have defined the possible mechanism

responsible for viral restriction. Rosa *et al.* have shown that SER5 overexpression in virus producing cells resulted in impaired release of the capsid content, without completely abrogating membrane fusion (159). Following this observation, it was hypothesized that SER5 prevented the release of the nucleocapsid from the virion by affecting the expansion of the fusion pore formed at the interface between cellular and viral membrane. However, subsequent data have shown that not only expansion, but also formation of the fusion pore is affected by SER5, and that this effect is the result of the reduced fusogenic activity of the Env glycoprotein (161–163). Consistent with the notion that SER5 may affect fusion, viruses harboring Env variants with strong fusogenic capacity may display resistance to SER5 irrespectively of Nef activity (162). Furthermore, HIV-1 virions pseudotyped with VSV-G or Ebola virus envelopes are not sensitive to SER5 activity.

Despite the lack of evidences of a direct interaction between SER5 and Env, colocalization of Env and SER5 in specific membrane districts has been shown to be crucial for restriction (163). Once localized at the cellular membrane, SER5 could either act as a physical barrier for functional Env clustering (161,162) or, alternatively, impair Env activity by triggering its conformational changes (163). Given the role of SER5 in lipid biosynthesis, perturbations of membrane fluidity have also been advocated as a possible mechanism of action of SER5; however, the observation that changes in lipid membrane composition do not correlate with Nef presence and activity does not support this hypothesis (164).

TRIM5α is a member of the tripartite motif (TRIM) protein family, which includes proteins that share an N-terminal domain consisting of a combination of a RING domain that functions as an E3 ubiquitin ligase, one or two B-boxes, and a coiled-coil domain. The C-terminal domain of TRIM5α is the SPRY-PRY domain, in which the RING/SPRY and coiled-coil/SPRY domains are separated by two linker regions, termed L1 and L2, respectively (165). Pull down and two-hybrid experiments using CA demonstrated that TRIM5α binds to the viral capsid (166–168), and that the SPRY-PRY domain governs the ability of TRIM5α to bind CA (169). However, this interaction is weak due to the millimolar affinity of the CA-TRIM5α binding (170,171), and higher order assembly is required to generate sufficient avidity to allow efficient capsid recognition. Dimerization of TRIM5α proteins was initially suggested by the observation that expression of a dominant negative defective TRIM5α harbouring only the SPRY-PRY domain results in the abrogation of the restriction factor activity (171,172). More recently, using a mutagenesis approach, Roganowicz *et al.* identified an α-helical segment that is responsible for the packing of the SPRY domain against the coiled-coil scaffold. This packing allows for a formation of a more precise supramolecular structure, by limiting the number of possible conformations that the SPRY domains can adopt relative to each other (171). Electron microscopy and biochemical studies have further confirmed this hypothesis showing that TRIM5α forms dimers, which in turn assembly into a hexagonal lattice which coats the viral capsid (168,173,174). The dependence of TRIM5α activity to the ability of the molecule to assembly in higher order structures is confirmed also by the observations made using the TRIM5α-CypA isoform. TRIM5α-CypA, expressed in owl monkeys, cynomolgus monkeys and rhesus monkeys, is a fusion protein resulting from the insertion of the coding region of Cyclophillin A (CypA) within the TRIM5α gene. The CypA domain, which replaces the SPRY domain in the

fusion protein, is known to bind CA (67) with high affinity and thus TRIM5α-CypA could potentially exert its effect without the need of forming a higher ordered structure. Nevertheless, as recently shown by Wagner et al., TRIM5α and TRIM5α-CypA are both characterized by self-assembly properties and share the same mechanisms for viral capsid binding (175).

The binding of TRIM5 to the viral capsid promotes premature disassembly of the viral CA lattice, thus preventing reverse transcription and integration (168,170). While the SPRY domain is responsible for the recognition of the viral capsid, TRIM5α effector function depends on the presence of the RING domain (176), which mediates the E3 ubiquitin ligase activity. Consistent with involvement of proteasome in TRIM5α activity, inhibition of proteasome prevents the premature disassembly of the capsid and restores HIV-1 reverse transcription (177,178). Autophagy pathway has been also implicated in TRIM5α activity based on the observation that TRIM5α associates with the autophagic adaptor protein p62, and that depletion of p62 improved infection efficiency in presence of TRIM5α (179).

An alternative mechanism through which TRIM5α exerts antiviral activity is the enhancement of IFN pathway activation. Crucial to TRIM5 α-mediated IFN activation is the synthesis of free K63-linked ubiquitin chains that bind to the transforming growth factor beta-activated kinase 1 (TAK1) kinase complex, promoting the translocation of restriction factors NF-kB and AP-1 to the nucleus and leading to the upregulation of type I IFN and other proinflammatory cytokines. The synthesis free K63-linked ubiquitin has been shown to depend on the presence of a SUMOylation domain (180,181), which modulates the activity of E3 ubiquitin ligase, promoting K63-linked ubiquitin chains at the expense of auto-ubiquitylation (180). Interestingly, in dendritic cells, where SUMOylation activity prevails over auto-ubiquitylation, TRIM5α lacks restriction activity but triggers the innate sensing of viral DNA by cGAS and robust IFN I production (182).

Restriction factors halting reverse transcription: SAMHD1 and APOBEC3G/F.

SAMHD1: Sterile alpha motif (SAM) histidine-aspartic (HD) domain protein 1 is a restriction factor that targets reverse transcription by reducing the pool of nucleotides available for RT. SAMHD1 converts dNTPs to the corresponding deoxynucleoside and inorganic triphosphate (183). Nucleotides bind to two allosteric sites present in each subunit of SAMDH1, inducing a conformation change and the multimerization of the protein into the active tetramer (184,185).

The role of SAMDH1 in determining the size of the dNTP pool within the cell has been identified in patients affected by Aicardi-Goutières Syndrome, a neurological disorder characterized by over expression of type I IFN and IFN-associated genes (135). In these patients, homozygous mutations in SAMHD1 gene, as well as in other genes involved in dNTPs metabolism, lead to the accumulation of free nucleotides in the cytoplasm, which, in turn, triggers IFN production (186).

The restriction activity of SAMHD1 against retroviruses was suggested by its isolation in pull down experiments targeting VpX, an HIV-2 protein which allows infection of non-dividing cells (187). Nucleotide availability is essential for completion of reverse transcription and HIV infection is more efficient in cycling cells, which are characterized by higher levels of free nucleotides in the cytoplasm. (188). Exogenous expression of SAMHD1 in U937 cells causes a drop in the size of dNTP pool, which is associated to resistance

to infection. In these experimental conditions, infection efficiency can be restored by supplementation of dNTPS to the culture media (189).

Although SAMHD1 expression is ubiquitous, HIV-1 restriction is observed only in monocyte derived macrophages, dendritic cells and resting CD4 T cells, indicating that the activity of the protein is regulated by a post-translational mechanism likely linked to cell cycle progression (190,191). Several lines of evidence suggest that modulation of SAMHD1 activity results from the interaction with the cyclin-dependent kinases (CDK) CDK1, CDK2, and CDK6, which are active only in dividing cells (192–195). CDK mediated phosphorylation of SAMHD1 C-terminal domain induces the inactivation of the protein resulting in the increase in intracellular dNTPs levels necessary for completing the S-phase (192). Phosphorylation of the threonine residue at position 592 has been specifically associated with SAMHD1 restriction activity. Consistent with a role in modulating susceptibility to HIV-1, stimulation of resting CD4+ T cells strongly induces T592 phosphorylation and a phosphorylation–defective mutant of SAMHD1 retains the restriction activity also in cycling cells (196,197).

Although clearly associated with the ability of SAMHD1 to limit HIV-1 infection, the effect of T592 phosphorylation on nucleotide metabolism is controversial. While some authors have described a substantial increase of the dNTP pool following T592 phosphorylation (195,196,198); others have reported that it does not affect triphospohydrolase activity of enzyme (199,200). The latter scenario would indicate an alternative mechanism responsible for the restriction effect of SAMHD1, independent of its dNTPase activity (191,200–202). Interestingly, SAMHD1 also binds single-stranded DNA and RNA and is reported to have nuclease activity against different substrates (203). Using SAMHD1 mutants that retain only one of the two enzymatic activities, Ryoo *et al.* have provided evidence that the restriction imposed by SAMHD1 to viral replication is the result of the viral RNA degradation rather than dNTP depletion. According to this model, switch between RNAse and dNTPase activity would be the consequence of increased dGTP levels, as dGTP is responsible for the tetramerization of the enzyme required for hydrolase activity (203). High dNTPs levels would also inhibit RNAse activity, explaining why addition of exogenous dNTPs can overcome the block to HIV-1 replication imposed by SAMHD1 (Figure 2A). Furthermore, the low-level of dNTPs in resting cells would slow down reverse transcription, allowing a sufficient time frame for the degradation of viral RNA by SAMHD1 (202).

APOBEC3G: Apolipoprotein mRNA editing catalytic polypeptide-like (APOBEC)-3 is a family of cytidine deaminases showing antiviral activity against HIV-1 and other retroviruses (204), as well as retrotransposons (205) and hepatitis B virus (206). The family includes seven members (A, B, C, D, F, G, and H), of which APOBEC3G (A3G) has been characterized as restriction factors.

Due to their ability to interact with the nucleocapsid protein and with viral nuclei acids (207,208), APOBEC-3 deaminases are incorporated into HIV-1 virions (209,210) and exert their antiviral activity in newly infected cells (211,212). HIV-1 has evolved to encode an additional protein, called Viral Infectivity Factor (VIF), to counteract this restriction activity. VIF decreases the amount of APOBEC-3 available for encapsidation by inducing its ubiquitylation and subsequent degradation by the proteasome (213).

In infected cells, A3G inhibits viral replication through three mechanisms: lethal mutagenesis, degradation of deaminated viral cDNA, and deamination independent block of reverse transcription (Figure 2C). Lethal mutagenesis occurs during reverse transcription and results from the deaminase activity of the enzyme, which converts the dCs to dUs in the (-) strand DNA thus determining a massive G-to-A mutation of the nascent viral DNA genome (214–217). The resulting viral DNA is correctly integrated in the host genome, however the high frequency of premature stop codons introduced by the massive hypermutation prevents the translation of viral messengers thereby terminating viral infection. Besides introducing stop mutations within the coding regions of the genome, A-to-G hypermutation can also target the regulatory portions of viral DNA; such as the trans-activation response (TAR) element, imposing an early block to viral gene expression and further affecting the transcriptional activity of the virus (218).

Degradation of deaminated viral DNA by the uracil base excision repair (BER) pathway has been proposed as an additional antiviral mechanism resulting from A3G activity. Under physiological conditions, the BER pathway is responsible for removing the uracil from DNA molecules, as their presence indicates DNA damage. Uracil is recognized and excised by the uracil–DNA glycosylases (UDGs), and the resulting DNA molecules harboring missing bases are then processed by the DNA repair enzymes. In the case of A3G mediated deamination, it has been hypothesized that the generation of abasic sites by UDGs would promote the degradation of viral DNA. The relevance of BER for the antiviral activity of A3G is controversial and contrasting observations have been made following knock down of UDGs in vitro (219–222). A recent work by Pollpeter *et al.* reported that, although excision of dUTPs from deaminated viral DNA takes place, it does not lead to the endonucleolytic cleavage of viral DNA, as shown by the substantial proportions of edited DNAs which remain detectable (222). However, inefficient UBER recognition of edited cDNA could still play a role in the interplay between HIV-1 and infected cells, for instance through sensing of aberrant cDNA fragments as a pathogen-associated molecular pattern.

Finally, A3G prevents the establishment of a productive infection by blocking RT elongation in a deaminase independent manner. Evidences for this mechanism of action derive from the observation that deaminase deficient A3G mutants are still able to affect cDNA production (223,224). Mechanistically, it has been shown that, within the virion, A3G initially binds the ssDNA strand in a monomeric form which is responsible for the deaminase activity. A3G monomers slide along the ssDNA and perform multiple cycles of association/dissociation leading to the editing of the (-) strand DNA. In a subsequent phase, A3G monomers associate in dimers, which have a slow dissociation rate and reduced mobility. Although the dimers lack the enzymatic function, they act as roadblocks for RT, preventing the elongation of the nascent DNA (225).

Restriction factors preventing integration: MX2

MX2: Myxovirus resistance protein 2 is a highly conserved protein belonging to the dynamin superfamily of large guanosine triphosphatases (GTPases) (226), whose expression is strongly induced by type I and type III IFN (227,228). It was identified as a lentivirus restriction factor in a screening comparing gene expression profiles in cell lines showing different susceptibility to HIV-1 infection (229,230). MX2 it is able to bind CA directly (231) or via the interaction with CypA (232) and thus is thought to act in the early phases of replication.

Similarly to TRIM5α, the interaction of MX2 with the viral capsid requires the formation of high-ordered structures (233,234), and loss of multimerization capacity is associated with the inability to block HIV-1 infection (235). Consistently, Alvarez *et al.*, using cryo-electron microscopy, have shown that oligomerization is a GTP-dependent process and that GTPase activity is essential for MX2 antiviral activity. GTP binding triggers conformational changes in MX2, causing a disruption in the oligomer assembly, while GTP hydrolysis leads MX2 to reverts back and assembly into competent conformation (236).

Blocking of uncoating and inhibition of nuclear import have both been implicated in the activity of MX2 against HIV-1. Stable expression of MX2 increases the amount of pelletable capsid obtained after infection suggesting that MX2 is able to prevent or delay uncoating (234). On the other hand, the observation that, in presence of MX2, the levels of integrated DNA and 2-LTR circles are increased despite stable levels of first strand cDNA synthesis suggests that MX2 impairs the nuclear entry of the viral genome (229,230). Exogenous expression of MX2 does not affect the functionality of PICs, supporting the notion that MX2 restriction activity occurs at the level of nuclear import rather than of reverse transcription (237). DNA destabilization was also hypothesized as a mechanism for MX2-mediated inhibition, based on the observation that the protein harbors a nuclear localization domain and could possibly exert its activity within the nucleus. However, mutagenesis experiments have shown that its antiviral activity is independent from nuclear translocation, and that the amino acid string containing the NLS instead plays a role in the oligomerization process (231).

Restriction factor preventing viral spread: BST2

BST2: Bone marrow stromal cell antigen-2 also known as Tetherin, is a restriction factor that impairs the release of many enveloped viruses, such as retroviruses, herpesviruses, filoviruses, rhabdoviruses, paramyxoviruses and arenaviruses. In HIV-1 infection, BST2 was initially identified as dominant factor whose activity was blocked by the viral accessory protein Vpu. Mature, fully formed particles are produced after infection with Vpu defective strains, however virions are trapped within intracellular vacuoles at cellular surface and cannot be released (238,239). The presence of Vpu is dispensable for infection in some cell lines, whereas others require it only after treatment with type I IFN, suggesting that the restriction factor is cell type specific and regulated by the IFN system.

Virions generated following infection with a Vpu defective strain accumulate on cell surface as wells as in intracellular compartments, suggesting the involvement of the endocytic pathway in BST2 mediated restriction (240), thus allowing the visualization of rod-like bodies which connect the virions with the plasma membrane. (241). Consistent with the identification of BST2 as the target of Vpu activity, it has been shown that the Vpu leads to BST2 degradation through the proteasome and the lysosomal compartments (209,242,243), as well as through a non-canonical autophagic pathway involving the recruitment of LC3 (244).

BST2 is a transmembrane protein whose two membrane anchors, one at N-terminus and one at C-terminus, act as hinges for a coiled-coil domain that protrudes on the outer side of the plasma membrane (245). Both membrane anchors are essential for restriction activity and either of them can interact with the membrane of the virions (246). Synthetic proteins made by combining domains structurally similar to the ones of BST2, but taken from unrelated proteins, retain their antiviral properties, suggesting that structural motifs, rather than

specific amino acid signatures, are required for the activity of this restriction factor (246). BST2 molecules act as a bridge between budding virions and cellular membranes causing the retention of the nascent viral particles at the surface of infected cells (247). Clusters of BST2 proteins have been visualized at the interface between the plasma membrane and the retained viruses as extended filamentous electron dense structures, which are specifically recognized by antibodies against BST2 (247,248).

