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The interleukin 21 (IL 21)/ microRNA-29 (miR-29) axis is associated with natural resistance to HIV-1 infection

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ABSTRACT

BACKGROUND: Interleukin-21 (IL-21) modulates HIV-1 infection through the elicitation of different antiviral mechanisms, including Th17 lineage commitment and induction of microRNA (miR)-29, a miRNA endowed with anti-HIV activity. As miR-29 expression is significantly increased in HIV-1-exposed seronegative individuals (HESNs), we investigated the role of miR-29/IL21 axis in the natural control of HIV-1 infection.

METHODS: Analyses were performed in two cohorts of sexually-exposed HESNs, one from Italy and another one from Colombia. Overall the two cohorts included 22 HESNs and 22 HIV-unexposed healthy controls (HCs) whose PBMCs were in vitro infected with an R5-tropic HIV-$1_{Ba\text{-}L}$ strain (Italian cohort) and an HIV-1-IIIB X4-tropic HIV-1 (Colombian cohort). Seven days post HIV-1 infection we evaluated: 1) p24 production (ELISA); 2) IL-21+/CD4+ and IL-17+/CD4+ T lymphocytes (FACS); 3) IL-17 concentration in supernatants (ELISA); and 4) IL-6, IL-17, and IL-21 mRNA and miR-29a, b, c expression by CD4+ T lymphocytes and PBMCs as well as perforin and granzymes in PBMCs (qPCR). The same analyses were performed on the 19 HIV-infected partners (HIV+).

RESULTS: At baseline IL-6 expression alone was increased in HESNs compared to HCs. Thus, IL-21+/CD4+ and IL-17+/CD4+ T lymphocytes, as well as IL-21 and IL-17 expression and production were significantly augmented in HESNs compared to HCs. Interestingly, IL-21 upregulation correlated with a significantly increased expression of miR-29a, b, c in Italian cohort and with a reduced susceptibility to in vitro HIV-1 infection in HESNs alone. Differences in the expression of perforin and granzymes were observed in the Colombian cohort.

CONCLUSIONS: The IL-21/miR-29 axis is upregulated by HIV-1 infection in HESNs suggesting its involvement in the natural resistance to HIV-1 infection in these individuals. Approaches that exogenously increase IL-21 production or prompt pre-existing cellular IL-21 reservoir could confine the magnitude of the initial HIV-1 infection.

SOMMARIO

INTRODUZIONE: L'interleuchina-21 (IL-21) modula l'infezione da HIV-1 attraverso l'attivazione di diversi meccanismi antivirali, compresa la stimulazione e la proliferazione e il differenziamento dei linfociti Th17 e l'induzione del microRNA (miR)-29, un miRNA dotato di attività anti-HIV. Poiché l'espressione di miR-29 è significativamente aumentata negli individui esposti a HIV-1 sieronegativi (HESN), abbiamo voluto valutare il ruolo dell'asse miR-29 / IL-21 nel controllo naturale dell'infezione da HIV-1.

METODI: Nello studio sono state incluse due coorti di individui sessualmente esposti ad HIV-1, una proveniente dall'Italia e l´altra dalla Colombia. Complessivamete esse comprendevano 22 HESN e 22 controlli sani non esposti ad HIV (HC) le cui cellule mononucleate di sangue periferico (PBMC) sono state infettate in vitro con due ceppi virali di HIV: HIV-1_{Ba-L} R5-tropico (corte italiana) e HIV-1-IIIB X4-tropico (corte colombiana). Sette giorni dopo l'infezione in vitro abbiamo valutato: 1) la produzione di p24 (ELISA); 2) la percentuale di linfociti T CD4+ producenti IL-21 e dei linfociti T CD4+ producenti IL-17 (FACS); 3) la concentrazione di IL-17 nei surnatanti (ELISA); e 4) l'espressione dell'RNA specifico per IL-6, IL-17 e IL-21 e miR-29 a, b, c da parte dei linfociti T CD4+ e delle PBMC, così come l'espressione di perforina e granzimi da PBMC (qPCR). Le stesse analisi sono state eseguite sui 19 partner HIV-infetti.

RISULTATI: Al basale, l'espressione di IL-6 è risultata superiore negli HESN rispetto ai HC. La percentuale dei linfociti T CD4+ / IL-21+ e dei CD4+ / IL-17+, nonché l'espressione e la produzione di IL-21 e IL-17 sono significativamente aumentate negli HESN rispetto ai HC. È interessante notare che la regolazione positiva della IL-21 correla con un'espressione significativamente aumentata di miR-29a, b, c nella coorte Italiana una ridotta suscettibilità all'infezione HIV-1 in vitro in tutti gli HESN. Nella coorte colombiana sono state osservate differenze nell'espressione di perforina e granzima.

CONCLUSIONI: L'asse IL-21/ miR-29 è regolato positivamente dall'infezione da HIV-1 negli HESN suggerendo il suo coinvolgimento nella naturale resistenza all'infezione da HIV-1 che caratterizza questi individui. Approcci volti ad aumentare la produzione esogena di IL-21 o a sollecitare le riserve cellulari preesistenti di IL-21 potrebbero limitare l'ingresso del virus e quindi ridurre la suscettibilità all'infezione da HIV-1.

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Abbreviation

AAT: Alpha-1-antitrypsin

Act 1: transcription factor NF-κB Activator 1

Ago: Argonaute

AICEs: AP1-IRF4 Composite Elements

AIDS: Acquired Immunodeficiency Syndrome

APCs: Antigen-Presenting Cell

APOBEC: Apolipoprotein B

APOBEC3G/ CEM15: Apolipoprotein B mRNA Editing enzyme Catalytic polypeptide-like Complex 3 (A3G)

ART: AntiRetroviral Therapy (ART)

ARVs: AntiRetro Virals

B10: regulatory B cells 10

BATF: Basic leucine zipper Transcription factor

BCL-3: B cell Lymphoma-3

Blimp-1: B Lymphocyte–Induced Maturation Protein-1

bp: base pair

CAF: CD8+ T lymphocyte Antiviral Factor

cART: Combinatorial Antiretroviral Therapy

CCL2: Chemokine (C-C motif) Ligand-2

CCL7: Chemokine (C-C motif) Ligand

CCL20: Chemokine (C-C motif) Ligand-20

CCL3L1: Chemokine (C-C motif) ligand 3-like 1

CCL3L1: Chemokine (C-C motif) ligand 3-like 1

CXCR3: CXC-Chemokine Receptor-3

CCR5: C-C chemokine receptor type 5

CCR5-Δ32: Deletion of CCR5- delta 32

cDNA: complementary DNA

CRFs: Circulating Recombinant Forms

CTLA: Cytotoxic T-Lymphocyte Antigen

CTLs: cytotoxic T lymphocyte cells

CVL: CervicoVaginal Lavages

CXCL1: Chemokine (C-X-C motif) Ligand- 1

CXCL2: Chemokine (C-X-C motif) Ligand- 2

CXCL10: CXC-chemokine ligand 10

CXCR-4: C-X-C chemokine receptor type 4

CXCR6: CXC-Chemokine Receptor-3

CypA: Cyclophilin A

DCs: Dendritic Cells

DC-SIGN: Dendritic Cell Specific Intercellular adhesion molecule 3-Grabbing Non integrin

DC-SIGNR: Dendritic Cell Specific Intercellular adhesion molecule 3-Grabbing Non integrin Related

DNA: DeoxyRibonucleic Acid

dsRNA: double-stranded RNA

EC: Elite Controllers

ECL1: first Extracellular Loop

ECL2: second Extracellular Loop

EDN: Eosinophil-Derived Neurotoxin

ELAFIN: Specific Elastase Inhibitor

Eomes: Eomesodermin

ERAP1: endoplasmic reticulum aminopeptidase 1

ERAP2: endoplasmic reticulum aminopeptidase 2

ERK: Extracellular signal–Regulated Kinases

ESCRT: Endosomal Sorting Complex Required for Transport

Foxp3: Forkhead box P3

GALT: Gut-Associated Lymphoid Tissue

G-CSF: Granulocyte Colony-Stimulating Factor

GLP-1R: Glucagon-Like Peptide-1 Receptor

GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor

GWA: Genome-Wide Association

Gzm: Granzyme

Gzma: Granzyme A

Gzmb: Granzyme B

HAB: Human Alpha-Defensines

HapMap: Haplotype Map

HAV: Hepatitis A Virus

HAVcr-1: Hepatitis A virus cellular receptor 1

HAVCR2: Hepatitis A virus cellular receptor 2

HBD: Human Beta-Defensines

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HESN: HIV Exposed SeroNegatives individuals

HIV: Human Immunodeficiency Virus

HLA: Human Leukocyte Antigen

HLA-DQB1: Major Histocompatibility Complex, class II, DQ beta 1

HSV: Herpes Simplex Virus

HTLV-1: Human Leukaemia Virus type 1

ICAM-1: InterCellular Adhesion Molecule 1

IDUs: Intravascular Drug Users

IDUs: Intravenous Drug User

IFNs: Interferons

IFN-α: Interferon-α

IFN-γ: Interferon- γ

Ig: immunoglobulin

IL: Interleukin

IL-1α: Interleukin-1 alpha

IL-1β: Interleukin-1 beta

IL-2: Interleukin-2

IL-4: Interleukin-4

IL-5: Interleukin-5

IL-6: Interleukin-6

IL-6R: Interleukin-6 Receptor

IL-10: Interleukin-10

IL-13: Interleukin-13

IL-15: Interleukin-15

IL-17: Interleukin-17

IL-18: Interleukin-18

IL-21: Interleukin IL-21

IL-21R: receptor of IL-21

IL-22: Interleukin-22

ILC3: Group 3 I Innate lymphoid cells

IN: Integrase

IP-10: IFN-γ induced Protein-10

IRF-1: Interferon Regulatory Factor 1

ISG: Interferon Stimulated Gene

JAK1: Janus Kinase 1

JAK3: Janus Kinase 3

KIR: Killer immunoglobulin-like receptor

LAG: Lymphocyte Activation Gene

LCMV: Lymphocytic Choriomeningitis Virus

LRA: latency-reversing agents

LTNPs: Long-Term Non-Progressors

LTR: Long Terminal Repeat

MAPK: Mitogen-Activated Protein Kinase

MHC: Major Histocompatibility Complex

MHC: Major Histocompatibility Complex

MIG: Monokine Induced by IFN-γ

MIP1-α: Macrophage Inflammatory Protein-1 alpha

MIP-1b : Macrophage Inflammatory Protein-1 beta

MIP3α: macrophage inflammatory protein 3α

miR: microRNA

miRNA: microRNA

MMPs: Matrix MetalloProteases

mRNA: messenger -RNA

MSCs: Mesenchymal Stem cells

MSM: Men who have Sex with Men

MX: Human Myxovirus resistance

MX2: Human Myxovirus resistance 2

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells transcription factor

NKs: Natural Killer cells

PAMPs: Pathogen-Associated Molecular Patterns

PBMCs: Peripheral Blood Mononuclear Cells

PD-1: Programmed Death-1

PI3K: PhosphoInositide 3-Kinase

PICs: Pre-Integration Complexes

Pol: Polimerase

PPR: Pattern Recognition Receptors

PR: PRotease

PtdSer: Phosphatidyl Serine

RA: Rheumatoid Arthritis

RANTES: Regulated on Activation Normal T Expressed and Secreted

RISC: RNA-Induced Silencing Complex

RNA: RiboNucleic Acid

RNases: Ribonucleases

RORγ: Retinoic acid receptor-related Orphan Receptor-γ

RRE: Rev Responsive Elements

RSV: Respiratory Syncytial Virus

RT: Reverse Transcriptase

RTCs: Reverse-Transcription Complexes

SEFIR: Similar Expression to Fibroblast growth factor genes, IL-17 Receptors and Toll–IL-1R

SerpinA1: Serine protease inhibitor, group A, member 1

SH2: SRC-Homology 2

sIL-6R: Soluble Interleukin-6 Receptor

SIV: Simian Immunodeficiency Virus

SLE: Systemic Lupus Erythematosus

SNP: Single-Nucleotide Polymorphism

SOCS1: Suppressor Of Cytokine Signaling 1

SOCS3: Suppressor Of Cytokine Signaling 3

SP: Spacer Peptides

SS: Sjogren's Syndrome

ssRNA: Single Stranded RNA

STAT: Signal Transducer and Activator of Transcription

STAT1: Signal Transducer and Activator of Transcription 1

STAT3: Signal Transducer and Activator of Transcription 3

STAT5: Signal Transducer and Activator of Transcription 5

TAR: Transactivation Response Element

T-bet: T-box transcription factor

TCR: T-cell receptor

TFH: T Follicular Helper cells

TGF-β: Transforming Growth Factor Beta

Th: T helper cells

TH17: T Helper 17 cells

TIM: T cell immunoglobulin and mucin domain-containing molecule

TLRs: Toll-Like Receptors

TNF-α: Tumor Necrosis Factor alpha

TNF-β: Tumour Necrosis Factor beta

Treg: Regulatory T cells

TRIM5α: Tripartite Motif proteins-5α

tRNA: transfer-RNA

UTR: UnTranslated Region

VDR: Vitamin D Receptor

VEGFR: Vascular Endothelial Growth Factor

Vif: Viral Infectivity Factor

VP: Viremic Progressors

XCL1: Lymphotactin

1. INTRODUCTION

1.1 The Human Immunodeficiency Virus

Human Immunodeficiency Virus (**HIV**) is the causative agent of Acquired Immunodeficiency Syndrome (**AIDS**) and represents one of the biggest health problems worldwide. Recent numbers estimate that approximately 76.1 million people have become infected with HIV since the beginning of the pandemic, and 35 million people had died as a result of AIDS-related diseases (1). The overwhelming majority of people living with HIV are in low-income countries being the fifth cause of death in the entire world (2).