The immune checkpoint proteins (ICPs)

ICPs are proteins that play pivotal roles in the immune disfunction and T-cells exhaustion. They have been described as inhibitory receptors expressed by immune cells during inflammation or chronic infection in order to activate the immunosuppressive signaling pathways that are crucial for maintaining self-tolerance, for modulating the length and magnitude of effector immune responses in peripheral tissues and minimize collateral tissue damage. Signaling via these molecules leads effector immune cells (especially T-cells) into a state known as "exhaustion". T-cell exhaustion is defined by reduced effector function, sustained expression of immune checkpoint molecules (such as TIM-3, LAG3, CTLA4, the PD- 1/PD-L1 axis or TIGIT), poor recall responses and a transcriptional state distinct from that of functional effector or memory T-cells. There are several types of activating and inhibitory interactions that occur between APCs and T cells that are relevant for immune responses. It is now established that pathogens and cancers promote inhibitory interactions between immune cells via ICPs to escape immune control. During untreated HIV-1 infection, an up-regulation of multiple immune checkpoint proteins, including PD-1 and PD-L1, have been observed on both CD4+ and CD8+ T cells. Following ART, the expression of ICPs declines but remains elevated compared to healthy subjects. Whether ART is started early (within 6 months of infection) or late (within 2 years of infection), similar levels of expression of immune checkpoint proteins persist (249). During the HIV-1 infection, this expression varies on different T-cell subsets (250). Therefore, it might be assumed that the expression profile of ICPs reflect the immune condition and activation. As ICPs are relevant in the changed environment built by pathogens, various studies have been done with the purpose to find good strategies to block the process of T-cells exhaustion and the absence of an immune response. Recently, Benci and colleagues observed that cancer cells chronically exposed to IFNγ showed an increment expression of ISGs and inhibitory receptors on T-cells such as PD-L1 with consequently T-cells exhaustion and tumor progression (251). They further observed a change in the ICPs profile, a restoration of immune cells and then an anti-tumor response after the introduction of therapeutically strategies based on the use of antibodies anti-ICPs and JAK-STAT inhibitors of IFNα signaling pathway. Moreover, in a different study on HIV-1 infection, Hoffmann and colleagues described the existence of a correlation between the expression of specific ICPs and the activation ability of CD8+ T-cells with HIV-RNA (copies/ml) in plasma sample. In particular, they showed a linear correlation between CD8+ T-cells activation (CD8+CD38+) and exhaustion (CD8+PD-1+) status. A similar correlation was further observed between the amount of HIV-RNA (copies/ml) and the percentage of CD8+ T-lymhocytes expressing both PD-1+ and CD38+PD-1+. Thus, during viral replication, an increment of T cells activation and exhaustion (referred to PD-1 expression) is crucial (252).

The balance between co-stimulatory and co-inhibitory signals is pivotal for the activation of immune response. During chronic HIV-1 infection, viral replication and the persistence of viral antigen exposition impairs immune functions. It has been observed that a prolonged antigen exposure during chronic infections give rise to T-cell exhaustion with a loss of proliferative capacity and effector function (253). Naïve T cells become

activated and an additional co-stimulatory signal promotes T-cell differentiation and effector functions. TCR signaling in the absence of co-stimulation induces T-cell anergy and weak co-stimulatory signals or a preferential engagement of co-inhibitory pathways during T-cell priming in vivo can lead to T-cell tolerance (254). The co stimulatory molecule CD28 and the co-inhibitory molecules cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) and programmed death 1 (PD-1; CD279) are particularly important for regulating T-cell responses (255). The co-inhibitory molecule PD-1 gained much attention in viral immunology as it plays a significant role in establishment of virus-specific CD8+ T-cell exhaustion.

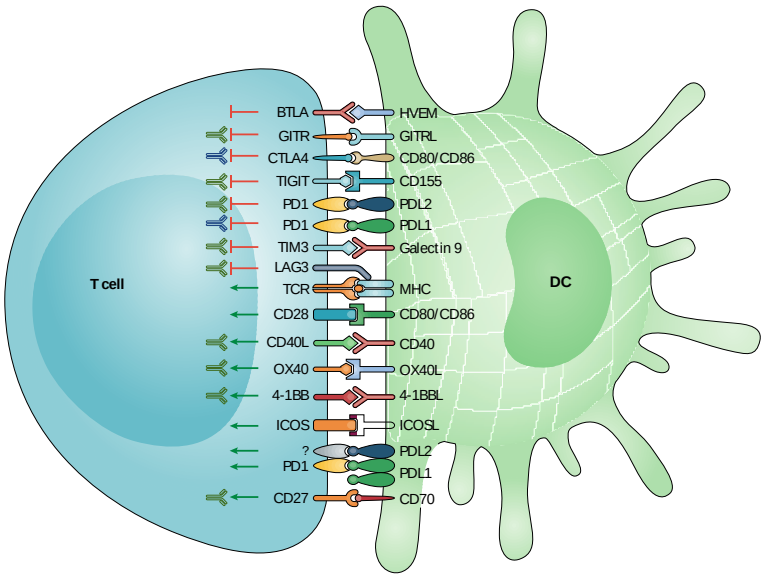


Figure 9: expression pattern of ICPs on immune cells surface (250).

Among the ICPs, this project gains much attention on TIGIT, PD-1, its ligand the PD-L1 and LILRB2 as molecules playing pivotal role in the immune cells disfunction (Figure 9).

TIGIT (T-cell Immunoreceptor with Ig and ITIM domains) is a coinhibitory receptor widely expressed on T and NK cells, as well memory T cells, follicular Th cells and Tregs (256,257). Two strategies have been described by which TIGIT can inhibit T-cell response: indirectly, by binding the CD155 (or PVR) and CD112 (or PVRL2) express on DCs, thereby inducing the production of IL-10 and preventing DC maturation, or directly through the recruitment of the phosphatases SHIP1 and SHP2. TIGIT counterpart is represented by the co-stimulatory molecule, the CD226, thus when PVR binds to TIGIT, it mediates inhibitory signals to T cells but transmits stimulatory signals while binds to CD226. Additionally, TIGIT-PVR ligation leads to a NK cytotoxicity reduction, granule polarization, and cytokine release (258). Recent studies revealed that TIGIT/PVR signaling had also been implicated in inhibiting the metabolism of CD8+ T cells, and therefore suppressing the effector function (259). Studies performed on cancer and inflammatory cells have shown high variability of TIGIT expression levels and frequent co-expression with PD-1 suggesting that the distinct function are involved most likely to prevent excessive immune responses (256,257). Moreover, higher expression of TIGIT and PD-1 in lymphocytes associated to areas of inflammatory diseases compared to areas

that are less populated by T-cells (such as connective tissues, intraepithelial areas) proves that downregulation of excessive inflammatory reactions through immune checkpoint upregulation (260).

PD—1 (Programmed Cell Death 1) has potent inhibitory effects on immunity and it is expressed on T, B, NK cells as well as on DCs and activated monocytes. It binds to PDL-1 and PDL-2 both mostly expressed on T-cells. PD-1 was identified as a gene up-regulated in a T-cell hybridoma undergoing apoptotic cell death, and was thus named programmed death 1 (261,262). PD-1 is inductively expressed on CD4+, CD8+, NK T-cell subsets, B cells and monocytic cell types upon activation. In close similarity to other CD28 family members, PD-1 transduces a signal when engaged along with TCR ligation. The cytoplasmic domain of PD-1 receptor contains two tyrosine signaling motifs, both of which may be phosphorylated upon receptor engagement. Phosphorylation of the second tyrosine, the immuno-receptor tyrosine–based switch motif, recruits the tyrosine phosphatase, SHP-2 and to a lesser extent SHP-1 to the PD-1 cytoplasmic domain. Recruitment of these phosphatases leads to dephosphorylation of TCR proximal signaling molecules including ZAP70, PKCθ, and CD3ζ, leading to attenuation of the TCR/CD28 signal (263). PD-1 signaling prevents CD28 mediated activation of phosphatidylinositol 3-kinase, resulting in reduced Akt phosphorylation and glucose metabolism. The PD-1 ligands have distinct patterns of expression.

PD-L1 (Programmed cell Death Ligand 1) is broadly expressed on both professional and non-professional APCs, whereas PD-L2 (B7-DC; CD273) is expressed in an inducible manner only on dendritic cells (DCs) and macrophages. PD-L1 is constitutively expressed on B cells, DCs, macrophages and T cells, and is upregulated upon activation. PD-L1 is also expressed on a wide variety of non-hematopoietic cell types, including vascular endothelial cells, kidney tubular epithelial cells, cardiac myocardium, pancreatic islet cells, glial cells in the brain, inflamed muscle, and keratinocytes and also immune privilege sites such as the placenta and eye. Interferon α, β, and γ are powerful enhancers of PD-L1 expression on APCs, endothelial cells, and epithelial cells. During pro-inflammatory immune responses, such as infection or transplant rejection, PD-L1 expression is intense and extensive. PD-L1 expression is found in many solid tumors, and high expression is associated with poor disease prognosis. Several recent studies suggested that PD-1– PD-L pathway plays an important role in exhaustion of antitumor as well as anti-viral CD8+ T cells during chronic infections (264,265).

HIV-1 chronic infection is characterized by a prolonged antigen exposure leading to T-cell exhaustion and towards a loss of proliferative capacity and effector function. Evidence show that pathogens successfully evade immunity by activating negative regulatory pathways that play an important role in maintaining peripheral tolerance and avoiding excessive immune activation under physiologic conditions. Complex mechanisms are involved in this T-cell dysfunction and PD-1 has been identified as a major regulator of T-cell exhaustion during chronic HIV/SIV infection. Blockade of the PD-1 pathway in non-human primate model of HIV infection can reinvigorate exhausted T cells, resulting in enhanced viral control during chronic SIV infection (249,253,266).

The presence of IFNα then leads to various effects: the induction of ISGs in infected and neighboring cells, the enhancement of the process of antigen presentation and chemokine production in innate immune cells, the boosting in the production of virus-specific antibodies and the increasing of effector T-cells responses. As the production and exposure of cells to IFNα becomes chronic, the scenario changes. In fact, it can be seen an induction of immunosuppressive pathways involving IL-10 and probably PDL1 (Programmed cell Death Ligand 1), which suppress the function of T-cells and feed back to also suppress innate immune cells. It can be assumed then that as the exposure to IFNα becomes chronic, there is evidence of immune dysfunction and T-cells decline.

LILRB2 (Leukocyte immunoglobulin-like receptors B2), also known as Immunoglobulin-like transcript 4 (ILT4), is an inhibitory receptor specifically expressed on myeloid cells that plays an important role in the regulation of DC functions. The inhibitory activity of this receptor is structurally and functionally related to their long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and to killer cell inhibitory receptors (KIRs) (267). Because these receptors mediate the inhibition of T-cell activation they have been proposed as a new group of immune checkpoint molecule (268,269). The potency of the LILRB2/MHC-I inhibitory axis can be modulated by genetic polymorphisms of MHC-I haplotypes. In fact, it has been observed as the natural progression of HIV-1 infection depends on genetic variation in the MHC-I, and the $CD8^+$ T cell response is thought to be a primary mechanism of this effect.

Moreover, antigen presenting function of monocytes and DC is modulated by the expression level of LILRB2 that appeared upregulated in monocytes and DCs upon their interaction with antigen-specific $CD8^+$ $CD28^-$ T suppressor cells, or upon exposure to inhibitory cytokines, such as IL-10. In fact, sera derived from HIV-1 infected patients induce the upregulation of LILRB2 on uninfected monocytes because of the elevated levels of serum IL-10 (270). Recently it has been analyzed tha LILRB2-HLA class I interactions influence viral load in HIV-1 infection. This effect appears to be driven by variations in LILRB2 binding affinities to HLA-B and is independent of individual class I allelic effects that are not related to the LILRB2 function (271). More recently, Aloui *et al* performed *in vitro* studies with the aim to investigate the strengthened interaction of LILRB2 with its MHC-I ligands induces the dysregulation of cDCs which is characterized by an altered capacity to stimulate CD4+ T cells and produce cytokines and consequently the rate of disease progression in HIV-infected patients (272). Moreover, the upregulation of both LILRB2 and MHC-I could be advantageous for HIV-1 to dysregulate cDC functions in early infection, a critical stage for the onset of a productive immune responses against viruses. The simultaneous expression of LILRB2 and its MHC-I ligands transiently increases at the surface of cDCs from blood and lymph nodes during the first days of infection. Early dysregulation of cDC functions hinders the initiation of an efficient host immune response to control viral infection and the enhancement of LILRB2 and MHC-I expression on cDCs during the acute phase of infection suggest a major role of the LILRB2/MHC-I inhibitory in the efficiency of immune responses attenuation and result in viral persistence (272).

Aim of the study

HIV-1 establishes latent infection in resting memory CD4+ T cells and macrophages. Latency occurs when the HIV-1 proviral DNA integrates into host chromosomal DNA but fails to express viral RNA and proteins (273). With currently available cART, most HIV-infected patients are able to achieve and maintain HIV viral suppression, nevertheless a latent form of HIV infection persists as integrated provirus in anatomical and cellular reservoirs. The elimination of the latent reservoir is critical to achieving HIV eradication (274).

One of the first lines of defense against HIV are Plasmacytoid Dendritic cells (pDCs) and Natural Killer (NK) cells: these cells can influence HIV pathogenesis as they are the major producers of type I Interferon (IFN-I), which regulates the expression of correlated genes that have antiviral activity, mediate cytolysis and produce numerous cytokines. Moreover, they can control the virus during the first stages of infection and shape the adaptive immune responses that will act later. As the infection occurs, it is registered an expansion of NK cells followed by a dysregulation partially driven by HIV, meaning that it can evade this defense mechanism.

The adaptive cellular response involves the action of CD4+ and CD8+ T cells. In particular, CD8+ T cells play a pivotal role in the control of infection during the course of acute phase. This leads to a decrease of viremia but also to viral escape mutants selection. In addition, CD8+ action is targeted against HIV epitopes by a mechanism that can be either non-lytic (secretion of soluble factors such as β-chemokines) or lytic (cytolysis of infected cells). However, this action is efficient only during the acute phase of HIV infection as its continuous replication provokes the progressive exhaustion of CD8+ T cells associated with the surface expression of negative regulatory molecules as PD-1 (265).
When HIV infects host cells, the production of IFNα is mainly due to myeloid cells and pDCs and triggers the transcription of ISGs in infected and neighboring cells, the enhancement of the process of antigen presentation and chemokine production in innate immune cells, the boosting in the production of virus-specific antibodies and the increasing of effector T-cells responses.
Among ISGs induction there are the production of Restriction Factors (RF), cellular proteins described as part of immune system and that are able to interfere with viral lifecycle by acting on different steps of viral lifecycle such as cellular entry and uncoating, reverse transcription, nuclear import, integration, budding and viral spread. Because of their interplay with viral replication, and in particular with reverse transcription and integration which are essential steps for the onset of latency, it is assumed a pivotal relationship between RF inductions and the viral amount in latently infected cells (209).
As part of my PhD project, on the basis of scientific data suggesting the crucial role of RFs in HIV infection , replication and latency, a group of RFs has been selected for this study: APOBEC3G, SAMHD1, MX2, SERINC3 and SERINC5, TRIM5, BST2) as well as the cellular sensor IFI16 associated to STING activation in the pathway that leads to ISGs induction.

The main aim of this study was the analysis of RFs expression levels during the course of infection in patients that are naïve to cART (characterized by high viral load and slow CD4+ T cells count) followed during the first year of follow up (4 and 8 months from the first diagnosis) and patients with undetectable levels of viral load from at least two years and under cART administration. By the end, correlation analysis between viral and immunological parameters was further performed in order to identify a link between the expression levels of one or more RFs and the size of viral reservoir.

The second part of this study was performed in Paris, at the INSERM laboratory where according to the notion that type I IFN production begins in the acute phase and its role changes during the course of infection, the induction of immunosuppressive pathways involving IL-10 and probably PD-L1 (Programmed cell Death Ligand 1) are further involved (275). It can be assumed then that as the exposure to IFNα becomes chronic, there is evidence of immune dysfunction and T-cells decline.

In this scenario, other cellular proteins are involved: in particular the immune checkpoint molecules (ICPs) are inhibitory receptors expressed on immune cells (mostly extracellularly) that trigger immunosuppressive signaling pathways, maintain self-tolerance and modulate the length and magnitude of effector immune responses in peripheral tissues, in order to minimize tissue damage (251).

In untreated HIV infection, ICPs appear up-regulated, including PD-1, CTLA4, TIM3 and LAG3 on both CD4+ and CD8+ T cells. Following ART, their expression declines but remain elevated compared to uninfected controls. Whether ART is started early (within 6 months of infection) or late (within 2 years of infection), similar levels of expression of immune checkpoint proteins persist (276).

Therefore, it might be assumed that the expression profile of ICPs reflect the immune condition and activation as well as the expression pattern of RF during the early phase of infection, before the beginning of ART, could influence the reservoir size.

The major aim of this project is to assess the clinical meaning of ICPs expression in HIV-1 chronically infected patients to better characterized their involvement in immune system disfunction.

Materials and methods

Chapter 1: RFs analysis

Sample collection

HIV-1 positive subjects were enrolled by the Infectious Disease unit and blood samples were collected by Retrovirus laboratory, Microbiology section of Sant'Orsola Malpighi hospital of Bologna, Italy. Buffy coat samples of healthy donors were collected by the Transfusion unit of Maggiore hospital of Bologna. This study was approved by the local Ethical Committees of Area Vasta Emilia Centro of Emilia-Romagna (CE-AVEC) and all subjects included in this study provided written informed consent.

Peripheral blood mononuclear cells (PBMCs) and nucleic acids extraction.

PBMCs from healthy donors (n=14) and HIV-1 positive patients at each time of collection (n=14) were isolated from fresh whole blood using density gradient centrifugation with Ficoll (Ficoll-Paque PLUS Pharmacia, Uppsala, Sweden). Cell samples where then stored at -80°C for the next nucleic acids extraction.

RNA was extracted from each cell samples by using the Quick-RNA^{TM} Miniprep Kit (Zymo Research Corp.) according with the manufacturer's protocol. This kit provided a double column filter system to perform a first separation phase between RNA and DNA from the same sample. Furthermore, a DNasi treatment is recommended to prevent the genomic contamination that could be present during data analysis. DNA extraction was carried out with Quick DNA™ Microprep Kit (Zymo Research Corp.) according with the manufacturer's protocol. DNA and RNA were both eluted in 50 µl of nuclease free water supplied by kits and stored at -80°C until the molecular analysis.

Real Time PCR for RF

Total RNA concentration and purity were measured using a NanoDrop2000 spectrophotometer (ThermoFisher Scientific) and 500 µg was reverse transcribed into cDNA using cDNA Synthesis Kit (Biotechrabbit) according with the manufacturer's protocol. A negative control of reverse transcription was included for each primer pairs to exclude nontranscribed genomic DNA contamination and to tests the efficiency of the reverse transcription reaction.

cDNA was diluted to a final concentration of 5 ng/µl and 25 ng were used in a 20 µl real time PCR reaction mix. The reaction mix includes 10 µl of 2X buffer (QuantiFast SYBR Green PCR kit, Primerdesign), 0.3 µM of forward and reverse primer. Real time PCR were performed on Rotor-Gene Q (Qiagen) at the following conditions: 95°C for 10 minutes, 40 amplifications cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The melting curve for the assay specificity was set up from 60°C to 95°C. The reference genes included for the normalization analysis were the β-actin (*ACTB*) and the glyceraldehyde-3-phosphate

dehydrogenase (*GAPDH*), according with previous studies where ACTB and GAPDH have been validated as the most stable reference genes in many species (277)(278)(279)(280). However, our preliminary studies revealed that the ACTB was the most stable and so it was selected to determine the effectiveness of extraction and purification of nucleic acids. Relative gene expression values of RF were calculated according to the 2ΔΔCt method.

Primers for RF were designed manually using SnapGene Viewer 4.1.8 and to test their specificity each primer was blasted and ordered at ThermoFisher Scientific. Primer sequences are listed in table 1.

GENE NAME	GenBank ID	PRIMER SEQUENCE (5' TO 3')	Position cDNA
APOBEC3G	NM_021822	FP: TAGCCGGCCAAGGATGAAGC RP: TCCGACGAGAAAGGATGGGTC	345-364 428-448
MX2	NM_002463	FP: AACACCGAGCTAGAGCTTCAGG RP: AGGTCAATGATGGTCAGGTCTGGAAC	627-648 756-781
SAMHD1	NM_015474	FP: AGGTGTGCTCCTTCCTCAGG RP: AAGGCAGTAATGCGCCTGTG	346-365 419-438
SERINC3	NM_006811	FP: ACCACCGTGTTAGAAAGCAGC RP: AGCAACTACACAGCAAACATGAGG	111-131 227-250
IFI16	NM_005531	FP: AAGGAGCAGAGGCAACTCCTG RP: CACCTTACTCCCTTTGGGTCCAG	643-663 700-722
BST2	NM_004335	FP: CAGAAGGGCTTTCAGGATGTGG RP: TTGTCCTTGGGCCTTCTCTGC	313-334 391-411
SERINC5	NM_001174071	FP: AGCACCCGCTTCATGTACG RP: CAGGTGTCACCAGCTTTAATGCC	260-278 383-405
STING	NM_001301738	FP: TGAACATCCTCCTGGGCCTCAAGG RP: GGATCAGCCGCAGATATCCGATG	482-505 585-607
TRIM5α	NM_033034	FP:GGGCAGAAGTAGGAAGTCTTTGGG RP: AGGAGTTCCAGGCAGATGGG	199-222 349-368
TRIM11	NM_145214	FP: CCGGAAGCAGATGCAGGATG RP: TCGAACTCACCCAGCACGTTC	693-712 783-803
ACTB	NM_001101	FP: GAC AGG ATG CAG AAG GAG ATT ACT RP: TGA TCC ACA TCT GCT GGA AGG T	1015-1038 1135-1156

Table 1: RFs primers sequences

Total HIV DNA analysis

Total DNA concentration was quantified with NanoDrop2000 spectrophotometer (ThermoFisher Scientific) and the HIV-1 reservoir was analyzed by quantifying the Total HIV-1 DNA. Total HIV-1 DNA was quantified by using a commercially available kit, the HIV-1 DNA test (Diatheva) a kit based on the amplification of a specific sequence with the use of a fluorescent-labelled probe. According to the manufacturer's protocol, 1 µg of purified DNA (the equivalent of 1.5 $x10^5$ PBMCs) was used for this analysis in 50 µl of reaction mix. The reaction was performed on Versant kPCR (Siemens) at the following conditions of temperature and cycles: 95°C for 3 minutes, 50 amplifications cycles of 95°C for 20 seconds, 60°C for 60 seconds. According to the manufacturer's protocol, the kit provides a 5-points calibration curve useful for the quantitative analysis and the HIV-1 detection is based on the fluorescent acquisition on green channel. The results were expressed as HIV-DNA/10^6 PBMCs copy number.

Statistical Analysis

For statistical analyses, the non-parametric unpaired analysis was selected. The intergroup comparisons were assessed using Kruskal-Wallis test and the Dunn's correction was performed. The threshold for statistical significance was set to $p < 0.05$. Correlation analyses were performed with the non-parametric Spearman test, two-tailed with a confidence interval of 95%. The Spearman's rank correlation coefficient (r) > 0,05 was considered. Data were analyzed using GraphPad PRISM5 software.

Chapter 2: ICPs analysis

Blood sample collection and PBMCs isolation

Fresh whole blood samples were collected after written informed consent. Plasma sample were than obtained by centrifugation 10 minutes at 1800 rpm and finally stored at -80°C for further analysis. To date 5 healthy donors and one HIV1 positive patients have been enrolled. PBMCs were isolated from fresh whole blood using density gradient centrifugation with Pancoll human (PAN-Biotech). Cells were counted by using Malassez counting chamber and seeded according to our experimental times of collection:

- RNA analysis
  - $1x10^6$ PBMCs were collected and harvested (*ex vivo* analysis)
  - $3x10^6$ PBMCs were seeded in a 48 multi-well plate at the concentration of $1x10^6$cells/ml and collected after 4 hours of IFNs stimulation
- Flow Cytometry analysis
  - $3x10^6$ PBMCs were divided into several tubes (500.000 cell/tube) (*ex vivo* analysis)
  - $9x10^6$ PBMCs were seeded in a 6 multi-well plate in order to have $3x10^6$ PBMCs/well at the concentration of $1x10^6$cells/ml; cells were stimulated with IFNs and maintained in culture 24 hours to be finally collected

IFNα and IFNγ were used at the final concentration of 500 U/ml and 10 ng/ml, respectively. A MOCK sample (unstimulated cells) was further included as control.

RNA extraction, reverse transcription and Real Time qPCR

RNA was extracted from all cell samples by using the NucleoSpin RNA Plus kit (Macherey-Nagel) according to manufacturer's protocol. Total RNA was eluted in 60 ul of RNasi/DNasi free water and concentration with purity were measured using a NanoDrop2000 spectrophotometer (ThermoFisher Scientific). 7 ul of extracted RNA was reverse transcribed into cDNA using the Enhanced Avian HS RT-PCR kit (SIGMA ALDRICH) according to the manufacturer's protocol.

cDNA was finally used to perform Real Time qPCR on IP-10, MX1, LILRB2 and PD-L1 genes by using ThermoFisher commercially available primers (reference Hs00171042, Hs00895608, Hs01629548 and Hs00204257 respectively). 18S (reference Hs03003631) was also included as housekeeping gene required for the normalization analysis. Real Time qPCR was performed in duplicate in a 20 ul of reaction mix. cDNA was diluted 1/10 and 4 ul was added at the reaction mix including 1 ul of primer, 10 ul of 2X TaqMan Gene Expression Supermix (ThermoFisher) and RNasi free water to final volume. Real time assays were run on a FX96 Cycler (Bio-Rad) at the following conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 49 amplifications cycles of 95°C for 15 seconds and 60°C for 1minute. Relative gene expression values were calculated according to the ΔΔCt method.

Flow cytometry analysis

Flow cytometry analyses were performed in order to analyze T-Lymphocytes and Myeloid cells. All antibodies were labeled with a specific marker and were firstly titrated for panel optimization, population identification and expression level measurements (see Table 2 for the antibody list optimized for Lymphoid and Myeloid panel).

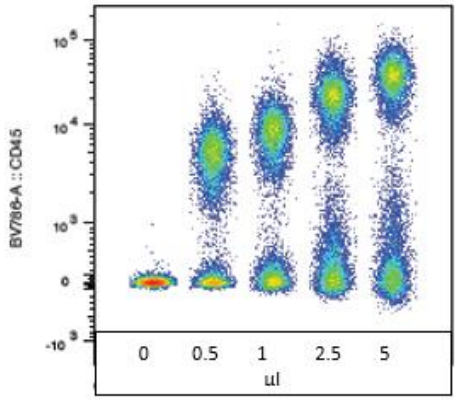
Antibody	Clone	Fluorochrome		
CD11c	3.9	BV421		Myeloid Panel
CD141	1A4	BV510		
CD14	M5E2	BV650		
CD1c	L161	BV711		
CD45	2D1	BV786		
CD3	SK7	FITC		
CD56	NCAM16.2	FITC	LINEAGE	
CD19	HIB19	FITC		
CD123	7G3	PE		
PD-L1	29E2A3	PE-Dazzle594		
CD16	3G8	PerCP-Cy5.5		
CX3CR1	2A9-1	PE-Cy7		
LILRB2	42D1	APC		

HLA-DR	G46-6	APC-R700		
PD-1	EH12-2H7	BV421		Lymphoid Panel (membrane staining)
CD38	HIT2	BV510		
CD3	SK7	BV605		
CD45RO	UCHL1	BV650		
CD8	SK1	BV711		
CD27	L128	BV786		
CD45	2D1	FITC		
CD4	SK3	PE		
PD-L1	29E2A3	PE-Dazzle594		
TIGIT	MBSA43	PerCP-eF710		
CCR7	G043H7	AF647		
HLA-DR	G46-6	APC-R700		
PD-1	EH12-2H7	BV421		Lymphoid Panel (intracellular staining)
CD38	HIT2	BV510		
CD3	SK7	BV605		
CD45RO	UCHL1	BV650		
CD8	SK1	BV711		
CD27	L128	BV786		
CD45	2D1	FITC		
GrB	GB11	PE		
PD-L1	29E2A3	PE-Dazzle594		
TIGIT	MBSA43	PerCP-eF710		
Perf	B-D48	PE-Cy7		
CCR7	G043H7	AF647		
HLA-DR	G46-6	APC-R700		

Table 2 : antobodies list

50.000 PBMCs were incubated with only one antibody and for each one a range of antibody amounts were tested. In particular the range included different antibody amounts above and below the manufacturer's recommended amount. In our experimental set up, all the antibodies used were obteined by Biolegend supplier and a volume of 5 ul per test was suggested for each antibody. Thus 0.5-1-2.5-5 ul were finally tested. Sample with no antibody was further included. Data for all samples were acquired on LSR Fortessa (BD biosciences) and analyzed using FlowJo software (FlowJo LLC, USA). For all concentration tested a separation index (SI) was calculated in order to find the concentration that is optimal for our analysis. In particular, thanks to SI,a separation between positive (upper part of graph) and negative (lower part of the graph) populations was obtained and the better separation is represented by a higher SI. Below is reported the SI formula with an example of separation cell populations.

$$Separation\ Index = \frac{MedianPositive - MedianNegative}{(84\%Negative - MedianNegative)/0.995}$$



Two types of staining were performed: a membrane staining and an intracellular one. About the *membrane staining*, 500.000 cells were firstly washed with 2 ml of Washing buffer (5 g of BSA dissolved in 500 ml of PBS 1X) and centrifuged 5 minutes at 1500 rpm. Supernatant was discarded and cells were resuspended in 200 ul of Washing buffer and antibodies mix. Cells and antibodies were then incubated 20 minutes in the dark and then washed with 2 ml of PBS 1X. To perform the *intracellular staining*, a membrane labeling was first done and a permeabilization/fixation steps were further included to allow the incorporation of antibodies targeting intracellular molecules. Cells were permeabilized 30 minutes at 4°C at the dark and washed in Washing buffer. Next, a second incubation step was performed by adding a new antibody mix for intracellular proteins. Thus, samples were washed with PBS 1X. Finally, each sample was fixed by adding 200 ul of PFA 1%.

For the lymphoid panel, two different staining were set up : a membrane staining, in order to analyse cell differentiation/activation and the expression profile variation of selected ICPs and an intracellular staining was to evaluate the CD8+ T-cells cytotoxic activity under IFNs stimulation. In contrast, only one membrane stain was considered for the myeloid population.

During the set-up of the different experiments, problems arose with the PD-L1 staining because it was difficult to assess the specificity of the extracellular signal. To confirm the signal specificity two different mix of antibodies were prepared for each panel (Lymphoid membrane, lymphoid intracellular and myeloid): one mix made by all the antibodies that are representative of one of the three panels plus the PD-L1 antibody and a second mix made by the same mix of antibodies, except for PD-L1, and containing the PD-L1 related isotype (IgG2bk from BioLegend) (Figure 10). We did not have any specificity problem with the other markers.