HIV virus belongs to the orden Ortervirales within the family of Retroviridae*,* subfamily Orthoretrovirinae*,* genus *Lentivirus* and is classified in two types, HIV-type 1 (**HIV-1**) and HIV-type 2 (**HIV-2**) (3). **HIV-1** is the retrovirus that has the greatest impact on humans being the responsible of most of the HIV infection cases worldwide. Its phylogenetic origin comes to a cross species transmission to humans from a Simian Immunodeficiency Virus (SIV) isolated from a Chimpanzee sub-species, *Pan troglodytes troglodytes*. HIV-2 is the second in a similar class of human retroviruses, but its distribution is particularly limited to West Africa and the primate reservoir is *Cercocebus atys* (common name: green monkey). HIV-1 and HIV-2 are firmly related viruses: their structure are analogous and diverge in the conformation of their genome with nucleotide sequence homology of 58% in ***gag***, 59% in ***pol*** and 39% in ***env*** genes (4). Additionally, both viruses have a specific complex combination of regulatory and accessory genes involved in

other important functions (3).

A principal cause for AIDS is the progressive decrease of CD4+ T lymphocyte cells count in infected patients, which are the main cell target for HIV-1. Since CD4+ T cells are critical for the induction of specific humoral and cell-mediated immune responses, this depletion increases risk of opportunistic diseases or malignancies, and the sufferer has a less effective response against other pathogens that enter the body (5), which is the hallmark of the illness.

1.1.1 HIV-1 groups and subtypes

Phylogenetic analyses have identified four different HIV-1 groups, called the **Major** (**M**), **Outlier** (**O**), **Non-M/non-O** (**N**) and **Putative** (**P**), based on differences of approximately more than 40% in some nucleotide sequences. The M group was the first to be found and represents the pandemic type of HIV-1 as it is responsible of over 90% of the world HIV-infections. This group has been sub-classified into nine distinct subtypes, also known as clades or genotypes, indicated with letters A, B, C, D, F, G, H, J and K, as well as more than 40 different circulating recombinant forms (CRFs), which are produced when different subtypes infect similar cell populations (4).

The HIV-1 subtypes C and A constitute the greater part of HIV cases in the pandemic, yet there are other viral forms circulating around the world. The most prevalent transmitted infection is Subtype C with 80% of all worldwide HIV-1 infection; its wide transmissibility mirrors the high viremia set point in an infection, and the higher proportion levels of this virus type, found more in genital fluids than clades that are prevalent in southern Africa and India. Subtype A is dominating in territories of central and eastern Africa (Kenya, Uganda, Tanzania, and Rwanda) and in eastern European nations. HIV-1 subtype B is overwhelming in North America, Western Europe, and

Australia and is likewise present in a few nations of Southeast Asia, northern Africa, and the Middle East, and among South African and Russian homosexual men. The subtypes B and D are more closely related, and clade D is viewed as the early clade B African variant (4) (6). Inter-subtype variety is around 30% regarding the *env* gene sequence and 15% for both the *gag* and *pol* genes sequences. In the Group O there are genetics variations around 10% called sub-subtypes discovered in 1990, this group is much less common than group M and represents less than 1% of global HIV-1 infections and is present in Cameroon, Gabon, and neighboring nations. Group N was identified in 1998, and is even less prevalent than group O; group P was found in 2009 in a Cameroonian woman living in France. All members of all this groups are able to cause CD4+ T-cell depletion and AIDS (7) .

How HIV-1 groups M, N, O, and P were transmitted to humans and diversified is currently not known; however, in view of the biology of these infections, transmission must have occurred through cutaneous or mucous membrane exposure to infected simian blood and/or body fluids. Such exposures happen most usually in the context of bush meat (wild meat) hunting (6).

1.1.2 HIV 1 genes

HIV-1 is an enveloped virus that encloses two identical 9.2 kb single stranded RNA (ssRNA) molecules, while the persistence form of the HIV-1 genome is preserved as a proviral double stranded DNA inside of infected cells. The HIV virion is spherical and has a pleomorphic shape with a diameter of approximately 100 to 120 nm, and possesses a lipoprotein-rich bilayer membrane that encompasses a thick, truncated cone-shaped nucleocapsid (**core**), wherein the RNA molecules are joined to a nucleoprotein containing as well the enzymes **Reverse Transcriptase** (**RT**),

Protease (**PR**) and **Integrase** (**IN**) (5).

The HIV genome is characterized by the presence of the structural genes ***gag***, ***pol***, and ***env***. The *gag-pol* gene codifies for a large 160 kd precursor molecule and is translated into the precursor protein gp160, it is glycosylated inside the endoplasmic reticulum, and subsequently cleaved by the HIV-1 protease into gp120 and gp41. The proteins coded by *pol* and *gag* genes shape the nucleus of the developing HIV particle and encode the viral enzymes protease, reverse transcriptase and integrase, from which the HIV protease cleaves the p24, p17, p9 and p7 Gag final products and the Pol proteins. The *env* gene codifies for the glycoprotein spikes of the viral envelope (3)(5).

The Gag precursor contains different domains: **Matrix**, **Capsid**, **Nucleocapsid** and **p6**, and also two spacer peptides, **SP1** and **SP2**. The Matrix domains target Gag to the plasma membrane and advances joining of the viral **Env** glycoproteins into the shaping virions. Capsid domain drives **Gag** multimerization during assembly. Nucleocapsid domain selects the viral RNA genome into virions and encourages the assembly and the p6 domain enlists the Endosomal Sorting Complex Required for Transport (ESCRT) complex, which catalyzes the membrane fission step to finish the budding process (8).

The proteins gp120 and gp41 remain non covalently connected with one another, as the external envelope of the virus and is promptly shed from the cell surface (7), making possible the identification of these in serum of HIV infected patients (3). Each viral particle membrane incorporates glycoprotein heterodimer buildings made out of trimers of the external surface gp120, and the transmembrane gp41 glycoproteins then sticks together.

HIV-1 viruses have other accessory and regulatory genes that play an

important role in the modulation of virus replication: ***Vif***, ***Vpr***, ***Rev***, ***Vpu***, ***Tat*** and ***Nef***. The *tat* gene codifies for the Tat protein that is expressed in the early moments after an infection, and advances the expression of HIV genes. The *Rev* gene codifies for the Rev protein that guarantees the transport from nucleus to cytoplasm of the accurately handled messenger and genomic RNA. All of the functions of the further accessory HIV proteins is less known. It is considered that the Vpr protein is engaged with the arrest of the cell cycle. This protein can be permit the reverse transcribed DNA to access the nucleus in non-dividing cells. Vpu is a protein codified by the *vpu* gene, important for the correct release of virus particles, allowing their maturation and trafficking; while the *Vif* gene codes for Vif protein, a little protein that improves the infectiveness of offspring virus particles. *Nef* gene encodes for the Nef protein that not only is important in cellular signal transduction, but also, in early phase, accelerates endocytosis and subsequent degradation of CD4 molecules as well as Major Histocompatibility Complex (MHC) class I and II molecules that reside on the cell surface, and in the late phase, down regulates the CD4 receptor on the cell surface to permit maturation and budding of virions. *Tat* gene encodes Tat protein which, in turn, also has regulatory functions including the activation of the transcription through binding to the TAR element of the LTR and to other transcriptional activators of cellular origin (3)(5).

1.1.3 The HIV-1 replication cycle

The HIV-1 replication cycle can be categorized into two phases: early and late (Figure 1). The early phase includes the events of infection that occur from virus binding to the surface of the host cell until the integration of the viral complementary DNA (cDNA) into the host cell genome (8); the late phase starts with the expression of viral genes and proceeds through to the release, development and maturation of progeny virions (9).

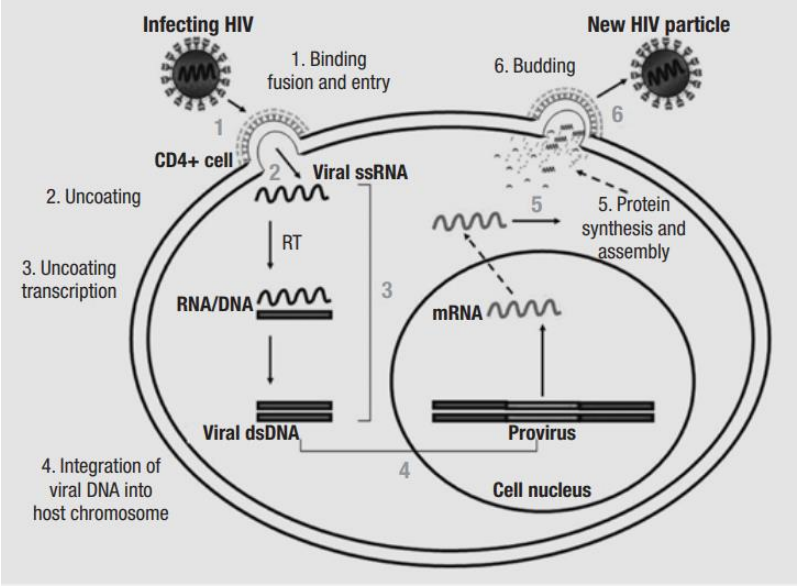

Figure 1. Early and late steps of HIV replication virus. *Early steps*: **1)** binding and entry; **2)** uncoating; **3)** reverse transcription; **4)** provirus integration. *Late steps*: **5)** virus protein synthesis and assembly and **6)** budding. RT: reverse transcriptase; dsDNA: double strand DNA (3).

The early phase is composed of different steps: **binding and entry**, **uncoating**, **reverse transcription of the viral RNA to DNA** and **DNA integration**. The initial step of the retroviral replicative cycle is the adsorption of viral particles to the surface of their target cells. The main HIV receptor is the CD4 molecule, a transmembrane glycoprotein representative of CD4+ T cells that interacts with MHC Class II antigens. In addition, HIV requires the binding to a co-receptor, which can be CCR5 (C-C chemokine receptor type 5) or CXCR4 (C-X-C chemokine receptor type 4) chemokine receptors. The early attachment of virions to the cell surface has been ascribed to various cell-surface molecules, including heparan sulfate, proteoglycan, the integrin LFA-1 and nucleolin (9).

The viral envelope trimeric complex, composed of the heterodimer proteins gp120 and gp41, is critical for virus recognition and passage into

target cells and binds to the CD4 receptor. The gp41 subunit contains a fusogenic hydrophobic peptide at its amino end, which is basic for combination of the viral and cell films (3). The interaction gp120-CD4 prompts a conformational change in the glycoproteins of the envelope, which uncovers a specific domain in the gp120 ready to the enlistment of co-receptors belonging to the seven transmembrane chemokine receptor family presents on the cell membrane, primarily the chemokines receptors CXCR4 and CCR5, yet other potential co-receptors have been described. As the affinity of HIV envelope glycoproteins for CD4 is moderately low, especially in the case of primary virus isolates, the presence of other attachment factors may serve to focus the virus on the target cell surface before specific receptor engagement (9).