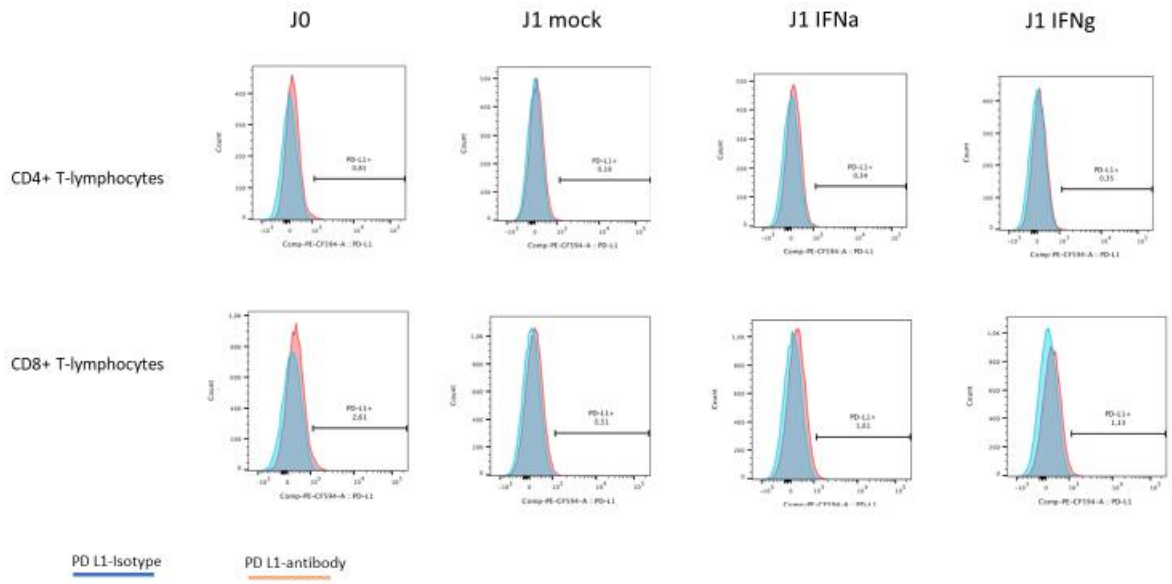


Figure 10: PD-L1 Signal validation in total CD4+ and CD8+ T-lymphocytes ex vivo (J0) and after 24 hours (J1) of stimulation with IFNα and IFNγ. Blue curve is referred to PDL1 Isotype and red curve represents the PDL1 antibody signal

Because PD-L1 give us a good specific signal and in the absence of a different signals between positive and negative cells, we plotted the mean fluorescence intensity (MFI) to represent the PDL1 (Figure 11).

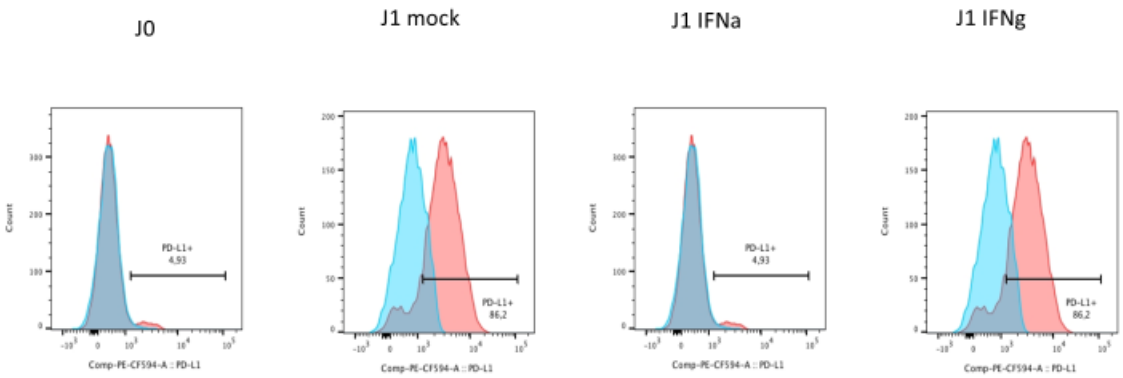
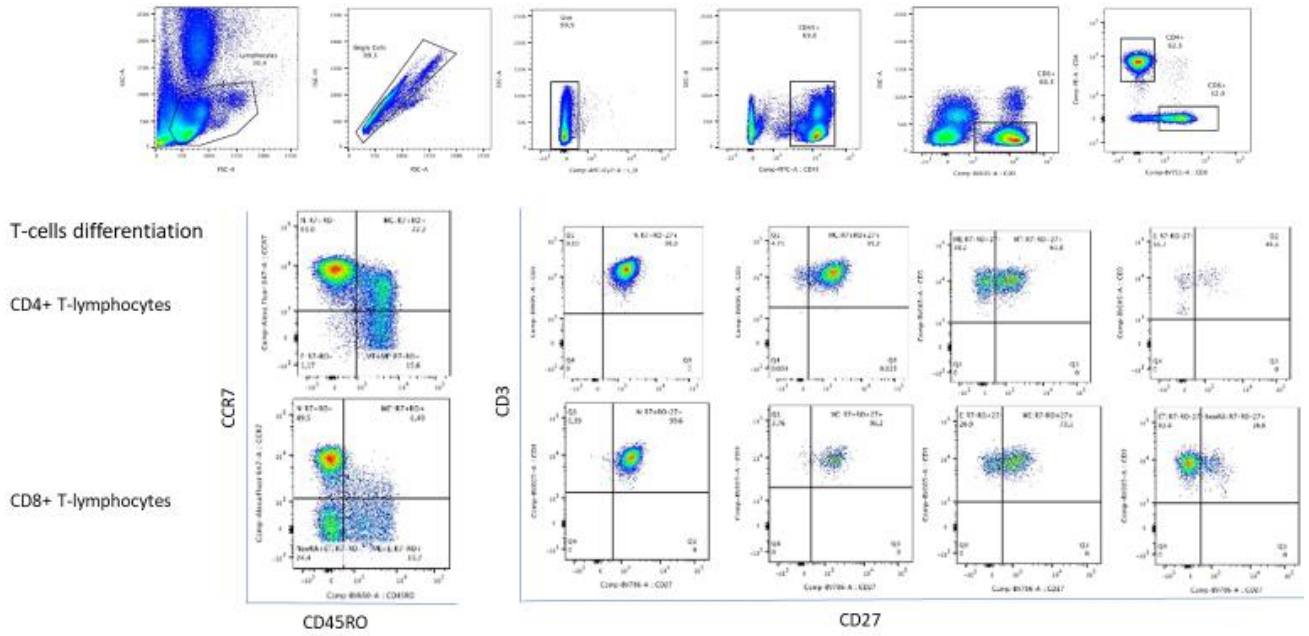


Figure 11: PD-L1 Signal validation in myeloid cells ex vivo (J0) and after 24 hours (J1) of stimulation with IFNα and IFNγ. Blue curve is referred to PDL1 Isotype and red curve represents the PDL1 antibody signal.

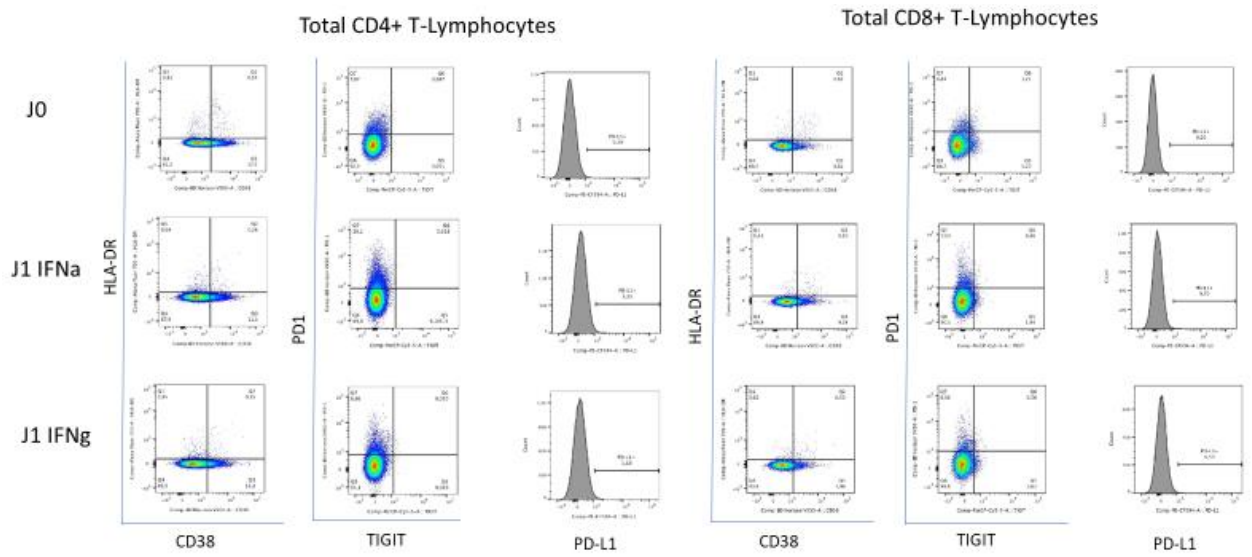
In myeloid cells PD-L1 showed a good specific signal with the identification of two different populations: positive and negative cells. Thus, for myeloid cells PD-L1 was plotted as the percentage of cells that are positive for PD-L1 as well as the MFI to further analyze the different expression pattern of PD-L1 on cellular surface.

Gating strategy: T-Lymphocytes

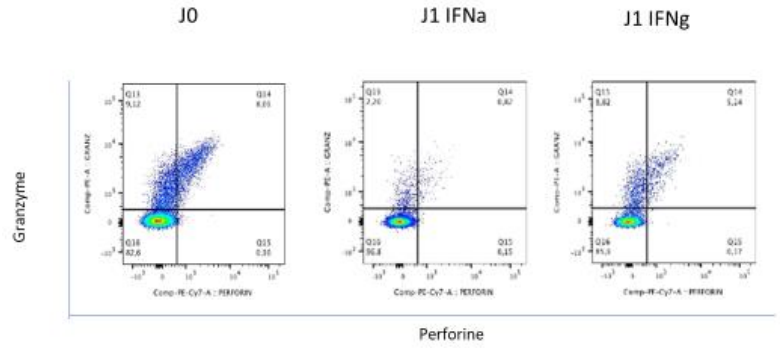
In Figure 12 is reported the gating strategy by which the CD4+ and CD8+ T-lymphocytes and related subpopulations were selected. Lymphocyte population were selected by SSC (side scatter) and FSC (forward scatter) and single cells were then gated by FSC-H (height) versus FSC-A (area). From this population, live cells were selected and CD45+ cells were further gated. CD3+ cells were then choosen to identify the CD4+ and CD8+ T lymphocytes as our cells of interest. CD45RO in combination with CCR7 and CD27 antibodies were used to phenotipically define subsets of CD4+ and CD8+ T-cells. In particular, CD4+ cells were divided in Naïve T-cells (CD45RO-, CCR7+, CD27+), Effector T-cells (CD45RO-, CCR7-, CD27-), Central Memory T-cells (CD45RO+, CCR7+, CD27+), Transitional Memory (CD45RO+, CCR7-, CD27+) and Effector Memory T-cells (CD45RO+, CCR7-, CD27-). Similarly, the gating strategy followed for CD8+ cells subsets identified the Naïve T-cells (CD45RO-, CCR7+, CD27+), Central Memory T-cells (CD45RO+, CCR7+, CD27+), Effector (CD45RO+, CCR7-, CD27-), Effector Memory T-cells (CD45RO+, CCR7-, CD27+) and Terminal Effector Tcells (CD45RO, CCR7-, CD27-) (Figure 12A). The pattern of activation or exhaustion for each subset was further analyzed by using the CD38 and HLA-DR antibodies for T-cells activation as well as TIGIT, PD1 and its ligand the PD-L1 for the exhaustion analysis (Figure 12B). With regard to cytotoxic activity, from CD8+ T-lymphocytes and subsets we further investigate cells that were positive for granzyme and perforin expression (Figure 12C).



A)



B)



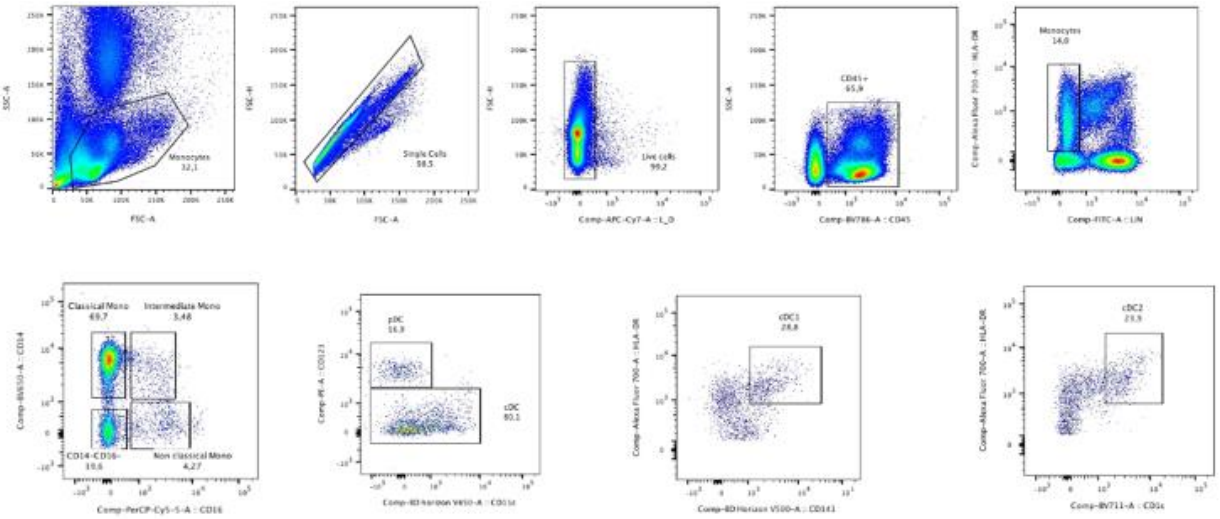
C)

Figure 12: gating strategy T lymphocytes. A) T-Lymphocytes differentiation analysis; B) Analysis of activation and exhaustion of CD4+ T-Lymphocytes, on the right, and CD8+ T-Lymphocytes, on the left, at J0 (ex vivo) and after 24 hours (J1) of stimulation with IFNα and IFNγ; C) cytotoxicity analysis of CD8+ T-lymphocytes at J0 and J1 after IFNα and IFNγ stimulation.

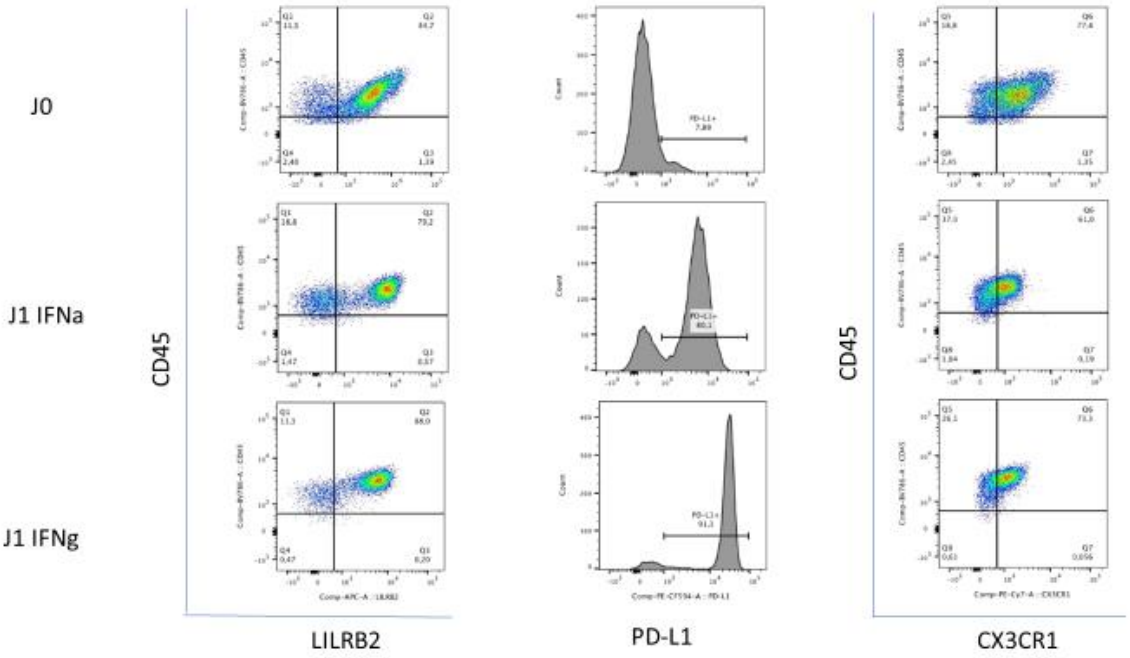
Gating strategy: myeloid cells

Figure 13 shows the gating strategy by which myeloid cells and the different subsets were selected. Myeloid population was selected by SSC (side scatter) and FSC (forward scatter) and single cells were then gated by FSC-H (height) versus FSC-A (area). From this population, live cells were selected and CD45+ cells were further gated. Monocytes population was identified using HLA-DR in combination with CD56, CD3 and CD19 (LINEAGE, LIN) and HLA-DR+ LIN- cells were gated. Different monocytes subsets were then characterized by CD14 and CD16 markers. At this regard, Classical (CD14+ CD16-), Intermediate (CD14+ CD16+) and non classical (CD14- CD16+) monocytes as well as conventional and plasmacitoid dendritic cells (DC) (CD14-CD16-) were distinguished. From CD14-CD16- cell subset, the characterization between cDC and pDC was allowed by CD123 in combination with CD11c : cells CD123+ were gated as pDCs in contrast to cells CD123-

that were selected as cDC. Moreover, from cDCs group it was possible a further distinction between cDC1 (CD141+) and cDC2 (CD1c) (Figure 13A). Myeloid cells and all subsets were further analyzed for the expression of markers related to cells exhaustion under IFNs stimulation, as LILRB2, CX3CR1 and PD-L1, too (Figure 13B).



A)



B)

Figure 13: Gating strategy of myeloid cells; A)myeloid cells differentiation analysis; B) markers of cell exhaustion at at J0 (ex vivo) and after 24 hours (J1) of stimulation with IFNα and IFNγ

Results

Chapter1: RFs analysis

Immunological and virological parameters in HIV patients

Immunological parameters of HIV patients enrolled in the study are shown in Table 3. Briefly, Group 1 patients presented at T0 a CD4+ T lymphocytes count of 235.5 cell/mmc with a significant increase ($p<0.001$) at T1 and T2 (406.5 cell/mmc and 489.5 cell/mmc, respectively).

CD8+ T lymphocytes percentage showed stable values prior and after cART (60.5% at T0, 54.5% at T1 and 44.25% at T2).

CD4/CD8 ratio appeared constant from T0 onwards (0.275, 0.345 and 0.480 at T0, T1 and T2 respectively).

Group 2 patients presented CD4+ T lymphocytes of 787 cell/mmc significantly higher when compared to group 1 patients at T0 ($p < 0.001$) and T1 ($p <0.05$).

CD8+ T lymphocytes percentage [44.25 (IQR =29.25-44.25)] showed lower values in comparison with group 1 patients at T0 ($p<0.001$), T1 ($p<0.001$) and T2 ($p<0.01$).

CD4/CD8 ratio showed elevated values (0.925) and significantly higher in comparison with Group 1 patients ($p<0.001$).

***Plasma HIV-RNA load*:** The viral load trend detected in plasma samples appeared drastically reduced after cART beginning (Group 1), decreasing from 4.67 log copies/ml at T0 to undetectable levels ($p<0.001$) at T1 and ($p<0.001$) at T2.

Group 2, based on inclusion criteria, showed undetectable levels (0 log) as previously described as patients under cART and with undetectable viremia from at least two years.

Total HIV-DNA. As expected, total HIV DNA amount showed higher values (3.192 log copies/10^6 PBMCs, IQR=2.901-3.665) at T0 in comparison to onwards controls [T1 (2.496 log copies/10^6 PBMCs and T2 (2.404 log copies/10^6 PBMCs)] ($p= 0.016$, $p= 0.003$).

TND patients presented a lower, but not significant, total HIV DNA amount (1.642 log copies/10^6 PBMCs), when compared with value obtained in Group 1 patients at T1 and T2 (for details see Table 4).

Patients Characteristics	Group 1			Group 2
	T0 (n=14)	T1 (n=14)	T2 (n=14)	TND (n=14)
Age	44 (35-47)			51 (43-60)
CD4+ T lymphocytes (cell/mmc) (IQR)	235,5 (94,75-436,5)	406.5 (289.5-629.5)	489,5 (324,3-726,3)	787 (575,5-1171)
CD8+ T lymphocytes (%) (IQR)	60,5 (51,50-70)	54.5 (49,75-69,25)	54 (48,75-65,5)	44,25 (29,25-44,25)
CD4/CD8 (IQR)	0,275 (0,125-0,465)	0,345 (0,127-0,592)	0,480 (0,177-0,625)	0,925 (0,69-1,56)
HIV-RNA load (cp/ml)*(IQR)	$4.73x10^4$ ($3.19x10^4$-$1.38x10^5$)	$2x10^1$ ($3x10^1$-$2x10^1$)	<20	<20
Total HIV-DNA (copies/10^6 PBMCs)(IQR)	$1.71x10^3$ ($8.51x10^2$-$3.24x10^3$)	$3.68x10^2$ ($2.23x10^2$-$7.22x10^2$)	$2.69x10^2$, ($7.46x10^1$-$4.30x10^2$)	$4.42x10^1$ ($3.50x10^1$-$1.18x10^2$)
* HIV-RNA load was detected by standard commercial viral RNA detection assay (COBAS® AMPLICOR, Roche Molecular Systems, Inc., Branchburg, NJ, USA). VL: Viral Load; TND: target not detected.				

Table 3: clinical and virologic characteristic of HIV patients (group 1 and 2). Values are reported as median (IQR); T0, blood sample prior to cART; T1, first follow up and blood sample collection at 4 months after cART administration; T2, second follow up and blood sample collection at 8 months after cART; T0, T1, T2 are referred at group 1 individuals. TND, target not detected, patients belonging to group 2, aviremic and under cART from at least 2 years.

Unpaired t-test	**T0 vs T1**	**T0 vs T2**	**T0 vs TND**	**T1 vs TND**	**T1 vs T2**	**T2 vs TND**
CD4 / mmc	ns	ns	P<0.001	P<0.05	ns	ns
% CD8	ns	ns	P<0.001	P<0.001	ns	P<0.01
CD4 / CD8	ns	ns	P<0.001	P<0.001	ns	P<0.01
Plasma HIV RNA	P<0.001	P<0.001	P<0.001	ns	ns	ns
Total HIV DNA	ns	P<0.01	P<0.001	P<0.05	ns	ns

Table 4: Statistical comparison of immunological and virologic parameters between Group 1 and Group 2 of enrolled patients. (NS in Non-Significant).

APOBEC3G, MX2, SAMHD1, SERINC3 expression is higher prior to cART

Each RFs were independently tested. Despite the restricted number of enrolled individuals and the inter-individual variability in RFs gene expression, APOBEC3G, MX2, SAMHD1 and SERINC3 showed higher levels in Group 1 patients at inclusion Time (T0) and 4 months later (T1) meanwhile at T2 (8 months later) RFs expression appeared decreased, with values comparable to those recorded in TND patients (Figure 14).

In particular, APOBEC3G appeared significantly upregulated at T0 and T1 compared to TND patients ($p<0.01$ and $p<0.001$ respectively). Meanwhile MX2 and SAMHD1 overexpression was registered at T0 compared to T2 ($p<0.05$ and $p<0.01$ respectively) and TND patients ($p<0.001$ for both MX2 and SAMHD1). Furthermore, SAMHD1 showed an overexpression at T1 compared to TND ($p<0.05$). SERINC3 was characterized by an increment at T0 compared to T2 and TND group ($p<0.05$ and $p<0.01$ respectively) (for details, see Table 5 and 6).

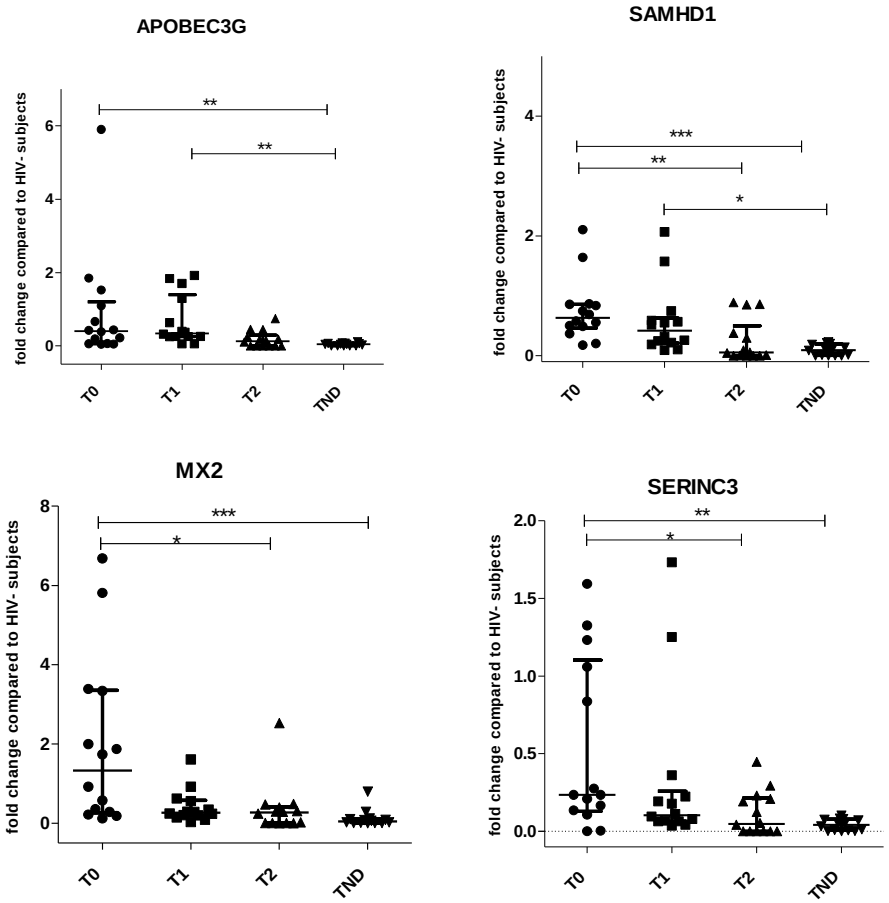


Figure 14: APOBEC3G, MX2, SAMHD1, SERINC3 expression profile in group1 and group 2 were analyzed at each blood sample collection.

RFS FOLD CHANGE	MEDIANS (IQR)			
	T0	**T1**	**T2**	**TND**
APOBEC3G	0.402 (0,059-1,199)	0,343 (0,251-1,394)	0,128 (0,002-0,296)	0,043 (0,001-0,071)
MX2	1,330 (0,264-3,350)	0,261 (0,186-0,576)	0,267 (0,004-0,418)	0,045 (0,006-0,108)
SAMHD1	0,633 (0,4599-0,858)	0,417 (0,211-0,627)	0,093 (0007-0,497)	0,052 (0,004-0,193)
SERINC3	0,235 (0,129-1,102)	0,103 (0,065-0,258)	0,048 (0,015-0,214)	0,042 (0,005-0,07)

Table 5: Median and interquartile range (IQR) of the fold change for each RF in Group 1 and Group 2.

RF name	**T0 vs T1**	**T0 vs T2**	**T0 vs TND**	**T1 vs TND**	**T1 vs T2**	**T2 vs TND**
APOBEC3G	NS	NS	<0.01	<0.001	NS	NS
MX2	NS	<0.05	<0.001	NS	NS	NS
SAMHD1	NS	<0.01	<0.001	<0.05	NS	NS
SERINC3	NS	<0.05	<0.01	NS	NS	NS

Table 6: Statistical comparison between RFs expression levels of Group 1 and Group 2 of enrolled patients. (NS is non-significant).

BST2, IFI16, SERINC5, STING and TRIM5 expression are higher after cART

BST2, IFI16, SERINC5, STING and TRIM5, similarly to RFs described before, appeared upregulated during the first period of follow-up, with an expression enhancement in T1 compared to T0, T2 and TND (Figure 15). Among them, BST2 expression is higher in T1 ($p<0.001$) than in T0 ($p<0.01$) compared to TND.

STING did not show a significant increment of expression in T1 compared to T0 but a statistically significant overexpression was registered in T2 compared to TND patients ($p<0.01$). Similarly, the expression levels of IFI16 were also higher in T1 than in T0 but not statistically significant p value was observed. However, IFI16 and TRIM5 expression appeared enhanced at T0 compared to TND ($p<0.01$ and $p<0.05$ respectively) and between T1 and TND ($p<0.001$ and $p<0.01$, respectively). The expression levels of SERINC5 appeared upregulated in T1 and T2 but the higher expression was registered in T1 compared to TND ($p<0.05$) (for details see Table 7 and 8).

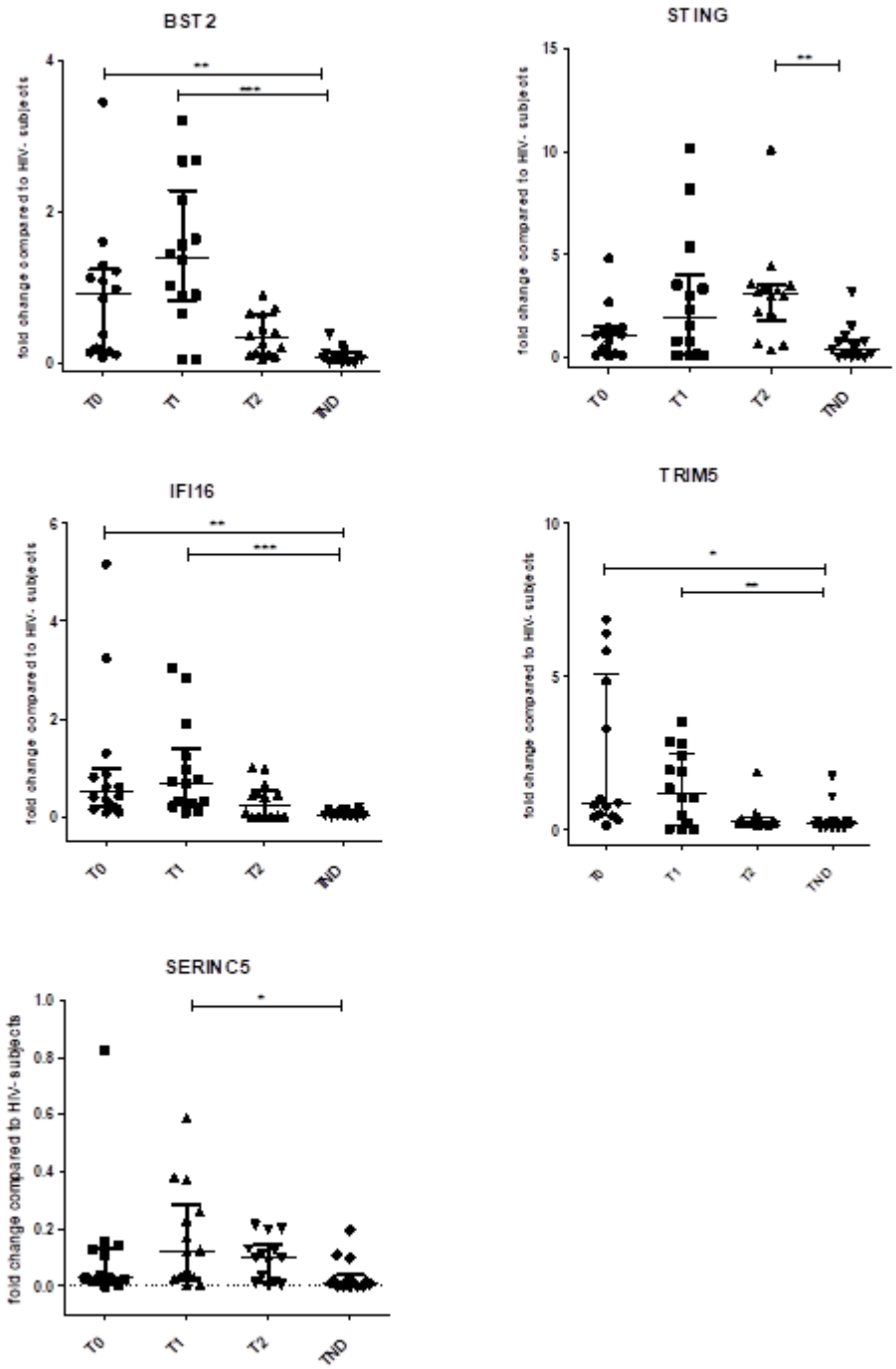


Figure 15: BST2, IFI16, SERINC5, STING and TRIM5 expression profile in group1 and group 2 HIV 1 were analyzed at each blood sample collection.