Depending on the specific co-receptor usage or tropism, different virus strains have been described. The viral strains that bind to the CXCR4 co-receptor are known as T-lymphocyte-tropic (T-tropic) or X4 viruses, as this chemokine receptor is highly expressed in T cells. While the viral strains that bind to CCR5 β-chemokine family receptors (RANTES, macrophage inflammatory proteins MIP-1-α and MIP-1-β) expressed by monocytes/macrophages, dendritic cells and activated T-lymphocytes are known as macrophage-tropic (M-tropic) or R5 viruses. There are HIV strains that can bind both CCR5 and CXCR4 receptors, and are known as dual tropic or X4/R5 tropic (3). HIV-1 infection is started by virus with tropism for the CCR5 co-receptor and, in 50– 60% of the patients, viral strains that can utilize the co-receptor CXCR4 develop over the course of infection; thus, the presence of X4 tropism is associate with disease progression (5).

There are different conditions that modify the interaction between HIV and cells targets and at the same time the disease progression. For instance, the natural ligands of CXCR4 and CCR5 have been shown to the ability to

inhibit the union of viruses to T cell tropic or monocyte tropic HIV strains. Diverse polymorphisms in the CCR5 gene have been associated with functional outcomes for HIV-1 pathogenesis. For instance, a deletion of 32 bp within the exon of the CCR5 gene (**CCR5-delta32**) confers protection against HIV-1 disease and a slower progression to AIDS in individuals homozygous for the allele. The phenotype of another chemokine receptor (**CCR2 V64I**) is also correlated with slower progression of AIDS. Since CCR2 is just utilized by uncommon HIV-1 variations, CCR2 V64I variant towards negative regulation of CCR5 may contribute to the beneficial impact on HIV-1 disease (5).

In the interplay of HIV with dendritic cells (**DCs**), the molecular interactions are different compared to that with CD4+ T cells. HIV binds the surface of dendritic cells through interaction of its envelope glycoproteins with the C-type mannose binding lectins DC-SIGN (Dendritic Cell Specific Intercellular adhesion molecule 3-Grabbing Non integrin) and **DC-SIGNR** (DC-sign Related). These interactions occur because HIV-1 contains high mannose structures on gp120 that are recognized by DC-SIGN. DCs capture virions at peripheral sites of infection and deliver them to the lymph nodes, thus promoting efficient *trans*-infection of CD4+ T cells (9).

In the context of the typical interaction between HIV and the target cells, the double interplay of gp120 with both CD4 and one chemokine receptor permits a stable attachment of the virus, which enables the N-terminal fusion peptide gp41 to penetrate the cell membrane. The HR1 and HR2 repeat sequences in gp41 connect, promoting conformational change of the extracellular part of gp41 right into a hairpin. This loop structure carries the virus and cell membranes unitedly, permitting the membrane fusion and entry of the viral capsid. The entrance of virions into the cell is reached by insertion of the gp41 fusion peptide into the target membrane, ensuing in the fusion of

viral and cellular membranes and the release of the viral core in the cytoplasm of the cell. Immediately after, the viral core undergoes a partial and progressive disassembly, the so called uncoating step (5)(9).

There are three proteins that modulate the early moments of the replicative cycle, **Nef**, **Vif** and cyclophilin A (**CypA**). However, their mode of action continues to be unclear. Nef possibly modulates viral entry only when it happens via fusion at the plasma membrane. Additionally, it has been proposed that Nef could enhance viral infectivity by augmenting the synthesis and incorporation of cholesterol into progeny virions (11). Vif neutralizes the antiviral action of CEM15/APOBEC3G by reducing its expression, this cell protein is a DNA deaminase that is incorporated into virions at the entry, and later exerts antiviral activity during reverse transcription, triggering G-to-A hypermutation inside the nascent retroviral DNA; also, Vif prevents its incorporation into progeny virions. CypA is integrated into virions through its interaction with viral capsid, and it is crucial in the appropriate disassembly of the HIV-1 core early after infection. In addition, CypA protects the viral capsid from the human restriction Ref-1 factor, increasing HIV-1 infectivity (9).

The uncoating results in the generation of subviral particles called **Reverse-Transcription Complexes** (**RTCs**) and **Pre-Integration Complexes** (**PICs**). RTCs interact with the host cytoskeleton after infection, likely via a direct interaction between the matrix protein and the actin network. PICs include a protease, a reverse transcriptase, an integrase and a Vpr protein (9). The pre-integration complex docks to the nuclear membrane directed through HIV-1 Vpr and enters the nucleus via the nuclear pore (5).

Reverse transcription normally occurs after the release of the viral core

into the cytoplasm of the cellular target. The transcription of viral RNA to proviral DNA occurs by the action of the RT and the IN. The viral RNA is reverse transcribed by the virion-packaged RT in the cytoplasm, through its Ribonuclease H active site. RT binds to the viral minus-strand to begin with the polymerization process at the primer binding site. The viral RNA is transcribed right into a RNA/DNA hybrid double helix (3)(8). Soon after, the Ribonuclease H site breaks down the RNA strand and the polymerase active site of the opposite transcriptase completes a complementary DNA strand to shape a double helix DNA molecule. This is incorporated inside the cell genome through the IN enzyme, which cleaves nucleotides of each 3´ ends of the double helix DNA, developing sticky ends and transferring the modified provirus DNA into the nucleus thus allowing its integration into the host cell genome (3). Previous to the integration, the viral DNA can be located inside the nucleus in three different structures: linear, 1-LTR or 2-LTR circles. Linear double-stranded DNA in the pre-integration complex is inserted into the host chromosome by the viral IN (5).

The proviral DNA integration and the expression of the provirus require that the target cell is in a metabolic activated state. This integration on different cells as monocytes/macrophages, microglial cells, and latently infected quiescent CD4+ T-cells, which are important HIV cellular reservoirs. Upon cellular activation, transcription of proviral DNA into a messenger RNA takes place (3). The first round of proviral transcription employs host RNA polymerase II and cellular factors which bind to the viral LTR, to synthetize the regulatory early such as Tat and Rev. Tat links to the TAR site (Transactivation Response Element) at the beginning of the HIV-1 RNA within the nucleus and promotes the transcription and the formation of longer RNA transcripts (3). Rev binds to RRE structure (Rev Responsive Elements) existing in the singly-spliced and unspliced viral RNA molecules and

facilitates their shipping to the nucleus membrane, the transcription of longer RNA transcripts and the expression of structural and enzymatic genes. Furthermore, it inhibits the production of regulatory proteins, consequently promoting the formation of mature virions. Viral RNAm coding for long fragments drift into the cytoplasm, where new viral structural proteins are synthesized (3). Subsequently, Env proteins migrate and insert into the plasma membrane. Gag and Gag-Pol polyproteins additionally pass to the cellular membrane and begin to assemble directed by means of the Gag polyprotein. Initially, in the infection process, Nef promotes the endocytosis and degradation of MHC I and II molecules at the cellular surface. Later, the envelope precursor gp160 traps the newly-synthetized CD4 molecules inside the endoplasmatic reticulum. Furthermore, Vpu induces the degradation of these CD4 molecules and releases the gp160 molecules allowing their maturation and trafficking, and additionally compose ion-conduction related to virus release (5).

The new virions formation is a stepwise process that continues with two viral RNA strands paired with replication enzymes, whilst core proteins gather over them forming the virus capsid and this immature particle migrates toward the cellular surface. The large precursors are cleaved by the HIV-1 protease, ensuing in new infectious viral particles, which bud through the host cellular membrane, thus acquiring a new envelope. Along of the budding process, the virus lipid membranes may additionally incorporate various host cell proteins including HLA elegance I and II proteins, or adhesion proteins that could facilitate adhesion to other target cells such ICAM-1 (Intercellular Adhesion Molecule 1) (3). As well as a matrix protein.

The budding of virions is different according to the cell type: in T-lymphocytes, budding occurs on the cell surface and virions are released into the extracellular field; in contrast, budding in monocytes and macrophages

result within the accumulation of virions in intracellular vacuoles which are then released (3).

One of the most important features of retroviruses include the ability of adaptation, immune-scape and therapy resistance, all through the capacity to introduce mismatches in the cDNA it synthesizes, which has a mutation rate of 3.4×10^{-5} mutations per base pair per replication cycle. Since HIV genome is approximately 10^4 base pairs in length, the rate of viral production is approximately 10^{10} per day (12). Moreover, other host mechanisms contribute to boost HIV rapid evolution. For example, the cellular apolipoprotein B mRNA editing enzyme catalytic-like family of enzymes (**APOBEC**), that induces hypermutation of cytosines to uracils in the viral negative single strand DNA (9), and recombination between diverse HIV-1 genomes (12). The high genetic variation provides HIV-1 with maximum adaptation efficiency and explains the challenges to develop an effective vaccine against it (8).

1.1.4 Latency & reservoirs

HIV-1 remaining at rest in memory or naive CD4+ T cells, macrophages or mononuclear cells leads to a non-productive infection. Latent infection is defined as a cell that at one moment lacks the expression of viral particles, but following a specific stimulus, is capable to express infectious viral particles (5)(13).

The most important latency cellular reservoir is represented by resting CD4+ T cells where the virus can establish two different forms of viral latency: a labile pre-integration form and a stable post-integration form.

The molecular mechanisms that allow to determinate whether a virus is to interrupt or to continue its life cycle are incompletely defined. There are several theories trying to explain this phenomenon, and for a long time it has been thought that resting T-cell infection occurs when an activated T cell is in the process of reverting to a resting state. At this stage, T cells can allow the early stages of virus infection, as reverse transcription and integration, but subsequent steps are attenuated once the cell achieves the resting state (14). Other studies demonstrated the virus capacity to infect resting cells even if weakly (15). It has also been described that HIV infection could promote the production of cytokine signaling following Nef stimulation thus promoting susceptibility of resting T lymphocytes (16). In the same way, cellular proliferation may contribute to latency favouring identical proviral sequences integrated at the same position in the host genome in multiple cells (17). Antigenic driven proliferation contributes to this phenomenon when the T cell receptor of a memory T cell is stimulated by a specific antigen that drives clonal expansion (18).

The latency happens even in the course of antiretroviral therapy (**ART**) that efficiently reduces plasma viremia to undetectable ranges. This therapy

is highly effective in controlling HIV peripheral replication but not to eradicate the virus totally. Furthermore, it may not be able to target HIV-1 that crosses the blood-brain barrier, although an early administration of ART is correlated with lower total and integrated HIV DNA, and minor frequency of latent infection. Latency decays very slowly with a half-life of 40–44 months, making essential lifelong ART suppress recognition of infection (19). HIV-1 sequences existent in reservoirs are not only wild type but also those that show resistances. Latently infected cells are long-lived, immunologically invisible, may undergo homeostatic proliferation, and are refractory to combinatorial antiretroviral therapy (cART); the majority of these cells contain a single copy of HIV-1 DNA that is stably integrated into the genome and is transcriptionally silent.

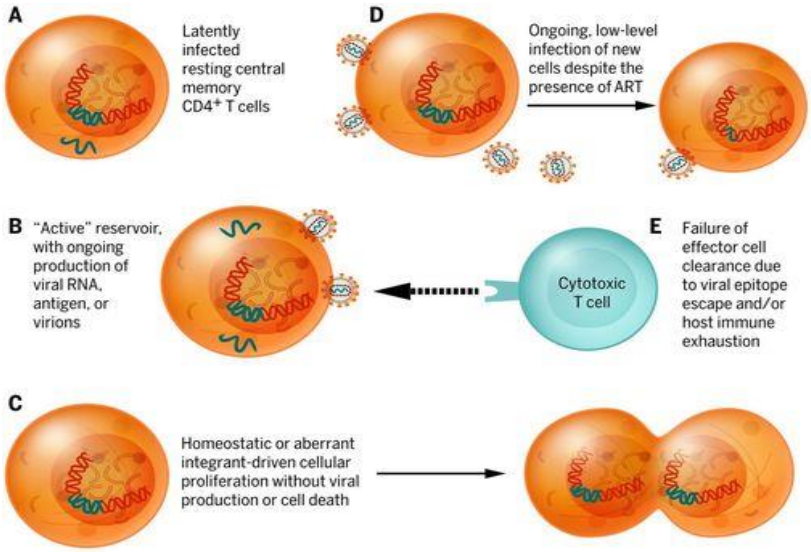

Figure 2. HIV latency. Potential obstacles to HIV eradication. (**A**) Latency virological and transcriptional, with low levels of HIV RNA expression, and no detectable HIV antigen presentation. (**B**) "Active latency" with continuous production of HIV RNA and antigens. (**C**) Proliferation of latently infected cells, driven by homeostatic forces, or by dysregulation of the host gene program by a viral integrant, without viral production. (**D**) The possibility that de novo infection occurs despite effective ART. (**E**) Failure of immune clearance owing to viral epitope escape or host immune exhaustion (19).