Table 7: Median and interquartile range (IQR) of the fold change for each RF in Group 1 and Group 2.

RFS FOLD CHANGE	MEDIANS (IQR)			
	T0	**T1**	**T2**	**TND**
BST2	0,921 (0,156-1,239)	1,406 (0,836-2,282)	0,329 (0,110-0,653)	0,091 (0,010-0,148)
IFI16	0,512 (0,198-0,985)	0,696 (0,249-1,396)	0,243 (0,005-0,528)	0,039 (0,003-0,128)
SERINC5	0,029 (0,018-0,133)	0,123 (0,027-0,287)	0,098 (0,015-0,147)	0,010 (0,001-0,038)
STING	1,105 (0,173-1,477)	1,945 (0,132-4,01)	3,107 (1,816-3,531)	0,422 (0,052-0,842)
TRIM5α	0,843 (0,437-5,083)	1,205 (0,154-2,503)	0,266 (0,181-0,376)	0,172 (0,055-0,208)

Table 8: Statistical comparison between RFs expression levels of Group 1 and Group 2 of enrolled patients. (NS is non-significant).

RF name	**T0 vs T1**	**T0 vs T2**	**T0 vs TND**	**T1 vs TND**	**T1 vs T2**	**T2 vs TND**
BST2	NS	NS	<0.01	<0.001	NS	NS
IFI16	NS	NS	<0.01	<0.001	NS	NS
SERINC5	NS	NS	NS	P<0.05	NS	NS
STING	NS	NS	NS	NS	NS	<0.01
TRIM5α	NS	NS	<0.05	<0.01	NS	NS

Correlation analysis

As restriction factors play pivotal role in the viral infection control especially in the first moments of viral replication and spread, we wished to evaluate the association between the selected RFs and the immunological and virologic parameters in group 1 of patients during the follow up. Surprisingly, we do not find any significant association between the RFs' expression levels with immunological parameters and the HIV-1 DNA, as reported in Table 9a, thus suggesting the complicated interaction between host and virus. On the contrary, the association analysis with viral load revealed a strong positive correlation for APOBEC3G (p=0.001, r= 0.491), TRIM5α (p=0.003, r= 0.446), SAMHD1 (p=0.005, r= 0.426), MX2 (p=0.002, r= 0.473), IFI16 (p=0.006, r= 0.414), STING (p=0.022, r= 0.352) and SERINC3 (p=0.004, r= 0.438) (Figure 16). Correlation analysis performed for Group 2 of TND individuals do not revealed any statistically significant correlation between RFs expression and the immunological and virologic parameters (Table 9b).

GROUP 1										
Spearman correlation analysis	**CD4 / mmc**		**% CD8**		**CD4 / CD8**		**Total HIV DNA**		**Plasma HIV RNA**	
	P value	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**
APOBEC3G	0,863	-0,027	0,408	0,130	0,267	-0,175	0,327	0,155	**0,001**	**0,491**
BST2	0,493	0,108	0,841	-0,031	0,579	-0,087	0,477	0,112	0,182	0,209
IFI16	0,294	-0,163	0,269	0,172	0,067	-0,281	0,403	0,130	**0,006**	**0,414**
MX2	0,360	-0,144	0,232	0,188	0,414	-0,129	0,222	0,192	**0,002**	**0,473**
SAMHD1	0,139	-0,231	0,811	0,037	0,277	-0,171	0,317	0,158	**0,005**	**0,426**
SERINC3	0,780	0,044	0,943	-0,011	0,901	0,019	0,719	0,057	**0,004**	**0,438**
SERINC5	0,805	0,039	0,785	0,043	0,793	0,041	0,773	-0,045	0,457	-0,117
STING	0,169	0,215	0,474	-0,113	0,366	0,143	0,127	-0,238	**0,022**	**0,352**
TRIM5α	0,910	0,017	0,878	0,024	0,736	-0,053	0,274	0,172	**0,003**	**0,446**

Table 9a: Correlation analysis between RFs expression and immunological-virologic parameters referred to Group 1 of enrolled patients (n=42) followed during the follow up (T0-T1-T2).

GROUP 2										
Spearman correlation analysis	**CD4 / mmc**		**% CD8**		**CD4 / CD8**		**Total HIV DNA**		**Plasma HIV RNA**	
	P value	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**	**P value**	**r**
APOBEC3G	0,418	0,235	0,373	-0,257	0,333	0,279	0,820	0,0667	**NA**	**NA**
BST2	0,759	0,090	0,887	-0,041	0,615	0,147	0,688	0,117	**NA**	**NA**
IFI16	0,872	0,049	0,516	-0,198	0,334	0,291	0,392	0,259	**NA**	**NA**
MX2	0,409	0,239	0,702	-0,112	0,691	0,116	0,532	0,182	**NA**	**NA**
SAMHD1	0,366	0,261	0,536	-0,180	0,409	0,239	0,682	0,120	**NA**	**NA**
SERINC3	0,970	-0,010	0,952	-0,017	0,994	0,002	0,478	0,206	**NA**	**NA**
SERINC5	0,771	-0,085	0,970	0,011	0,970	0,010	0,53	0,180	**NA**	**NA**
STING	0,366	0,261	0,981	0,006	0,669	0,125	0,268	0,317	**NA**	**NA**
TRIM5α	0,958	0,015	0,440	-0,224	0,483	0,204	0,957	-0,015	**NA**	**NA**

Table 9b: Correlation analysis between RFs expression and immunological-virologic parameters referred to Group 2 of enrolled patients (n=14). Correlation analysis with viral load cannot be analyzed because viral load is undetectable and it represents either a vertical or horizontal line. NA: not analyzed.

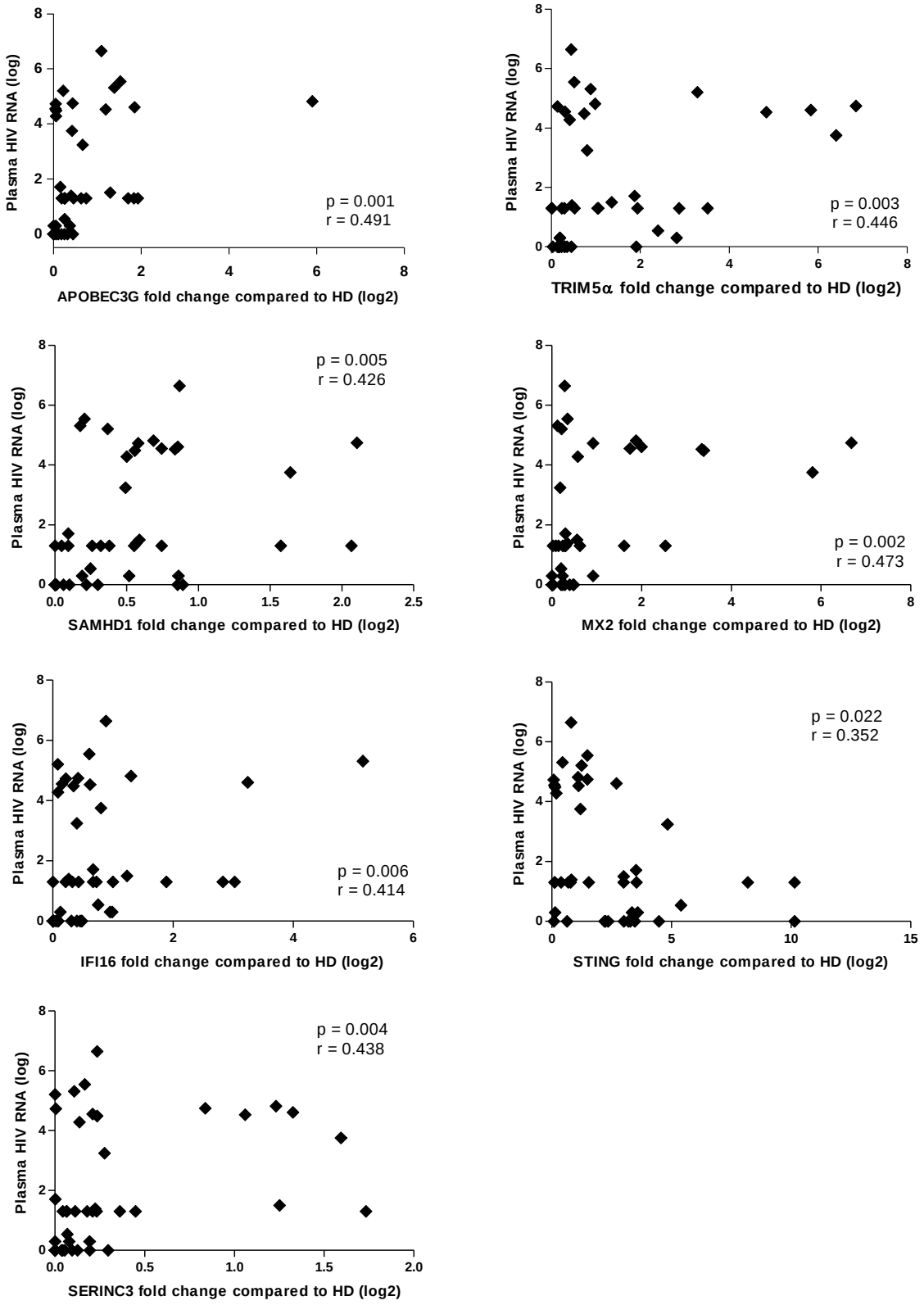


Figure 16: Correlation analysis between RFs and plasmatic viral load referred to group 1(n=42) of patients followed at each follow up (T0, T1, T2). Plasma HIV RNA levels was expressed as Log of copies/ml. P and r values were calculated using Spearman correlation test.

Chapter 2: ICPs analysis

IFN treatment and Real Time PCR analysis

Real Time qPCR was performed on PBMCs of healthy donors, collected *ex vivo* and at 4 hours post stimulation with IFNα and IFNγ. MX1 and IP10 are two candidate genes induced by IFNs pathway activation. Under our experimental conditions, MX1 appeared upregulated after 4 hours of IFNα stimulation compared to *ex vivo* analisys (p=0.0021) and in a less extentent after IFNγ stimulation compared to J0 (p=0.0277). Similarly, at 4 hours of IFNγ stimulation a statistical significant increment of IP10 expression was registered compared to *ex vivo* sample (p=0.0021) and a mild increment was further observed after IFNα induction (p=0.0245). MX1 and IP10 enhancement after stimulation with 500 U/ml of IFNα and 10 ng/ml of IFNγ confirmed the good performances of our exprimental conditions. On this basis, we further analysed the expression pattern variation of LILRB2 and PDL1 during IFNs induction as well as our gene of interest of immune checkpoint. Compared to MX1 and IP10, LILRB2 did not appear upregualted under either IFNα or IFNγ stimulation whereas PD-L1 expression analysis showed a statistical significant overexpression at 4 hours of IFNγ stimulation compared to *ex vivo* analysis (p=0,0009) (Figure 17).

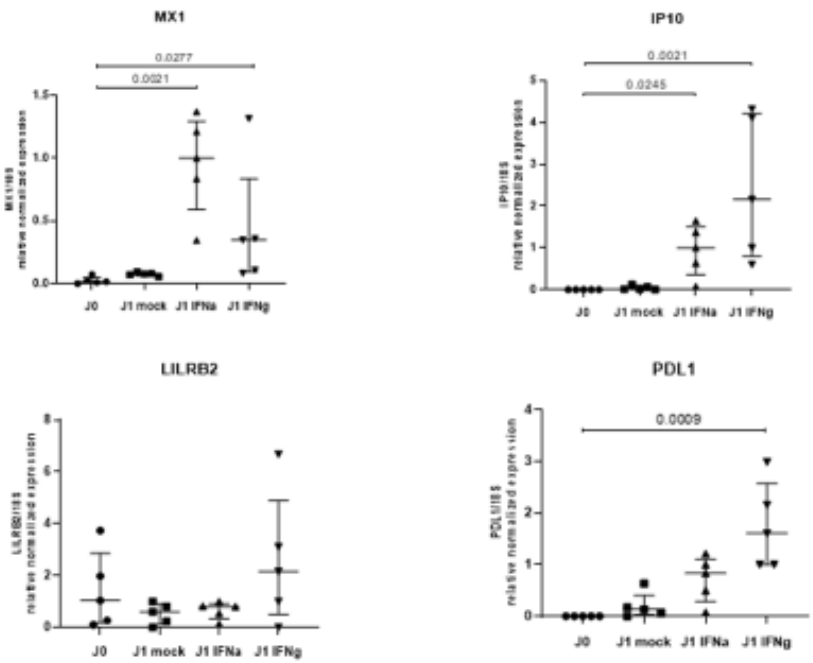


Figure 17: MX1, IP10, LILRB2, PDL1 expression profile. Scattered dot plots showing on Y-axis the relative expression of each gene. Each dot represents separate healthy donors (n=5). The median and interquartile range (IQR) are indicated.

Flow Cytometry analysis: T-Lymphocytes

At 24 hours post stimulation, flow cytometry analysis was performed on T-Lymphocytes derived from healthy subjects with the aim to investigate the different expression profile of specific markers associated to cellular activation and/or exhaustion under IFNs stimuli. Total CD4+ and CD8+ T-Lymphocytes were analyzed separately and each subset were further characterized for TIGIT, PD1 and its ligand PD-L1expression as the main immune check point proteins associated to immune cells exhaustion and disfunction (Figure 18). Contrarily, HLA-DR and CD38 together were studied in order to examine the pattern of cellular activation under one or both IFNs stimulation. Despite IFNs stimulation, in healthy subjects, positive cells percentage for TIGIT and PD1 did not show any significant increment compared to mock and ex vivo sample as well as PD-L1 MFI analysis. Similarly, the HLA-DR+CD38+ T lymphocytes amount appeared the same with or without stimulation. In Figure 6 are represented results obtained on Total CD4+ and CD8+ T cells. Analyses performed for each T- lymphocytes subsets are omitted because any significant difference was observed in MFI and percentage of cells that are positive for markers of activation and exhaustion.

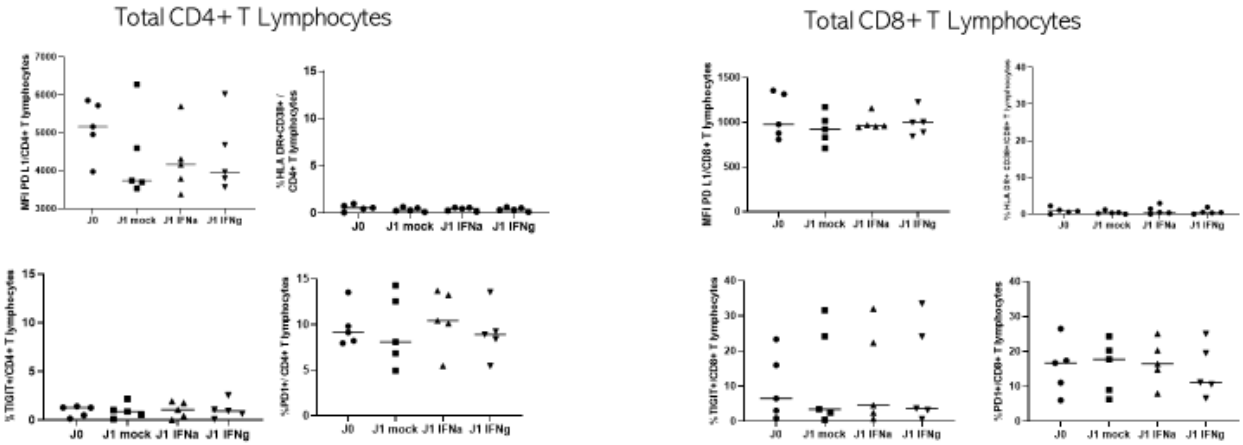


Figure 18: PDL1, HLA DR-CD38, TIGIT and PD1 analysis on total CD4+ T-Lymphocytes, on the right, and total CD8+ T-Lymphocytes, on the left, at J0 (ex vivo) and after 24 hours (J1) of stimulation with IFNα and IFNγ or without stimulation (MOCK). Each dot represents separate healthy donors (n=5). PDL1 is indicated as MFI meanwhile HLA DR-CD38, TIGIT and PD1 are graphed as positive percentage of T-cells.