Anatomical sanctuaries include cellular reservoirs in circulation, gut-associated lymphoid tissue (**GALT**), central nervous system in perivascular macrophages, microglia, bone marrow hematopoietic progenitor cells, secondary lymph nodes, and the spleen. Additionally, some investigations have shown that the lung is a potential reservoir for the virus, as HIV-1 has been found in alveolar macrophages in patients on ART with undetectable plasma viral loads (18).

HIV-1 latency and reservoirs represent the major barrier to virus eradication and is one of the most difficult and significant medical research problems of the modern era, as illustrated in Figure 2 The total elimination of the viral reservoir presumably needs a combination of pharmacological agents with different mechanisms of action with no adverse effects and highly effectively target against circulation latent viruses and those located at anatomical reservoirs (20). Some trials propose to target persistent HIV infections through latency reversal using small molecules capable of inducing HIV-expression. One of this proposed strategy aims to reactivate viral transcription with various latency-reversing agents (LRA) that would drive to the death of the productively infected cells by the virus itself, or by the host immune system, and dodge new infections by antiretroviral therapy. There are several LRA that have been extensively investigated, most notably Histone deacetylase inhibitors (HDAC inhibitors), which are an emerging class of therapeutics with potential as anticancer drugs (21).

New gene methodologies can be produced utilizing gene editing strategies to completely eradicate the HIV-1 genome from infected cells in whole animal models and then in a clinical setting. To achieve this purpose, it is necessary to comprehensively study viral genetic variations, and improve on the delivery mechanisms of the editing materials to reach latently infected circulating cells, and those cells that become anatomical sanctuaries (19).

1.2. HIV Immunology

1.2.1 Immune responses to HIV

During early phases of infection, the immune responses to HIV is quite similar to that to other viruses, but it fails in providing a complete protection and in eradicating the infection.

1.2.1.1 Innate Immune Responses

The innate immune responses are the first line of defense against pathogens. Cells of the innate immune system identify pathogens by pattern recognition receptors (**PPR**), which are able to recognize pathogen-associated molecular patterns (**PAMPs**), evolutionary conserved structures. The family of Toll-like receptors (**TLRs**) are the most well characterized PPR. Viral infections are detected by TLR9, TLR7, TLR8 and TLR3 that recognize viral DNA or single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA). Recognition of viruses by TLRs induces the production of a cascade of cytokines. At the beginning, there is a sudden increase in many cytokines, which is triggered by Dendritic Cells (DCs), infected $CD4^+$ T cells, monocytes, macrophages and Natural Killer cells (NKs). In particular, a rapid increase of Interferon-α (IFN-α), which is followed by a transient up-regulation in interleukin-15 (IL-15), IL-18, IL-22, IL-10, Interferon- γ (IFN-γ), Tumor Necrosis Factor α (TNF-α), and CXC-chemokine ligand 10 (CXCL10) levels (22), are observed. IFNα directly inhibits HIV replication at different stages of the viral life cycle and by the activation of the Interferon Stimulated Genes (ISG) (23). During acute infection an expansion of NK cells has been reported. This phenomenon is probably consequent to high levels of pro-inflammatory cytokines secreted by monocytes and DCs (24). Then, progression of HIV infection is characterized by NKs activity decrease.

1.2.1.2.a Adaptive Immune Responses: Cell-mediated Immune Responses

Adaptive humoral and cellular immune responses play an important role in the fight against HIV infection. T lymphocytes are involved in cellular immune responses and they can be subdivided into two main types: T helper (Th) $CD4^+$ T cells and cytotoxic $CD8^+$ T cells (CTLs). $CD4^+$ T cells can then have classified into different T cells subsets: Th1, Th2, Th17, and Treg. Th1 cells produce type 1 cytokines (IFN-γ, TNFα and IL-2), which are important for T cells proliferation and activation and for CTL differentiation into memory or terminally differentiated CTLs. Th2 cells activate the humoral immune responses by producing IL-4, IL-5 and IL-13. Th17 cells produce IL-17 promoting inflammation and the clearance of pathogens, especially at mucosal sites (25).

The main function of CTLs is to detect and eliminate virus-infected cells. CTLs recognize viral antigens associated with MHC I molecules on HIV-infected cell surface and once activated, they release perforines and granzymes that can directly kill infected cells (26). CTLs may also induce apoptosis of infected cells by Fas-L/Fas interaction (27). During the early phases of HIV infection, the adaptive immune response is characterized by the appearance of HIV-specific $CD8^+$ T-cells. It has been established that they play a key role in controlling viral replication (28). These $CD8^+$ T cells are specific for viral antigens **env** and **nef**, while $CD8^+$ T cells against other viral proteins appear later during infection (29). Cellular immune responses are critical in controlling HIV infection. Indeed, the evolution of the virus resulted in viral isolates that are able to escape to CTLs and $CD4^+$ T cells having lost their original CTL and Th epitopes (30). Persistent viral replication leads to a functional exhaustion of HIV-specific T cells, a condition characterized by lose of proliferative and cytotoxic abilities and death by

apoptosis (31). Several markers of T-cell exhaustion have been identified such as Programmed Death 1 (PD-1), cytotoxic T-Lymphocyte Antigen (CTLA)-4, lymphocyte activation gene (LAG)-3, T cell immunoglobulin domain and mucim domain (TIM)-3 (32).

1.2.1.2.b Adaptive Immune Responses: Humoral Immune Responses

Humoral responses are another important arm of the adaptive immune system. DCs uptake viral proteins and process them into small peptides. DCs present viral antigen /MHC II complexes to $CD4^+$ Th cells, which in turn stimulate naïve B cells by producing specific cytokines and by expressing co-stimulatory receptor molecules. B cells that recognize specific epitopes by membrane antibodies, differentiate into plasmacells, that produce immunoglobulins, and into memory B cells.

Antibody to HIV can be measured within 6 to 9 weeks' post-infection and this process is commonly referred as seroconversion (33). Envelope glycoproteins are the most immunogenic proteins of HIV, and high titers of anti-gp120 and anti-gp41 can be detected in HIV-infected patients. The first produced antibodies usually are not neutralizing and are not efficient in controlling the infection. Neutralizing antibodies have been detected after 3 months from primary infection, however, they are not usually able to cope with the evolving virus. Interestingly, broadly neutralizing antibodies have been found in patients who have been infected with HIV-1 for a few years (34). These antibodies which are effective in clearing the infection have been selected after extensive somatic hypermutation, implying that naïve B cells bind weakly to antigenic epitopes. Many vaccine strategies are based on the induction of broadly neutralizing antibodies, even though so far very little has been achieved.

1.3. Natural resistance against HIV

Since 1989, human individuals exposed to HIV-1 have been repeatedly described to be seronegatives, i.e. HIV-uninfected. The acronym consensus frequently used to designate this population is **HIV Exposed SeroNegatives individuals** (**HESN**), which are subjects who although repeated exposure to the virus, by different ways, remain persistently HIV seronegative. Notably, despite the absence of HIV antigens as well as viral DNA/RNA the exposure of these individuals to HIV is confirmed by the presence of humoral and cellular HIV-specific elements (35), some of these are illustrated in Figure 3.

HENS individuals are relatively rare, approximately 10%–15% of all HIV-exposed subjects, living in different regions of the world (36) and they have been identified in all the so called at risk cohorts including: heterosexual serodiscordant couples, Men who have Sex with Men (MSM), healthcare workers accidentally parenteral exposed to HIV, Intravenous Drug User (IDUs), sex workers, and seronegative newborns of HIV-seropositive mothers. Somehow, these groups have characteristics that confer them protection or less susceptibility against HIV-1 suggesting the existence of mechanisms that lead to natural resistance, which are not fully identified until now. Therefore, to study these populations is important to identify which are these mechanisms that determine their immune responses to the virus. Actually, more than 80% of the infections around the world are transmitted by sexual intercourse. Hence the study of serodiscordant couples, generally monogamous couples where only one partner is infected, is considered essential for understand in depth the characteristics of immune responses that might be associated with protection from HIV (37).

The mechanisms involved in natural resistance or reduced susceptibility to HIV-1 infection are multifactorial and are influenced by distinct variables which have to do with the host but also with the virus. Among others these include: way of exposure, virus' intrinsic characteristics as tropism, viral subtype, viral input, viral fitness and host characteristic as mucosal integrity, genetic background, immune system efficiency and concomitant diseases at the moment of contact with HIV (35)(38). Another variable to take into account may be the degree and continuity of exposure to the virus, which may be essential for this protection to continue to be effective. In particular it has been demonstrated that viral exposure results in the exclusive priming of HIV-specific CD4+ T cell and CTLs (39), which would be long-lasting maintained by the immune system regarding to continuous viral exposure (35).

1.3.1 Immunological factors associated with natural resistance to HIV-1 infection

Different and specific immunological factors have been described in the different HESN cohorts contributing in generating the natural resistance profile or in reducing the susceptibility to HIV-1 infection. Many of these factors have been identified regarding to comparative studies among HESN, healthy controls and HIV-infected individuals. These observations give an insight of inter-specific responses to infectious process in each group.

The resistance or reduced susceptibility to infection mechanisms belong to both natural and adaptive immunity. The frequency of different immune cellular subsets (24), along with production of cytokines leads to a more effective earl response to control of HIV infection and to neutralize of the virus, an event that mainly occurs in the mucosal barriers where the first encounter with HIV takes place (33)(36-38). Cytokines and secreted factors

are able to activate antiviral responses and block the first steps of infection. These components reduce the ability of the HIV to enter the target cells and to induce a defense against the virus. The cells of the immune system, principally those with cytotoxic functions recognize more efficiently the HIV-infected cells activating their cytotoxic mechanisms and potentiating the capacity to eliminate them (23-24)(28). Regarding to these mechanisms the infection is blocked and subsequently the spread of the infection to other cells is inhibited.

Besides that, the production of specific humoral response to recognize viral epitopes and the elements to potentiate the rapid neutralization of virions, has been evidenced in several studies in the different cohorts of HESNs (33-35). These antibodies recognize particular cellular proteins implicated in early steps of infection and IgG HIV-1 specifics (39).

All these factors have been discovered throughout the years of study in HESNs, suggesting the prevalence of specific profile of natural resistance to infection that is of great interest to design strategies of prevention, therapeutic implementations or possible vaccines for the population worldwide.

1.3.1.1 Innate Immune Factors

High levels of anti-inflammatory and neutralizing proteins, such as anti-proteases are detected in the genital mucosa of HESN individuals. Lower levels of pro-inflammatory cytokines (**IL-1α**, **IFN-γ** and **TNF-α**), as well as Monokine Induced by IFN-γ (**MIG**) and IFN-γ induced Protein (**IP**)-**10** chemokines have been observed in cervicovaginal lavages (CVLs) of HESNs. IFN-γ induces MIG and IP-10 production, and polymorphisms in the

IRF-1 regulating IFN-γ were associated with protection to HIV. Moreover, HESN individuals presented higher levels of **IFN-α** in their CVLs when compared to those observed in HIV-infected, which could be critical to sustain immune homeostasis, antiviral activity and restriction factors such as **APOBEC3**, in cells at mucosal site that are the portal of entry for HIV. Indeed, type I interferons are crucial in host protection against viruses. During HIV infections type I IFNs promote inflammatory responses, recruit target cells, and also induce a plethora of IFN-stimulated genes (ISGs) (40). HIV ssRNA triggers TLR7 and TLR8 that in turn induce type I IFNs synthesis. High levels of **TLR7** has been reported to be overexpressed in genital epithelial and myeloid cells of some HESN cohorts (41). The immunoregulatory cytokine IL-10 is elevated in the CVLs of HESNs, but lower compared to HIV-infected. In HIV infection the production of IL-10 is often elevated and this excessive may sustain chronic activation and dysregulation associated with HIV disease progression, and may impede on viral eradication. A different scenario characterizes HESNs, in which a modest increase of IL-10 may be beneficial and may promote an immunoregulatory microenvironment preventing HIV attempts to establish infection by reducing availability of activated target cells. Therefore, a delicate balance between IL-10 and IFN-α production and as consequence a peculiar equilibrium between immune activation and immunoregulation characterizes HESN individuals (42).

1.3.1.1.a HIV- Specific Cellular Immunity

As mentioned above, factors secreted by innate immune cells may play a relevant role in the regulation of susceptibility to HIV infection. It's mandatory to relieve the production profile of these factors in different cohorts of HIV-exposed seronegatives to identify common patterns that may

give insights to the phenotype of resistance to HIV-1.

Regarding to many researches carried out in the different HESN cohorts, unique cellular profiles have been identified with distinct cytokines and secreted factors. In addition, it has been possible to identify peculiar cellular responses showing enhanced cytotoxic activity that permit to kill HIV itself or HIV-infected cells as well as characteristic cellular subset distribution.

In previous studies from our group, performed on a HESN cohort, higher levels of Tumour Necrosis Factor-α (**TNF-α**) and **TNF-β** mRNA was observed in cervical sample of the exposed women. These data indicated that HIV-specific T-cell responses in HESN individuals could be dampened by an excess of Regulatory T (Treg) cells. In the same study, significantly higher levels of mRNA for **IL-6**, **IFN-ɣ**, **IL-10**, **IL-12** in peripheral blood mononuclear cells (PBMCs), likewise CCR5 and CXCR4 mRNA in cervical mucosa biopsy specimens were reported. Moreover, HIV-specific IFN-γ–secreting CD8+ T lymphocytes were augmented in cervical vaginal washes. In addition, it was found that CD4+CD25+ and CD8+CD38+ T lymphocytes were significantly augmented in this HESN cohort compared to healthy controls (43). Taken together, these results suggest that a Th1 response characterized by a high production of IFN-ɣ and IL-12 might be protective against HIV-1 infection.

RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) and **MIP-1β** (Macrophage Inflammatory Protein-1 beta) are produced by macrophages, NKs, γδT lymphocytes and CD8+ T lymphocytes (44) and are CCR5 ligands that inhibit infection by blocking interaction of the co-receptors with HIV gp120 and induce co-receptor internalization, blocking HIV-1 infection by R5 viral strains (45). High levels of these chemokines, and significantly higher mRNA levels of MIP-1β, RANTES were observed in

vaginal and endocervical mucosa and in oral mucosa of sexually exposed HESNs (46). This is also supported by a previous *in vitro* study where levels of these chemokines were elevated in cultures of HIV-specific in CD4+ T cells from HESN, compared to those from healthy controls. Interesting, CD4+ T cells of these HESN individuals are less susceptible to infection by R5 tropic HIV virus (47).

In addition, CCL3L1 (C459T SNP in the intron 1), a genetic variant of MIP-1α, showed a higher frequency in HESN compared to long-term non-progressors (**LTNPs**), suggesting that it could increase the efficiency of mRNA processing, thus increasing protein expression (38).

In several studies in HESN cohorts many authors have described HIV-specific CTLs and they postulated that these cells make an important contribution in modulating resistance to HIV infection. The presence of HIV-specific CTLs suggest that HIV has managed to infect the host, but that its further propagation has been contained by immune mechanisms. The presence of these specific cells in HIV-exposed individuals would be the results of an alternative and apparently more efficient processing pathway of HIV antigens within dendritic cells (35). **CD8+ CTLs** of the HESNs recognize epitopes of HIV that are different from those recognized by CTLs of HIV-infected patients (48).

CD8+ lymphocytes are believed to be important in host defense against HIV infection, inhibiting viral replication through cytolytic and non-cytolytic pathways. The cytotoxic activity of these cells have shown strong non cytotoxic anti-HIV responses, mediated by other soluble factors different than β-chemokines (49). This work also reported that CD8+ T cells inhibit HIV replication in acutely infected CD4+ T cells, indicating that antiviral soluble factors with non-cytotoxic activity may be involved in HIV resistance

in exposed uninfected individuals. Specifically, the CD8+ T lymphocyte Antiviral Factor (**CAF**) produced by CTLs is a soluble factor that has a non-cytotoxic anti-HIV activity blocking viral transcription (50). In another study CAF was identified to be the main actor in suppressing HIV- 1 replication (51).

Additionally, higher intracellular levels of **perforin** and **granzyme B** expression in CD8+T cells after stimulation with *gag* p24 antigen were reported in HESN individuals compared to healthy controls (52).

In different HESN cohorts other factors have been described to have particular properties, such as NKs that show a higher production of **IFN-δ** compared to cells of healthy controls. mRNA levels of this cytokine augmented in PBMCs and genital mucosa biopsies of HESNs exposed by sexual intercourses (53). Moreover, in uninfected children born to HIV-1 positive mothers an increased concentration of this same cytokine was found in cells separated by umbilical blood, suggesting its important role in protection from vertical transmission of HIV (54).

Among injection drug users (IDUs), NKs have higher lytic activity compared to healthy controls or seroconverters. In addition, in exposed but uninfected individuals, NKs produce increased levels of **TNF-α** and of **CCL3**, **CCL4**, and **CCL5** (55). An increased proportion of Killer immunoglobulin-like receptor (**KIR**)-3DS1 homozygosis was observed in HESNs, compared to HIV-1 infected individuals (56).

All these evidences were associated to an augment in NKs activation, induction of an effective anti-HIV-1 response, and an impediment of the establishment of infection, thus supporting the anti-HIV-1 activity of these cells could contribute to generate a resistance profile.

In addition to the aforementioned cytokines, there is another one that is also widely related to HIV infection in different studies carried out: Interleukin-21 (**IL-21**). CD4+ T lymphocytes, that are the target cells of HIV, are the main source of IL-21. IL-21 modulates natural and adaptive immune responses. Its effects in limiting early HIV-1 infection in humanized mouse model (57), the negative correlation between IL-21 levels and CD4+ T cell counts in HIV-infected patients, the decreased levels of circulating IL-21 in HIV-infected AIDS patients, and the encouraging therapeutics impact in SIV and HIV infections observed in clinical trials make this cytokine a good candidate involved in resistance to HIV infection.

1.3.1.1.b HIV- Humoral protective factors

HIV-exposed seronegative individuals show a characteristic humoral immune response that could be a powerful protective mechanism against viral infection contributing to HIV-1 resistance. Two distinctive types of HIV-1 related humoral immune responses have been described to date: first, the presence of antibodies that recognize specific cellular proteins implicated in early steps of infection, and second, HIV-1-specific immunoglobulins at mucosal sites (35).

Some studies identified immunoglobulins that recognize epitopes in the envelope glycoprotein **gp120** in serodiscordant couples, which were different from that observed in HIV-positive individuals (58). Similarly, **IgAs** directed to gp41 recognizing different epitopes were detected in different HESN cohorts (sexual exposed and newborns of HIV-1-infected mothers (59). A repeated and long term exposure to HIV would either promote the production of new protective antibodies and/or strengthen the pre-existing natural's antibodies (35).

Another epitope against which antibodies have been identified in

HESNs corresponds to the first extracellular loop (**ECL1**) and second extracellular loop (**ECL2**) of the CCR5 receptor, immunodominants regions involved in chemokine binding and HIV binding. Interestingly, anti-CCR5 IgA and IgG were found in mucosal secretions, such as saliva, breast milk, and cervicovaginal fluids from different HESN cohorts (sexual exposed individuals and newborns) (60). These immunoglobulins compete for chemokine binding, blocking HIV docking and preventing cell fusion and virion entry. It has also been described that these antibodies can induce the internalization of the receptor by clathrin-dependent pathway, or via cholesterol-rich raft domains (60-61).

In a previous study from our group, HIV-specific mucosal IgA antibodies was observed in cervical secretions of HESNs (62). These antibodies have also been observed in other HESN cohorts including Kenyans and Thai commercial sex workers (63-66). HIV-specific **IgAs** were also detected in the seminal fluid of male HESN partners of HIV-infected women (67), indicating the possible protective role of mucosal IgA in HIV-seronegative individuals exposed to this virus independently of their genetic background.

Moreover, higher levels of HIV-1 gp160-specific IgA were found in saliva of HIV-1 negative children, born to HIV-1 positive mothers, who have been breastfed, implying that a specific IgA response is important for inhibiting transmission also in the vertical exposure condition (38)(68).

However, the mechanisms concerned with the production of those antibodies with high specificity and neutralizing capability are not completely clear. It has been hypothesized that during HIV-1 exposure some viral particles are processed and presented to T cells, indirectly enhancing specific humoral responses (38)(69). This humoral immune response is

really dependent on exposure to HIV. Low level of ongoing virus replication (70) or a previous event wherever single round of HIV replication occurred, followed by totally viral clearance may be compatible with the stimulation of HIV-specific antibodies production.

1.3.1.1.c Other Soluble Factors

In addition to cytokines, there are other soluble factors that are able to block viral replication. These molecules and systems intervene at different steps of the infection and replication of HIV. For example, Ribonucleases (**RNases**) have the capacity to inhibit all steps of viral replication by inhibition of protein production, degradation of tRNAs, dsRNA and HIV-1 RNA without degrading ribosomal RNA (71).

Specifically, RNase-1 inhibits the accumulation of early, late and 2-LTR products of reverse transcription. In HESNs, increased levels of **RNase-1** mRNA compared to healthy controls were reported in vaginal mucosa (46).

Another ribonuclease, RNase-2, also known as Eosinophil-Derived Neurotoxin (**EDN**), is an antimicrobial protein with activities of chemotaxis for dendritic cells, and was showed to have some inhibitory capacity against HIV (72). HESN subjects have increased values of RNase-2 in their genital mucosa, suggesting a protective activity during sexual exposure to HIV-1 (46).

Ribonuclease-7 (**RNase-7**) is a potent antimicrobial peptide expressed by intercalated cells in the renal collecting tubules that contribute in maintaining sterility of the urinary tract (73). In a HENS cohort, in endocervical and vaginal mucosa, an increment in these antiviral proteins was observed compared to healthy controls (71).

Specific Elastase Inhibitor (**ELAFIN**) is a serine protease inhibitor that

functions as an anti-inflammatory mediator at mucosal surfaces and also as an antimicrobial molecule against Gram-positive and Gram-negative bacterial and fungal pathogens. During HIV infection, it affects the virus attachment and transcytosis in epithelial cells in a dose-dependent manner (74). An over-expression of this ELAFIN protein was measured in genital samples from female HESN sex workers and compared to healthy non exposed females, with same results in Colombian heterosexual serodiscordant couples HENS cohort (71).

Serine protease inhibitor, group A, member 1 (**SerpinA1)** is another serine protease inhibitor also known as Alpha-1-antitrypsin (AAT), produced mainly by hepatocytes and, to a smaller extent, by mononuclear phagocytes, neutrophils, and airway/intestinal epithelial cells (75). This protein regulates immune responses by controlling inflammation and the cytotoxic response mediated by CD8+ T cells. In genital mucosa of HESNs, women had higher levels of SerpinA1 compared to healthy non-exposed and HIV-infected women (76).

Tripartite motif proteins 5α (**TRIM5α**) is a restriction factor with RING, B-box, and coiled-coil (RBCC) domains that binds to viral capsids mediated by C-terminal domains, degrading them and preventing reverse transcription as it blocks the infection at the post-entry pre-integration phase (77). Different polymorphisms are described capable of modulating the activity of this protein, such as the allele TRIM5α 136Q, specifically associated with higher anti-HIV activity *in vitro*, and granting protective effects: The haplotype TRIM5α- CypA showed *in vitro* protective functions against HIV (78). The SNP rs11601507 has been reported to be negatively correlated with HIV-1 viral loads, because a lower frequency of this allele was found in HIV+ individuals (79). It is necessary to deepen an evaluation of this polymorphism on HESN cohorts to determine if there is effective protection against HIV-1

infection or contribution to resistance HESN profile.

Vitamin D, besides being a steroid prohormone that plays a fundamental role in the regulation of calcium homeostasis, is a pleiotropic and immunomodulatory vitamin that induces the production of microbicide factors against bacterial infections and in the same time has immunosuppressant activity that controls the cellular immune responses (80). Different evidences supporting the importance of vitamin D in control or progression of HIV infection. For example abnormal levels of 1,25 (OH)2D3 (Calcitriol, active vitamin D3) have been described in symptomatic HIV-infected patients, also contributing to bone metabolism dysregulation (81) (82), with lingering controversies. Its effect in viral infection and replications depend on several parameters, like concentration, activation, genetical regulation of the Vitamin D Receptor (**VDR**) and other proteins that intervene in its metabolic pathway.