Perforin and Granzyme B expression were further analyzed as two cytotoxic molecules used by CD8+ T-lymphocytes to induce apoptosis and their overexpression has been reported in several chronic diseases as well as chronic inflammation or infection. However, our analyses performed on PBMCS derived from healthy donors and stimulated 24 hours with IFNα and IFNγ, did not show any differences in cells that are positive for Perforin or/and Granzyme B in presence or not IFN stimuli. In Figure 19 is reported the expression profile of

Perforin and Granzyme B on total CD8+ T cells and similarly, any differences were observed in all CD8+ subsets.

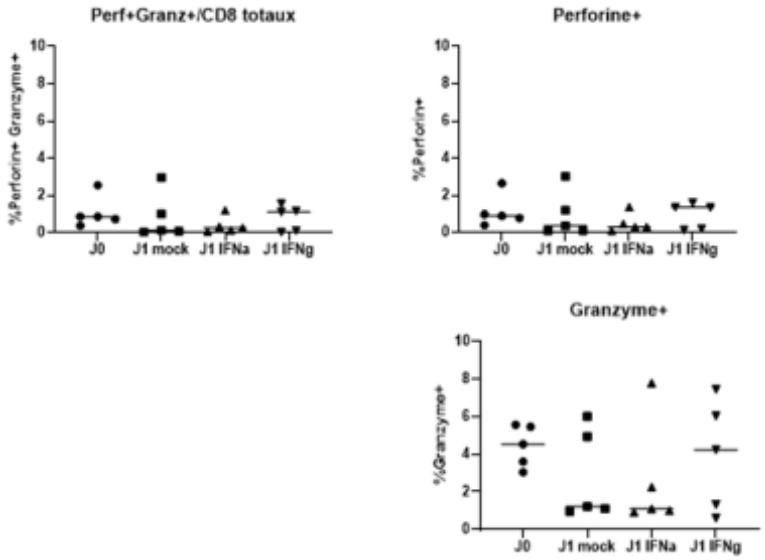


Figure 19: Granzyme and Perforine analysis on total CD8+ T-Lymphocytes, at J0 (ex vivo) and after 24 hours (J1) of stimulation with IFNα and IFNγ or without stimulation (MOCK). Each dot represents separate healthy donors (n=5). The positive percentage of CD8+ T-cells is represented.

Flow Cytometry analysis: Myeloid cells

Myeloid cells were studied for the expression of markers associated to exhaustion cell status described during chronic inflammation and infection. Flow cytometry analysis was performed 24 hours post stimulation with IFNα and IFNγ for LILRB2, PD-L1 and CX3CR1. Analysis were divided into monocytes subset and dendritic cells (DC). Our analysis shown interesting results at 24 hours in PBMCs derived from healthy donors (Figure 20). In detailed, LILRB2 and CX3CR1 did not show any significant increment after IFNs stimulation. On the contrary, we observed a percentage increment of cells that are positive for PD-L1 at 24 hps compared to ex vivo sample. In particular the percentage of Monocytes expressing PD-L1 was statistically incremented in cells stimulated with IFNα and IFNγ compered to *ex vivo* sample (p=0.0452 and p=0.0031 respectively). Similarly, in the other monocytes subsets we observed the same increment in IFNs stimulated samples compared to *ex vivo* one (p- values are reported in Table 10).

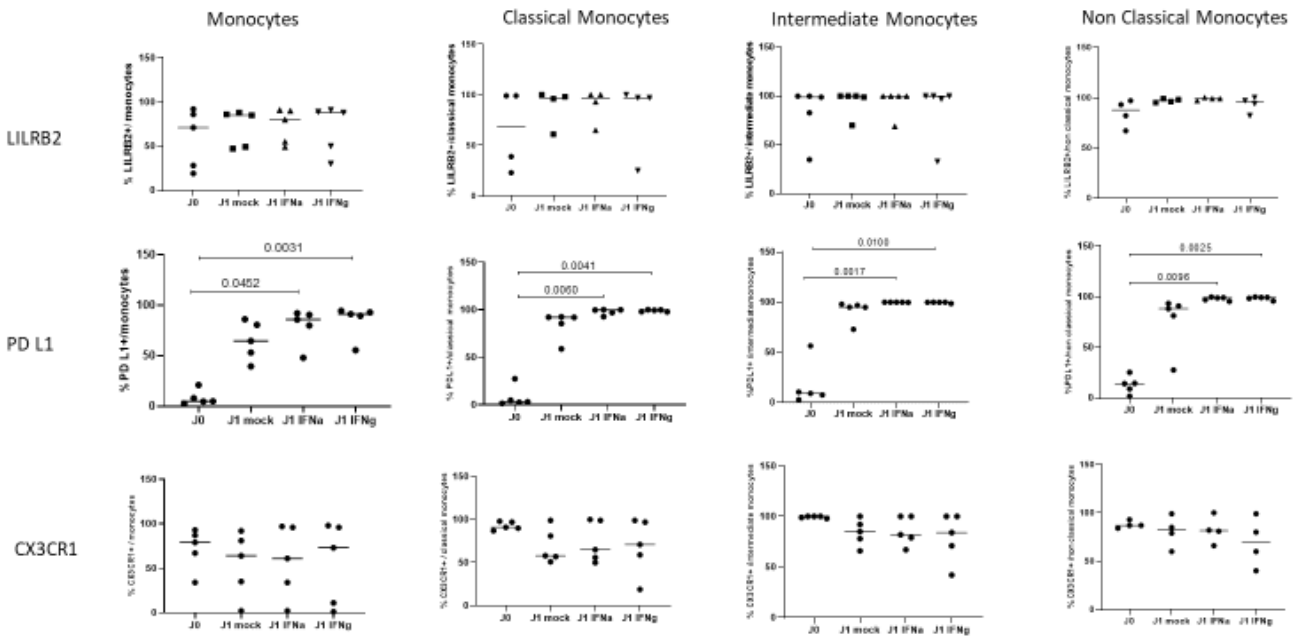


Figure 19: LILRB2, PDL1 and CX3CR1 analysis on Monocytes, Classical, Intermediate and Non Classical monocytes at J0 (ex vivo) and after 24 hours (J1) of stimulation with IFNα and IFNγ or without stimulation (MOCK). Each dot represents separate healthy donors (n=5). Results are represented as positive percentage of cells expressing LILRB2, PDL1 and CX3CR1.

p-value	Monocytes	Classical	Inermediate	Non classical	pDC	cDC1	cDC2
IFNα vs J0	0.0452	0.006	0.0017	0.0096	0.0165	0.0095	0.0277
IFNγ vs J0	0.0031	0.0041	0.01	0.0025	ns	0.0194	ns

Table 10: p-value calculated for PD-L1 positive cells percentage

Dendritic cells displayed a similar pattern of expression observed in monocytes and related subsets. CX3CR1 expression did not change after IFNs induction in all DC subsets as well as LILRB2 analyzed in pDC and cDC2. However, contrary to monocytes populations, LILRB2 appeared increased in cDC1 after IFNα stimulation compared to ex vivo samples (p=0.0326). The percentage of pDC and cDC2 that are positive for PD-L1 expression appeared augmented in IFNα stimulated samples (p=0.0165 and p=0.0277 respectively). PD-L1 expression in cDC1 increased in a statistically significant manner after either IFNα and IFNγ stimuli (p=0.0095 and p=0.0194 respectively) (Figure 20).

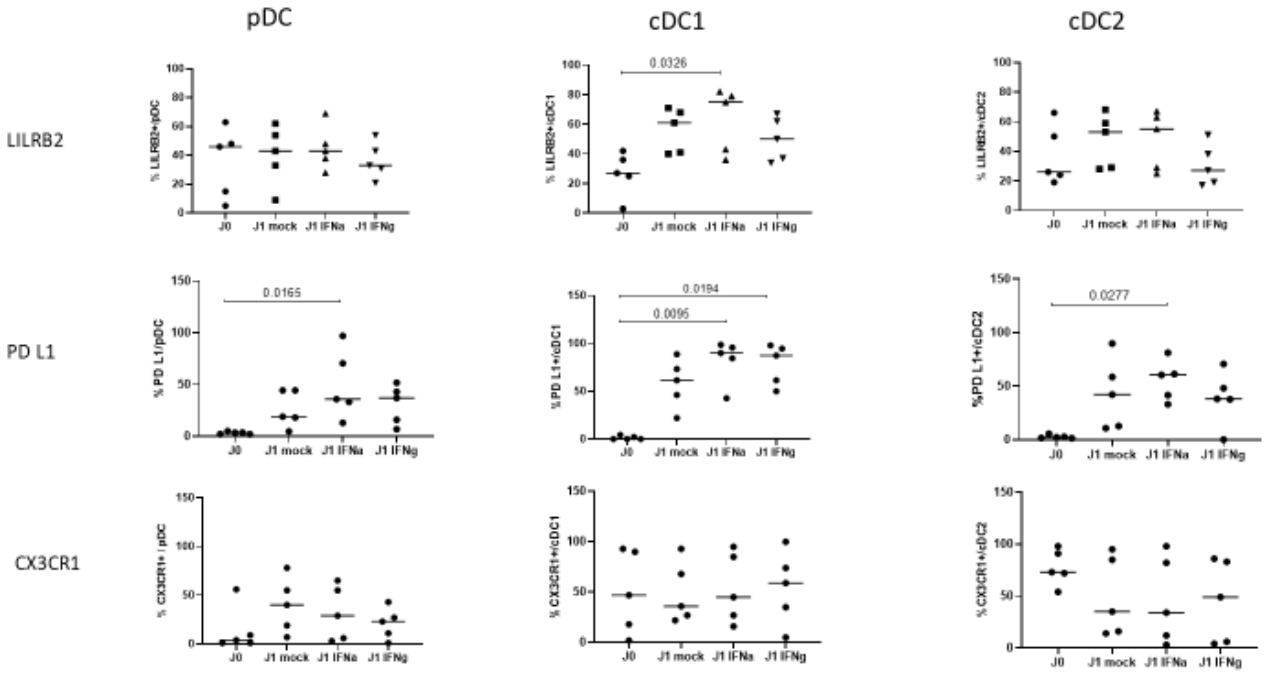


Figure 20: LILRB2, PDL1 and CX3CR1 analysis on pDC, cDC1 and cDC2. IFNγ or without stimulation (MOCK). Each dot represents separate healthy donors (n=5). Results are represented as positive percentage of cells expressing LILRB2, PDL1 and CX3CR1.

Interestingly, PD-L1 MFI analysis showed that under IFN stimulation, the expression pattern of PD-L1 on cellular surface is different between cells and the PD-L1 enhancement appeared mostly évident after IFNγ stimulation, thus according with what observed in Real Time PCR where after 4 hps PD-L1 overexpress after IFNγ induction. As reported in Figure 10 and in Table 11, in monocytes, classical and non classical monocytes subsets there is a statistically significant increment of MFI PD-L1 after IFNs stimulation compared to *ex vivo* sample and Mock control too. Intermediate monocytes registered instead a significant increment after IFNγ stimulation compared to ex vivo sample and mock control (p=0.0005 and p=0.0529 respectively). Dendritic cells MFI analysis showed a different pattern of expression compared to monocytes and related subsets. In particular in pDCs the PD-L1 MFI did not enhance after IFNs induction. On the contrary, among cDCs subsets the cDC1 and cDC2 appeared more susceptible to IFNγ stimulation (p=0.0004 and p=0.0116 respectively). Moreover, PD-L1 MFI appeared statisticaly incremented in cDC2 group after IFNα stimulation (p=0.0277).

p-value	Monocytes	Classical	Inermediate	Non classical	pDC	cDC1	cDC2
IFNα vs Mock	ns	ns	ns	ns	ns	ns	ns
IFNγ vs Mock	0.0452	0.0452	0.0529	0.0452	ns	ns	ns
IFNα vs J0	0.0452	0.0452	ns	0.0452	ns	ns	0.0277
IFNγ vs J0	0.0004	0.0004	0.0005	0.0004	ns	0.0004	0.0116

Table 11: p- value calculated for MFI PD-L1

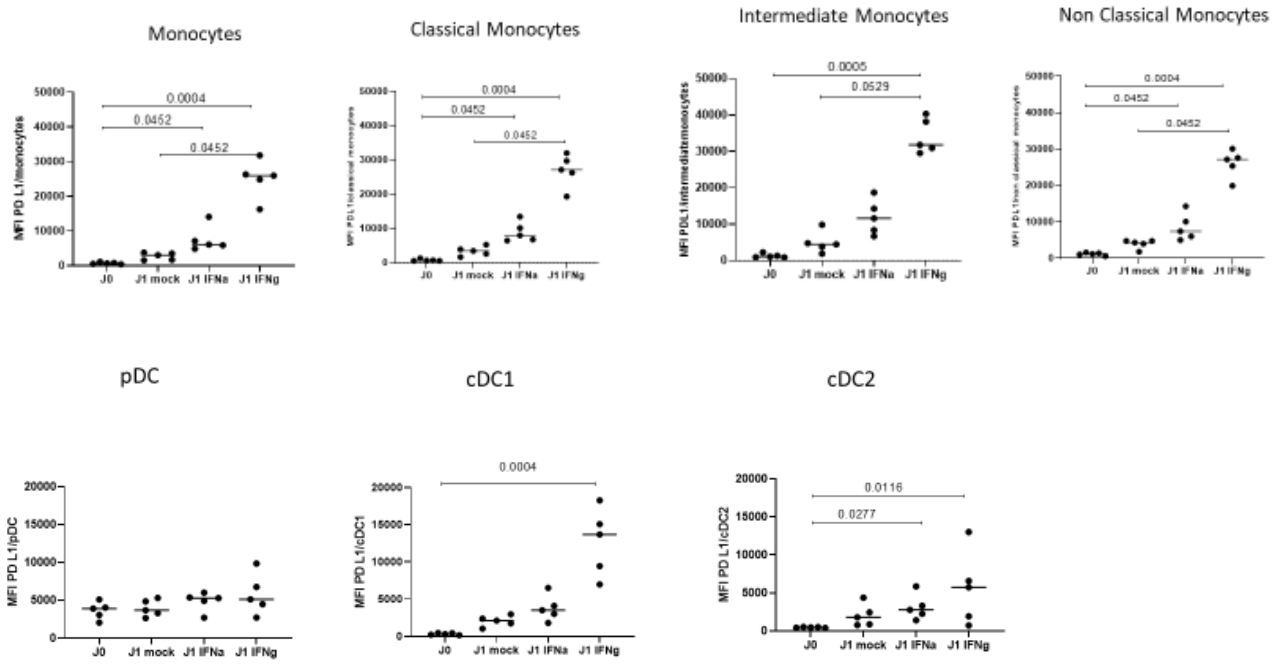


Figure 21: PDL1 MFI analysis on myeloid cells subsets. Each dot represents separate healthy donors (n=5).

We further analyzed the MFI for LILRB2 in order to assess the significant increment observed in the percentage of cells expressing LILRB2 (Figure 22). MFI analysis confirm that upon IFNs stimulation, the expression pattern of this protein on the cellular surface is different in cDC1 subset and LILRB2 enhancement appeared mostly evident after IFNα stimulation. On the contrary, any significant overexpression was observed in other myeloid subpopulations.