Sequence variations and polymorphisms in the transcriptional regulator gene Interferon Regulatory Factor 1 (**IRF-1**) in Vitamin D receptor gene was correlated with reduced susceptibility to HIV infection in HESN individuals exposed by injection drugs (83) which is reinforced by the fact that high vitamin D levels in plasma as well as higher mRNA expression of VDR in blood and mucosa from Colombian HESNs compared to healthy controls was observed (84). Here, Calcidiol (25 (OH) D, no active vitamin D) was added to HIV infection *in vitro* assays, and it was found that an increment of different antivirals as well as an increase of mRNA expression of HAVCR2 (Hepatitis A virus cellular receptor 2) stimulate Calcitriol to also increase, allowing a percentage of CD4+ T cells-expressing TIM-3 protein to rise, too.

Similarly, a higher Vitamin D receptor was correlated to higher mRNA expression of **Beta-Defensins** and **IL-10** in Mucosa of Seronegative

Individuals exposed to HIV-1 (85).

Defensins have antiviral effects on enveloped and non-enveloped viruses. Plausible action mechanisms against enveloped viruses are membrane disruption, and the specific binding to some viral proteins or the non-specific lectin-like binding to the envelope glycoproteins of viruses blocking the interaction with cellular receptors or interfering with the cell signaling necessary for replication of the viruses (86).

Beta-Defensins are cationic and Cys-rich small proteins (3-5 kDa), first in line of defense against pathogens at mucosal surfaces and skin, produced predominantly by epithelial cells, and expressed at high levels in several mucosae, including the mouth (where it reaches a very high concentration until 100g/ml) and urogenital tract (87). Their production and secretion is regulated by responses of innate and adaptive immunities, so it is stimulated by microbes and by the release of cytokines such as IFN-*γ*, IL1, IL17A, IL22, and TNF-*α*. Due to its high concentration in the oral mucosa, it is hypothesized that it has a fundamental role in the prevention of oral transmission. The specific effects of these molecules in HIV infection have been observed in a study that used recombinant HBD (Human Beta-Defensins)-2 and HBD-3. Inactivation of HIV-1 was possible by a direct interaction with virions. Specifically, HBD2 blocks HIV-1 replication by preventing an accumulation of reverse transcription products, and modulates both CCR5 and CXCR4 coreceptors without affecting cellular proliferation (87-89).

In the field of vertical transmission, studies have demonstrated a strong association between high HAB (Human Alpha-Defensins) concentrations and depressive risk of partum and postnatal HIV transmission, while others show that lack of HBD expression allows

transmigration of virions within oral mucosa, increasing the risk of mother-to-child HIV-1 transmission (88)(90).

In HESN populations, an increased number of copies of HBD1, HBD2 and HBD3 mRNA in vaginal and endocervical mucosa, and HBD2 and HBD3 in oral mucosa, have been observed comparing them with healthy controls, as well as a more frequent homozygosity for the A692G polymorphism in HBD1. This SNP is located in position -20 of the 5′UTR of this gene, codifying for a nuclear factor NF-κB binding site, allowing the up-regulation of numerous immune genes that potentiate the innate response. All this suggests an increase in HBD expression, and specific polymorphisms reflect a genetic trait correlated with HIV-1 infection resistance (91).

The Alpha-Defensins have been found to be less relevant in the HIV field than Beta-Defensins, but there are some studies about them, as well: HADs are six, HNP1, HNP2, HNP3, HNP4, HD5, and HD6; found in neutrophils, NKs, B cells, γδ T-cells, monocytes/macrophages, and epithelial cells. These molecules display immune-stimulatory activities as chemotactic effect for T lymphocytes, monocytes, immature dendritic cells and the induction of cytokine production (92).

Specific HADs correlated with HIV infection and natural resistance, and research observed that HNP1, HNP2 and HNP3 -produced by CD8 +T cells- had a 10-fold difference in HESN individuals, compared with low-risk controls in blood and cervicovaginal lavage, as well as HAD mRNA was increased on PBMCs and cervical biopsies of HESN versus healthy controls (93).

1.3.1.1.d Immune activation

Immune activation is essential in AIDS progression and pathogenesis, but on the other hand it could contribute to the profile of resistance to HIV-1 infection. Although several other studies suggest that immune quiescence is a trait that could confer resistance to HIV infection (94), the evidence found in our studies, performed since 1992, on a cohort of sero-discordant couples, shows that immune activation has a fundamental role in the profile of resistance to infection for this HIV virus. This activation in the immunological system can be conferred to induce an immune resistance to primary HIV exposure in HESN subjects, contrary, during the course of HIV infection, where it contributed to the viral replication and the consequent progression of the disease (35).

In several evaluations throughout the years, in our laboratory and in others where the HESN cohorts are followed, an augmented levels of memory and activation of T lymphocytes CD4+ and CD8+ , B cells, NKs and monocytes, a superior production of **pro-inflammatory** cytokines (**IL-1β**, **IL-6**, **IL-18** and **TNF-α** among other) and chemokines as **MIP-1α**, **MIP-1β**, **RANTES**, and a diminution of naïve cells percentage, have been observed in the HESN subjects compared to healthy controls (43)(95-96).

The possible interpretation about this contribution in HIV resistance profile is that this potent immune response could be more effective in limiting HIV infection, impeding the successive steps of HIV virus infection as replication and viral spread. All these immune elements promote the production of a more potent adaptive immune response induced by virus exposure (36).

1.3.2 Genetic factors

The host genetic background influences the immune response against different environment conditions, among these, the contact with pathogenic microorganisms and the response generated before them. Specifically, there are host genetic elements that could confer resistance to HIV acquisition at different steps in viral infection inclusive of virions penetration through surface barriers of the body, their attachment to target cells, integration, and viral gene expression (35).

The deletion of CCR5- delta 32 (**CCR5-Δ32**) has been the most investigated mutation correlated to natural resistance to HIV infection, because it efficiently inhibits access of R5 strains which results in the lack of cell-surface expression of the coreceptor for macrophage tropic HIV (35)(38). This deletion is a 32 base-pair deletion from nucleotide 794 to 825 that introduces a premature stop codon encoding a protein severely truncated and renders the CCR5 receptor non-functional, and it is present in Caucasian populations with a frequency around 10%. Homozygous individuals for this mutation are highly resistant to HIV-1 infection. It has been hypothesized that it became favored by natural selection because the mutation could have conferred resistance against the Black Death or other infections like smallpox and hemorrhagic diseases similar to the Ebola-like virus (97).

It was observed that there is an increase in the frequency of specific haplogroups, HHC/HH-G1 and HH-C/HH-F2 genotypes, in IDUs of South East Asia HESN cohort, indicating that these could be associated to HIV resistance (98). In the same way, there are other polymorphisms in the promoter region of gene or specific substitution that have an influence on HIV-1 transmission and disease progression as is the -2459A/G (99).

These evidences have contributed to the therapeutic approach of this

HIV co-receptor, for example using CCR5 gene editing with engineered nucleases or factors as interference RNA that cause a gene disruption or less expressing protein (100) or the using of allogeneic hematopoietic stem cell transplantation with CCR5∆32 donor cells to HIV infecting individual post leukemia treatment (101).

Other important mutation that have been implicated in HIV resistance is a deletion in the 23 amino acid repeat units within the neck region of **DC-SIGN**. The frequency in HESN from Thailand was evaluated, evidencing an augmented presence in these individuals. This receptor plays an important role in enhancing trans-infection of CD4þ T cells from HIV-carrying dendritic cells in the regional lymph nodes (102).

Some studies focus on the gene encoding **CCL3L1**, a natural ligand of CCR5, and this gene has been seen to modify the susceptibility to HIV depending on the copy number of the gene. A lower CCL3L1 copy number is associated with markedly enhanced rates of HIV infection and AIDS progression, whereas a higher copy number is associated with reduced vertical HIV transmission in HIV infected South African pregnant (103-104). Both evidences indicate that variations in the dose of immune response genes can also represent a genetic basis for variable responses to infectious diseases.

Several studies connected the polymorphism inside the Human Leukocyte Antigen (**HLA**) class I and class II loci with HIV acquisition, control of viral replication, and disease progression. HLA class I, principally HLA-A, HLA-B and HLA-C, situated on the short arm of chromosome 6 —the most polymorphic of the entire human genome-, encodes various molecules that have the ability to bind antigenic epitopes normally derived from intracellular pathogens and present them to CD8+ T cells permitting the outset of

cytotoxic T cell response (105).

Specifically, **HLA-B** alleles have been strongly associated with either slow or rapid progression exerting the strongest selection pressure on the virus, and becoming a clinical marker of disease progression. Specific polymorphic variants showed protective effect in HIV infection control, for example HLA-B57 and HLA-B27 have been associated with better control of an infection, lowering viral loads, and restricting dominant Gag-specific responses, which results in a significant reduction of viral replication capacity (106). Other HLA-B polymorphisms, B*35, B*5802, and B*18, have been associated with inefficient viral replication control and rapid disease progression. All these evidence has made HLA-B types the strongest predictor of viral load and CD4 count in HIV infection context. Among other alleles, HLA-C type has been connected, to a lesser extent, with viral control (107).

The HLA class II loci, *HLA-DR, HLA-DQ*, and *HLA-DP*, specify for molecules that bind extracellular peptides and present them to CD4+ T cells, deriving in production of cytokines, and helping the production of antibodies. A study with a Pumwani Sex Worker cohort published that various DQB1 alleles are correlated with resistance to HIV infection (35).

The Endoplasmic Reticulum Aminopeptidase 1 (**ERAP1**) and Endoplasmic Reticulum Aminopeptidase 2 (**ERAP2**) genes are located on chromosome 5q15 in opposite orientations, are multifunctional enzymes and ubiquitously expressed; these are strongly induced after stimulation with type I and type II Interferons (IFNs) and Tumor Necrosis Factor-alpha (TNF-α). Both aminopeptidases have important roles during antigen processing for presentation by MHC I molecules, trimming down the N-terminal residues of proteasome-digested peptides, and they shape the antigenic repertoire

presented to CD8+ T cells modulating global innate immune responses (108).

Genetic variants of ERAP1 and ERAP2 have been associated with cancer and different inflammatory disorders as Chron's disease (109), ankylosing spondylitis (110), psoriasis (111), multiple sclerosis (110), infectious diseases, for example, a correlation with HCV chronic infection in a Chinese Han population (112), and also associated with natural resistance to HIV-1 infection. One study in collaboration with our laboratory, a HapMap (Haplotype Map) showed a real association between SNP genotype of both ERAP1 and ERAP2 with resistance against HIV infection, specifically HapA that encodes canonical ERAP2 full-length protein was found to be significantly overrepresented in HESN individuals (95).

T cell Immunoglobulin and Mucin domain-containing molecule (**TIM**) is a family proteins that include TIM-1, TIM-3, and TIM-4, and are type I cell surface glycoproteins with similar structure as Ig-like, mucin, with transmembrane and cytoplasmic domains, and hold diverse functions in immunity regulation (113). Its variable region domains allow highly specific recognition of phosphatidylserine (PtdSer), exposed on the surface of apoptotic cells. TIM-1 is principally expressed on Th2 cells, originally identified as the receptor for hepatitis A virus (HAV), a powerful costimulatory molecule to activation of T cell and has been correlated with the susceptibility for asthma and allergy. In addition of this, it promotes the entry of many enveloped viruses in host cells (114).

TIM-3 is expressed on DCs, CD8+ and promotes an inhibitory signal deriving in the apoptosis of Th1 cells (115). TIM-4 is only expressed on APCs and is important in immune tolerance.

The gene that codifies for TIM-1 is HAVCR1, mainly in exon 4 which

encodes a portion of the mucin-like domain, highly polymorphic in humans. This region is constantly over selection pressures, so it serves to oversee some viral infection susceptibility and disease progression. The insertion or deletion of 18-base pair to produce a six amino acid insertion/deletion variant (157ins/del MTTTVP), has been correlated with susceptibility of HIV-1 infection and AIDS progression (116). Studies in different HESN cohorts showed that the S allele protects from infection and is associated with a delay in AIDS progression in HIV-1 infected women in Thailand cohort (117).

TIM 1,3 and 4 could be blocking the release of HIV-1 from infected cells, resulting in diminished viral production and replication, possible by the interaction with virion-associated PtdSer (118). Also, expression of TIM-1 produces HIV-1 Gag and mature viral particles to accumulate on the plasma membrane in *in vitro* assays.

In the case of TIM-3, codified by HAVCR2, the polymorphism (rs4704846) frequency is significantly higher in different HESN cohorts compared to HIV-infected individuals. It has been observed that HIV-1 infected subjects more frequently show the G allele than HESN and healthy controls showing moderate frequency (119).