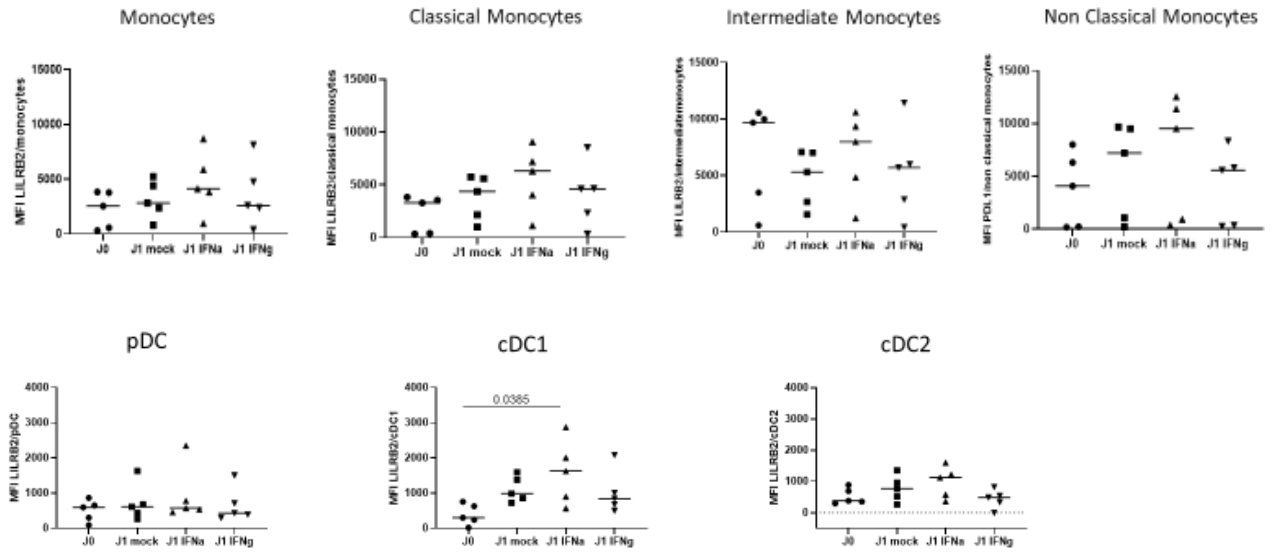


Figure 22: LILRB2 MFI analysis on myeloid cells subsets. Each dot represents separate healthy donors (n=5).

Discussion

Chapter1: RFs analysis

This study investigated the variations of the expression levels of a selected group of RF in a small cohort of patients, prior and after cART administration, with the aim to analyze the possible role of this cellular defense protein in the onset of latency and how cART could play or modulate their role.

Despite the huge variability between patients and the small number of enrolled individuals, we observed that during the first year from the beginning of cART, the levels of these cellular defense proteins appeared upregulated prior and after four months to cART administration suggesting that an ongoing upregulation was maintained during cART to decrease lately, during chronicization of infection.

In particular, among the selected RFs, APOBEC3G, MX2, SAMHD1, SERINC3 were mostly upregulated before the cART administration than BST2, IFI16, SERINC5, STING and TRIM5α that increased mostly after four months of antiviral treatment. Moreover, we observed that the increment of all RFs analyzed, excepted for BST2 and SERINC5, was associated with higher plasma HIV RNA levels, thus suggesting their potential contribution in viral load control and the central role in the interplay virus-host. Our findings are consistent with previous works describing the increment levels of RFs during HIV-1 infection, mostly in cART naïve patients and elite controllers, a very interesting group of HIV-1 infected individuals able to control viral infection without antiviral therapy. High expression of RFs still in the first months after cART suggests that some viral elements persist in the cellular landscape which in turn could trigger the RFs transcription activation through IFNα pathway involvement.

Moreover, in our study, we did not find any correlation with immunological parameters. It is known that untreated HIV infection has opposing effects on circulating CD4+ and CD8+T lymphocytes (117,281,282). Probably the absence of any correlation with CD4+ or CD8+ T lymphocytes or with the CD4+ and CD8 ratio, could be linked not only to the limited cases number but also to the fact that our study were conducted on total PBMCs without considering the CD4+T lymphocytes activation according to a recent study where R.T. Gandhi and colleagues described the changes in markers of HIV1 persistence in relation to inflammation and immune activation (283).

Despite that, previous studies are discordant on this matter. Van Hecke et al. recently described a positive correlation between CD4+T cell count and the expression levels of some of the RFs like BST2, SAMHD1 and APOCEG3G (279), suggesting that ART treated patients present increased levels of RFs which in turn they associated to a better immune preservation. Discrepancy were further described by Bachtel et al. studies where observations have been made between uninfected healthy donors and ART treated patients. They did not found any significant correlation between RFs increment and CD4+ T lymphocytes count or CD4/CD8 ratio (284). According with their studies, differences could depend on several factors, one of them not only linked to study cohort characteristic but also on sample of total CD4+ T lymphocytes cell population. It is in fact largely described that several stimuli like HIV 1 infection, elicit a modification in CD4+ T cell composition, in favor

of memory T cell subset which appears overrepresented in ART-suppressed individuals but with lower expression of restriction factors. Moreover, Abdel-Mohensen et al. described a negative correlation between MX2 and CD4+T lymphocytes activated, similarly for SAMHD1, while APOBEC3G correlated positively with this activated subset of CD4+ T cell (285). Furthermore, they suggested a role of ART in the modulation of RFs' expression, as well as the time of ART administration. An immediate treatment initiation after the acquisition of HIV-1 infection is important and recommended by current WHO guidelines (https://www.who.int/hiv/topics/treatment/en/). Several studies have instead underlined that the timing of treatment initiation influences the RF expression levels and this could lead a differentiation between early treated and late treated subjects. Early treated individuals maintained increased levels of RFs despite a decade of ART and viral suppression, compared to late treated subjects where RFs expression profile is similar to uninfected subjects, indicating that when ART is initiated during chronic infection, expression levels of antiviral factors are normalized to levels observed in HIV negative individuals (279,286,287). Based on this assumption, TND individuals enrolled in this study as group 2 could be considered as late treated because the expression levels of RFs did not appear increased compared to group1. The fold-change calculated referred to healthy donors did not reveal significant differences in RFs expression. Furthermore, a prosecution of this study by collecting blood samples during the follow-up of the same enrolled individuals could allow and/or confirm the role of ART in the maintenance of innate antiviral responses.

In this study, the quantification of total HIV DNA, used to assess the reservoir size, overestimates the size of the replication competent cells. Several studies agree with the notion that total HIV DNA is predictive of the response to cART and it declines during cART but remains quantifiable despite plasma HIV RNA levels significantly decrease under undetectable levels (288). It was largely described that reservoir size in viremic non-suppressed patients have an excess of unintegrated HIV DNA that contributes to the total HIV DNA signal, while unintegrated DNA contributes very little to the viral reservoir due to its limited transcription potential. This is true for patients on suppressive ART where total HIV DNA level is reflective of the total HIV proviral reservoir size (289). Our data showed that the RFs upregulation are not associated to total HIV DNA increment in the early months of infection and prior to ART treatment even in presence of a drastic reduction of viral load. Nevertheless, the persistence of viral elements maintains the activation of immune system and the RFs' role in infected cells. This notion is supported by a recent study where Himamichi and colleagues described the existence of some "zombie" proviruses, that is, defective provirus that are unable to give rise intact to viruses but have the capacity of transcribing novel unspliced HIV-RNA molecules in HIV-infected patients on cART. Thus, this could explain the constant persistence of inflammation and immune activation in vivo. Immune activation and inflammation associated to HIV-1 persistence trigger RFs overexpression, and it was observed in viral suppressed individuals. Contrarily, our analysis did not show an overexpression in TND patients and this is consistent with recent observations who associated a different pattern of RF expression to ART timing beginning (279). Another consistent consideration is linked to the impact of RFs'gene polymorphisms with disease progression and a major or minor ability in HIV-1 acquisition (290–293). Differences in the production of RFs between individuals have been correlated to single nucleotide

polymorphisms (SNPs) and it was further described correlations between differences in the production of host defense proteins with SNPs (291). It is also now established that HIV developed several strategies to overcome host surveillance by the expression of its viral proteins that enhance viral infection and infectivity but can lead RFs degradation by proteasomal complex (136,209,294).

In this study we did not focus on the nature of pharmaceutical administration, but it could be useful in future to improve our data associated to cART. It was described that cART could interfere or modulate the RFs role during viral infection. This suggestion is in part supported by some studies who investigated the role of SAMHD1 in the modulation of dNTPs levels during retroviral transcription and in the increase of the NRTI efficacy (285,295). More recently, clinical studies suggested also the ability of HIV 1 to exploit the hypermutation activity of APOBEC3G in the induction of new drug-resistances (296). Differently, Liu and colleagues described the role of cART in the restoration of JAK-STAT pathway involved in the induction of the anti-HIV-1 cellular factors and HIV-1 inhibition in macrophages (297). They observed that HIV 1 infected subjects had a compromised JAK-STAT pathway mediated antiviral immunity and anti-HIV 1 factors. cART beginning restore some RFs expression in infected individuals (298).

Chapter2: ICPs analysis

This study focused on the analysis of IFNs signature during chronic infection as HIV1. The characterization of this signature was based on the use of flow cytometry and molecular biology assays on IFNα and IFNγ stimulated PBMCs derived from healthy subjects and HIV1 positive individuals. In the present study we analyzed levels of markers associate to inflammation and immune cells activation/exhaustion that lead to immune disfunction during chronic inflammation and infection. Despite the huge variability between samples, RNA expression analysis performed at 4 hours post stimulation with IFNα and IFNγ showed that RFs as MX1 and IP10 are significantly activated by IFNs signaling and among the ICPs analyzed, PD-L1 expression was most influenced by IFNγ stimuli. Despite that, T-Lymphocytes as total CD4+, CD8+ T-cells and related subpopulations, after 24 hours did not show any variation in the expression of PD-L1 as well as PD1, TIGIT and markers of activation as HLA-DR and CD38. Presumably time of stimulation was enough to appreciate in lymphocyte populations the enhancement of activation and exhaustion under IFN stimulation. On the contrary, myeloid cells showed a significant increment in cellular percentage positiveness for PD-L1 and it was further surprisingly to observe the increment of MFI upon IFNγ contribution thus supporting what observed at 4 hours post stimulation by molecular biology.

In the present study, we have carefully characterized different subsets of monocytes. Monocytes and macrophages are heterogeneous in nature and have the ability to exert different functions. Based on the expression of CD14 and CD16 different monocytes subsets have been described as classical, intermediate, and non-classical subsets. Intermediate monocytes are the most pro-inflammatory subtype of monocytes, and their perturbation has been reported in a variety of inflammatory and autoimmune diseases (299–301). The impact of IFNs stimulation appeared more significant in DCs subsets as observed in the increment of positive cells

percentage for markers associated to immune exhaustion and disfunction. It is known that cDCs and pDCs play different specific role in the antigen recognition and immune activation. In particular, it has been described that cDCs are specialized in in antigen presentation to naïve T cells and segregates into cDC1 and cDC2, excelling in MHC class I- and class II-mediated antigen-presentation, respectively. cDC1 possess specialized mechanisms to mediate efficient antigen recognition, antigen transport to appropriate endosomal compartments and subsequent processing for the presentation to CD8+ T-cells in a process known as cross-presentation. cDC1 can also activate CD4+ T lymphocytes through MHC class II antigen presentation. cDC2 are specialized in MHC class II-mediated antigen presentation and are the most efficient antigen presenting cells (APCs) for activation and expansion of CD4 T cells. Mounting evidence suggests cDC1s to be the critical antigen presenting DC subset for the generation of anti-viral as well as anti-tumor immunity. It is possible that cDC1s are the main DC subset that orchestrate an optimal CTL generation through antigen presentation via MHC class I as well as MHC class II (302). The co-stimulatory pathway consisting of the PD-1 receptor and its ligand, PD-L1, delivers inhibitory signals that regulate the balance among T-cell activation, tolerance, and immune-mediated tissue damage (4–7). In fact, as demonstrated in cancer biology, activation of this pathway contributes to T-cell exhaustion and lack of resolution during chronic infection (251). Interestingly, in our study we found high expression of PDL1 in monocytes, classical, intermediate and non-classical monocyte subpopulation in stimulated and mock control compared to ex vivo sample. MFI analysis revealed that only in stimulated sample we can appreciate a different pattern of expression on cellular surface between cells meanwhile mock sample did not show any increment of PD-L1 since its expression it's like *ex vivo* analysis. However, MFI analysis did not show any increment of PD-L1 in pDC and among the cDCs, the cDC1 subset showed the most evident MFI enhancement under IFNγ stimulation compared to cDC2 subset where PD-L1 increment appeared influenced by IFNα, too. Moreover, contrarily to other monocytes and DC subsets, cDC1 was the only subpopulation where LILRB2 expression is associated to IFNα stimulation, this further confirmed by MFI analysis for LILRB2. Our findings were supported by different studies describing the role of LILRB2 as an inhibitory receptor specifically expressed on myeloid cells playing a pivotal role in the regulation of cDC functions (268,269). The main ligands of LILRB2 are MHC-I molecules and their interaction trigger an inhibitory axis that it has been described as crucial in the early dysregulation of cDCs and, consequently, attenuation of immune responses (272). *In vitro* studies indicate that in the early phases of HIV1 infection, LILRB2 and MHC-I expression on cDCs surface increases during the first days of infection and their interaction induces the dysregulation of cDCs thus leading to an altered capacity to stimulate CD4+ T cells and produce cytokines (305). It was also reported that the strength of the LILRB2/MHC-I interaction is correlated with the level of cDC dysregulation and consequently the rate of disease progression in HIV-infected patients (271). Future studies are needed to elucidate the role of PDL1-expressing intermediate monocytes in regulating the interplay between host defenses aimed at eradicating inflammation and chronic infection, and possible involvement of those cells in reduced T cell function.

To date our analysis were performed only on healthy subjects even if HIV1 infected patients were further included in the planned experiments. Differences in the expression pattern of activation and exhaustion

markers could be appreciate in comparison with subjects affected by chronic inflammation or infection such as HIV1 positive individuals. Thus, the main limit of this study was the inability to perform ex vivo and in vitro analysis on infected patients in order to define the impact of IFN signatures in the differentiation of immune response and T-cells disfunction. Moreover, any results have been obtained on PBMCs isolated from HIV1 patients treated with Baricitinib, the JAK-STAT inhibitor.

A second observation concerning the small number of enrolled subjects that limited the possibility to perform correlation analysis between the expression levels of our genes of interest and the percentage or MFI of markers of activation and exhaustion. Furthermore, it will be interesting to correlate immunological and viral parameter with Real Time PCR and flow cytometry data obtained on infected individuals.

Finally, based on our preliminary results on CD4 and CD8 T-Lymphocytes, the absence of significant differences in the expression of activation or exhaustion markers could be associated with time of stimulation. Our previous results showed that PBMCS stimulated 48 hours with IFNs were characterized by higher percentage of T-cells positive for HLA-DR and CD38, both total C4+ and CD8+ T-lymphocytes and cognate subsets. It will be interesting to analyze this pattern of markers in cells stimulated 48 hours and observe myeloid cells as the first line of defense that initiate and orchestrate the innate and adaptive immunity by interacting with T-cells.

In conclusion, PD-L1 appeared overexpressed in HDs myeloid cells and not in T-lymphocytes under IFNs stimulation and any increment of markers reflecting immune activation and exhaustion was evident on T-cells. On the contrary, myeloid cells and related subpopulations appeared more susceptible at IFNs stimulation through PD-L1 upregulation and in a less extent LILRB2 that appeared upregulated in cDC1 stimulated with IFNα. Thus it is still unclear the role of cDCs during infection and our unanswered questions deal with the possible role of ICPs and RFs upregulation after IFNs treatment in presence of HIV1 infection, thus supporting the hypothesis if a synergy between IFNs and HIV-1 replication synergy could be pivotal in the immune disfunction and dysregulation.

Conclusion

In conclusion, this study offers a contribution in the characterization of host-virus interplay. Despite the limitations that encourage future studies, we observed a pivotal and initial support by the host in HIV-1 infection control, especially during the beginning of cART. Our data suggested also that antiviral treatment administration not only guarantees the control of viral replication and prevent viral rebound, but in addition it might modulate the RF expression, especially in a landscape where the virus drives or could be driving the immune activation and inflammation persistence and in a complex network where RFs and cART share some viral targets. Meanwhile, immune activation and response is crucial during the onset of infection. Restoration by using IFN-inhibitors could represent a good therapeutic strategy that, associated with ART administration, may lead toward a more efficient cure for HIV-1 infected patients and good life expectancies.

By the end, the controversial findings suggest the virus-host interaction *in vivo* is complicated.

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