Human Myxovirus resistance (**MX**) 2 is GTPase that is part of the interferon-inducible factors family, with antiviral action of a broad range of RNA and DNA viruses. Studies suggest that **MX2** is an effector of the anti-HIV-1 activity of type-I IFN, blocking infection at a late post-entry step by inhibiting HIV-1 nuclear import, or destabilizing nuclear HIV-1 DNA and suppressing nuclear accumulation and chromosomal integration of nascent viral complementary DNA (120).

In the research performed on HESN cohorts, MX2 was significantly overexpressed in Kenyan sex workers HIV seronegative, compared to high-

risk negative controls. A single nucleotide polymorphism (rs2074560) that induces a possible overexpression of MX2 was related with control to HIV infection and replication in *in vitro* assay (36).

The Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like Editing Complex (**APOBEC**) proteins are very important in the field of antiviral response. Human APOBEC3 includes seven members, A, B, C, D, F, G and H, localized in cluster on chromosome 22. These genes encode cytidine deaminases able to inhibit retroviruses and retrotransposons, capable of reducing viral fitness (36). **APOBEC3G** (A3G) antiviral function is exerted by introducing detrimental levels of cytosine to uracil in the minus-sense single-strand DNA during reverse transcription in proviral genome, thus resulting in hypermutations. This mutated proviral DNA may developed defective or truncated viral polypeptides that could be easily recognized by MHC I to activate HIV-1-specific CTLs. APOBEC3G also prevents proviral integration into the cellular genome through a deamination-independent mechanism and neutralizes the HIV protein Viral Infectivity Factor (Vif) (35). In an Italian HESN group, in monocyte/macrophage-lineage cells and PBMCs, a higher basal and IFN-alpha-induced APOBEC3G mRNA and protein levels were detected (35). In a Colombian HESN cohort, the researchers found higher expression of APOBEC3G mRNA in cervical tissues, compared to heathy controls (71).

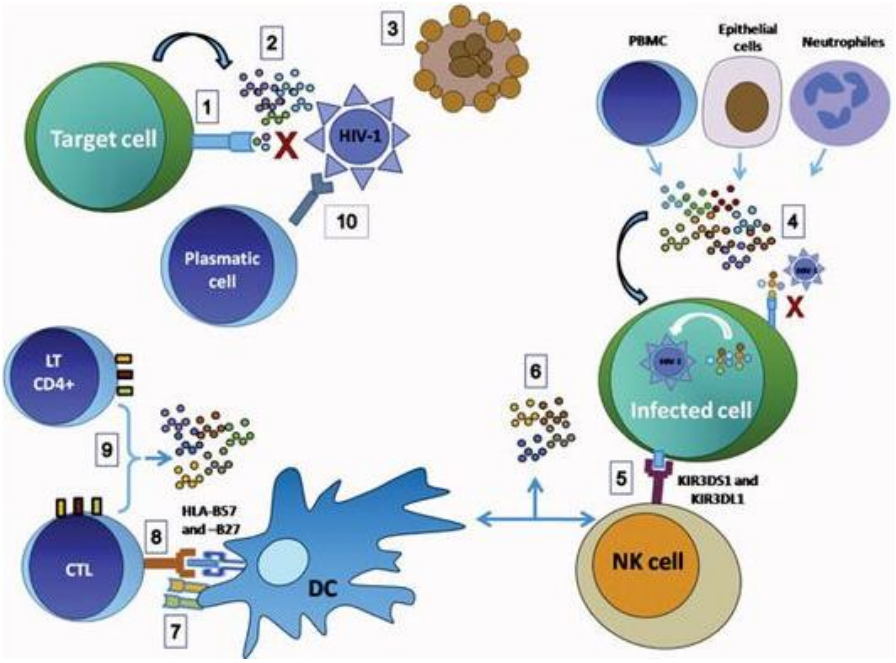

Figure 3. **HESN´s protective factors against HIV-1-infection**. (**1**) Target cells have viral coreceptors as CCR5, low or non-expression of these inhibiting viral entry. Also high coreceptors ligands production, MIP-1α/β or RANTES, can be blocked this step. (**2**) In HESN has been reported high level of monocytes apoptosis and other HIV-1-target. (**3**) Augmented production of soluble antiviral factors block viral entry or replication (RNases, CAF, α- β defensins, APOBEC3G, type I IFN, MIP-1 α/β, RANTES, TRIM5 and Elafin). (**4**) HESN subjects show high activity in NK cells and DCs (**5**) and can delivery IFN-γ (**6**). DCs express the co-stimulatory molecules CD80 and CD86 (**7**) and produce IFN-α. Type I IFN induces HLA expression, block viral replication, apoptosis of infected cells, and production of antiviral molecules. The specific immune response is characterized by the presence of CTLs restricted to HLA-B57 and – B27, (**8**) production of cytokines to increase the immune response and expression of markers associated with T cell activation such as RANTES, MIP-1, IL-6, IFN-γ, TNF-α and IL-2. (**9**) B-cells produce neutralizing IgA antibodies, detectable in serum, mucosa and breast milk (**10**) (38).

1.3.3. MicroRNA (miRNA/miR)

Besides to all factors of innate and adaptive immunity that have been related to the resistance profile to HIV-1 infection, there are other factors identified as possible mechanisms which may contribute to this condition. Among those elements are the microRNA or **miRNA/miR**. MiRNAs are endogenous conserved noncoding RNAs of small size, from 18 to 25 nucleotides, but enough length to be quite specific (121). Whose function is gene expression regulation at post transcriptional level and repress the target messenger through binding to their 3- untranslated region (UTR), producing gene silencing mainly through degradation of target mRNA or inhibition of protein translation (122). In this manner, miRNAs exercise a potent control on biological processes to provide homeostasis in transcript copy number and post transcriptional control, contributing to the normal development, cell differentiation, apoptosis, innate immunity and molecular metabolism, also intervene in deregulatory circumstances, viral infections, cancer and others abnormal conditions. In the human genome hundreds of miRNAs have been described with the ability to regulate thousands of mRNAs. Their expression levels are specific according to kind of tissue and organs, depending on where they exercise their regulatory effect (122). MiRNAs are originated from long primary transcripts precursors called pre-miRNA, approximately of 75 nucleotides, and produced in the nucleus by Drosha, microprocessor complex that contains an enzyme RNase-III-type, from endogenous transcript of protein-coding or non-coding transcription units in intronic or exonic regions (123). Whereupon pre-miRNA is being exported to the cytoplasm by exportin-5 and further processed by a cytoplasmic RNase-III-type protein known as Dicer, generating the final product approximately of ~22-nucleotide. The mature miRNA along with other proteins, as well as Argonaute (Ago) 1 and 2, forms RNA-Induced Silencing Complex (**RISC**),

which recognized the target sequence and is responsible for the degradation of mRNA or retarding the synthesis of the respective protein (124).

In the field of viral infections, miRNAs assume crucial part in complex regulatory network of virus-host interaction, not only of the miRNAs from the host, but also from virus. Have been reported mRNAs from DNA and RNA viruses, which generate them to interact with those of the host to favor the infectious process, auto-regulating viral mRNAs transcripts and down-regulating host mRNAs transcripts to facilitate the evasion of viruses, preventing host cell apoptosis or modifying host cell cycle. Viral miRNAs are closely homologies and similar capacities compared with cellular miRNAs, which allows them to mimic some of its functions and regulate to the intrinsic mechanisms of infected cells (125).

In the infection process host´s miRNAs profile is modified by the effect of the virus and by the activation of the immune system against to viral attack. Specific miRNAs are produced to support the different functions of the cells and to modulate proteins necessary to immune response, activing o silencing particulars elements that contribute to regulate transcriptional patterns of inflammatory network. In the same way that miRNAs production patterns have been identified in inflammatory cascades in response to non-specific viral infection, miRNAs profiles have also been recognized that are produced to counteract specific viruses. Additionally, miRNAs keys have also been identified that could be used as related biomarkers in progression and viral pathophysiology (126-128).

MiRNAs´ evaluations on HIV infection demonstrated the presence of cellular miRNAs which modulate directly and indirectly of HIV replication and other vital step to the virus infection, for example, inhibiting Cyclin T1 expression in resting CD4+ T lymphocyte, miR-198, miR-27b, miR-29b, miR-150, and

miR-223 (129) ,or targeting the Tat cofactor Pur-α mRNA in monocytes to diminished the HIV-1 infection susceptibility, miR-15a, miR-15b, miR-16, miR-20a, miR-93, and miR-106b . There are cellular miRNAs to target the HIV 3 LTR region contributing to the latency, miR-28, miR-125b, miR-150, miR-223, and miR-382 (130). And others decreased to HIV-1 infection, miR-92a, miR-133b, miR-138, miR-149, and miR-326 (131).

Profiles of miRNAs have been developed in HIV-1 infected individuals, Elite Controllers (EC) and Viremic Progressors (VP), also demonstrating specific patterns, for example, have been observed up-expressed levels of miR-221, miR-27a, miR-27b and miR-29b in EC compared to VP (132).

In our laboratory we have performed a miRNAs expression screening in our HESN cohort comparing it with HIV-1 infected patients and healthy controls. We have observed differential expressions between groups in some miRNAs, principally, on *in vivo* HIV-1 infection of HESN PBMCs were significantly augmented of miR-29a and miR-223, which probably contributed with resistance phenotype. It was also observed in basal conditions an up-regulation in HESN and HIV-infected individuals of same miRNAs, suggesting that exposure to HIV-1 impairs the miRNA profile independently of the establishment of overt infection (133).

1.4. Interleukin-21

Interleukin-21 (**IL-21**) is a pleiotropic cytokine composed of four α helical bundles and produced primarily by NKs, T Follicular Helper (TFH) cells and T Helper 17 (TH17) cells, with lower levels of production by several other populations of lympho haematopoietic cells, as illustrated in the Figure 4. (134-135). This cytokine has a broad spectrum of action on different cell types, among these, lymphocytes T CD4+ and CD8+, B cells, macrophages/ monocytes, and DCs with autocrine, paracrine and endocrine signaling. In humans, IL-21 is encoded on chromosome 4, specifically on the 4q26-q27 region producing a polypeptide of 131 amino acid residues, similar to IL-15 (135-136).

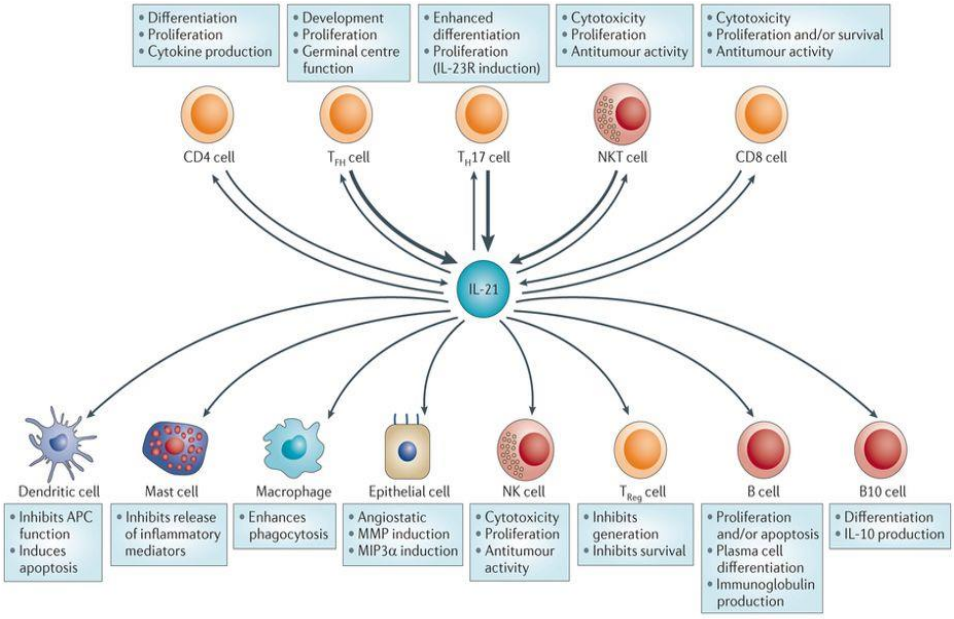

Figure 4. **Interleukin-21 (IL-21) pleotropic functions**. IL-21 is produced by $CD4^+$ T cell, $CD8^+$ T cells, T follicular helper (T_{FH}) cells and T_H17 cells, and in lower levels by natural killer (NK) cells. IL-21 exerts different paracrine and autocrine actions on multiple lymphoid and myeloid populations as well as on epithelial cells. APC, antigen-presenting cell; IL-23R, IL-23 receptor; MIP3α, macrophage inflammatory protein 3α; MMP, matrix metalloproteinase; Treg, regulatory T (137).

IL-21 is part of the one of most important families of cytokines which includes IL-2, IL-4, IL-7, IL-9 and IL-15, colony-stimulating factors and other important molecules such as erythropoietin, growth hormone, and prolactin, all of these appertained to the same cytokine type-I-family (134).

The receptor of IL-21 (**IL-21R**) was discovered in 2000 as an orphan receptor by genomic and cDNA sequencing, located on chromosome 16, contiguous to IL4RA gene and encodes a 538-amino acid protein, similarly to IL-2Rβ (136). IL-21R is heterodimeric, formed by the IL-21R α and γ chains, γc subunit, that which is common with cytokines of the same family. In fact, the functional receptor for IL-21 is IL-21R + γc (134).

This receptor is broadly expressed on lympho haematopoietic populations, in lymphoid tissues like the spleen, thymus and lymph nodes. Production of this receptor has been detected on CD4+ T cells, CD8+ T cells, B cells, NK cells, DC, macrophages after their activation. Also found in non-immune cells, for example on keratinocytes, intestinal epithelial cells, endothelial cells and fibroblasts (134)(136).

The IL-21 target genes are associated with different important cell regulation functions as cell cycle progression, cellular activation and differentiation, trafficking and cell survival and its own regulations of expression. Among these are Granzyme A (Gzma), Granzyme B (Gzmb), gene encoding BLIMP1, Eomesodermin (Eomes), gene encoding Retinoic acid receptor-related Orphan Receptor-γ (RORγ), Interferon-γ (IFN-γ), B cell Lymphoma-3 (BCL-3), Suppressor Of Cytokine Signaling 1 (SOCS1), SOCS3, CXC-Chemokine Receptor-3 (CXCR3), Cyclin A/B/E, CXCR6, Janus Kinase 3 (JAK3), IL-21 and the own receptor IL-2 (136).

Mutations in IL-21 or in its receptor were associated with immunological

disturbs and primary immunodeficiency, including recurrent pulmonary infections, opportunistic pathogens infections and inflammatory disorders, reduction or abnormal functions of various cellular subsets, for example diminution of proliferation and citotoxity in NKs and in CD8+ cells, impaired immunoglobulin class-switching in B cells and less production of cytokines (136)(138-140).

1.4.1 IL-21 Transductional signal pathways

IL-21 transduces molecular signals by the Janus kinase (**JAK**), JAK1 and JAK3, Signal Transducer and Activator of Transcription (**STAT**), predominantly STAT3 and STAT5 but also STAT1, mitogen-activated protein kinase (**MAPK**) and phosphoinositide 3-kinase (**PI3K**) pathways(136) (141).

Intracellular signaling begins with the union of this cytokine to its receptor which activates JAK1 and JAK3. JAKs are cytoplasmic tyrosine kinases whose function is activation of cellular substrates binding by their SRC-homology 2 (SH2) domains. Depending of cellular subset, via this signaling pathway, JAK1 & JAK3 phosphorylate STAT1, STAT3, and STAT5, proteins that then dimerize and acquire high-affinity DNA-binding activity to later translocate to the nucleus. These transcriptional trans-activators initiate the specific gene transcription and effector function in responding cells (142).

Beside the JAK-STAT pathway and similarly to other γ_c-dependent cytokines, IL-21 could transmit the intracellular signal activating the MAPK and PI3K-pathway. These kinases are known to contribute to proliferation signaling (143). In the figure below, the activation pathways of IL-21 and some of its gene effects are observed, as illustrated in the Figure 5.

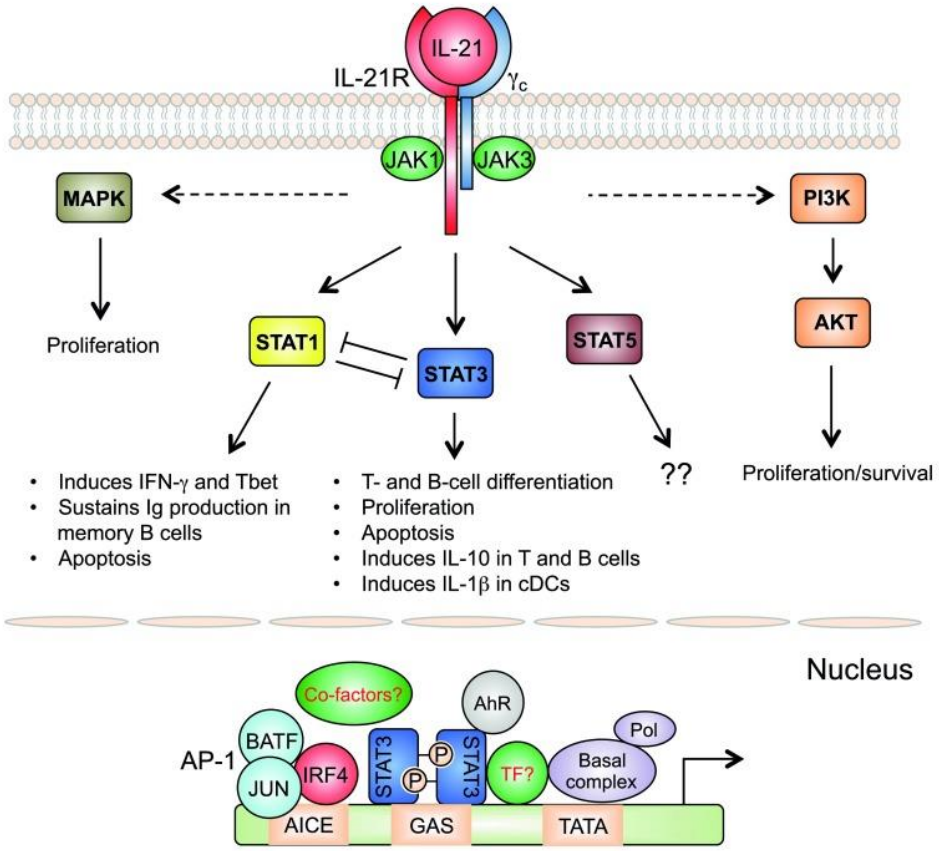

Figure 5. IL-21 Signaling pathways. IL-21 activates JAK-STAT, PI3K, and MAPK pathways. STAT3 plays a major role in the biological actions of IL-21, but STAT1 also contributes to IL-21-regulated gene expression. The importance of IL-21-activated STAT5 is not known. MAPK and PI3K pathways contribute to the proliferative effect of IL-21. In T cells, after IL-21 stimulation, optimal STAT3-mediated gene regulation requires functional cooperation with IRF4, which binds together with AP-1 family proteins (predominately BATF and JUN family proteins), to regulate genes containing AP1-IRF4 composite elements (AICEs). AhR can also cooperate with STAT3 for gene regulation in T cells after IL-21 stimulation. Additional transcription factors (TFs) and co-factors may also be involved (141).

1.4.2 IL-21 and T cells

The CD4+ T and CD8+ T cells represent the majority of T-lymphocytes linage. CD4+ T cells are activated by T-cell receptor (**TCR**) engagement by antigen/ MHC II complex and differentiate in distinct effector subtype: T Helper 1 (TH1), TH2, TH17, T Regulatory CD4+ cells (Tregs) and T Follicular Helper cells (THFs), in response to different stimulus and cytokines. They produce several cytokines that play an important role in immune response activation (144).

CD8+ T cells, often called Cytotoxic T Lymphocytes (**CTLs**), are very important for immune defense against intracellular pathogens and to tumour surveillance. This subset recognize peptides presented by MHC Class I molecules and becomes activated which can trigger cytokines secretion, primarily TNF-α and IFN-γ, and producing and releasing of cytotoxic granules which mainly contain **perfonin** and **granzime** (145).

IL-21 promotes the functional differentiation of several CD4+ T cell subsets. In particular, IL-21 production by T helper 17 cells (**TH17)** cells can further stabilize and enlarge this cellular population (146) through the induction of **RORγt**, a transcription factor that works as a master regulator of the TH17 phenotype and redirection cell differentiation from the TREG cells to the TH17 cell pathway. TH17 subset cells is produced principally in inflamed tissues and has a protective function against infectious diseases and pathogenic inflammatory responses in autoimmune disorders (147).

At the same time TH17 cells were the most noteworthy producers of IL-21 proposing that there may be an enhancement loop in which IL-21 delivered by TH17 cells takes part in upgrading further differentiation of TH17 cells. **STAT3** is an important signaling molecule in TH17 differentiation (148) and as described above IL-21 activates this signaling way, driving the

differentiation of this subset (149).

Some studies demonstrated that this cytokine stimulates the expansion of T cells co-stimulated with anti-CD3 and in combination with Transforming Growth Factor Beta (TGF-β) induced IL-17 production from naive T cells also suppressed Forkhead box P3 (Foxp3) expression (135)(149).

Focusing on IL-21 in autoimmune diseases it may consequently equilibrate the balance between pathogenic TH17 and Foxp3+ TREG cells, which is hypnotized to be defective in these kind of disorders (149).

IL-21 promotes CD8+ T cell proliferation and functional responses. For example, stimulation of CD4+ T cells with IL-27 can induce the IL 21-release, which then leads to autocrine production of Granzyme B (150) and promotes CTL function *in vitro* thought activation of **STAT1** (151).

Furthermore, IL-21 has been correlated in memory CD8+ T cells differentiation after acute viral infection and vaccination (152)(153) and cooperating with IL-10 to induce their memory and promote the maturation of CD8+ T cell in LCMV infection (154).

Relating to HIV infection, it is described that IL-21 is required for the generation and maintenance of functionally competent CD8+ T cells and viral containment. Additionally, IL-21-producing HIV-1-specific CD8+ T cells were a very good indicator of functional of CD4+ T cells (155). In HIV-infected patients, IL-21 could upregulate Perforin production in the absence of cell activation in memory T cells (134).

1.4.3. IL-21 and B cells

B lymphocytes are a population of cells that participate in the adaptive immune system, expressing clonally diverse cell surface immunoglobulin (Ig) receptors that recognize specific antigenic epitopes, secreting pathogen-

specific antibodies and cytokines. Additionally, this subset also presents antigens and are classified as Antigen Presenting Cells (APC) (156) .

The principal IL-21 effects on B cells are proliferation induction of mature B cells in secondary lymphoid organs via CD-40; stimulation of the immunoglobulin responses or/and class switching; increment the number of plasma cells but also potently inducing B cell apoptosis via caspases, a mechanism that could determine the elimination of improperly activated auto-reactive B cells. All of these depend on the surrounding environment costimulatory signals and antigenic stimuli (157-158).

The IL-21 capacity to induce differentiation of B cells into plasma cells is done by encourage expression of B Lymphocyte–Induced Maturation Protein-1 (**Blimp-1**) via STAT3 activation, a transcription factor that operates as a controller to terminal differentiation of these cells and is fundamental in developing and maintaining the germinal center (134). Moreover, IL-21 acts as switch factor to the production of IgG1 and IgG3, and also prompts isotype class switch to IgA in CD4+ -stimulated human naive B cells; and promotes plasma cells homing to mucosal zones collaborating with TGFβ (157-158).

The presence of IL-21 at the moment of B cells receiving a polyclonal nonspecific signal by TLRs, avoids the production of no antigen-specific and auto-reactive immunoglobulins. In opposition to this, when a B cell receives specific T cell and interacts specifically by BCR, IL-21 acts as a positive costimulatory signal (134).

1.4.4 IL-21 and Natural Killer cells (NKs)

Natural Killer cells are lymphocytes with remarked functions related to both innate and adaptive immunity, involved in self-recognition by surveilling self-proteins and stress markers to avoid tumor transformation or stranger cells due to cytotoxic functions. NKs produces pro-inflammatory and immunosuppressive cytokines including Interferon-γ (IFN-γ) in high levels, Tumor Necrosis Factor–α (TNF-α) and Interleukin (IL)–10, and growth factors like Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), Granulocyte Colony-Stimulating Factor (G-CSF), and IL-3. In addition, NK cells secrete large number of chemokines such as CCL2 (MCP-1), CCL3 (Macrophage Inflammatory Protein 1-alpha, MIP1-α), CCL4 (MIP1-β), CCL5 (Regulated on Activation Normal T Expressed and Secreted, RANTES), XCL1 (lymphotactin), and CXCL8 (IL-8) (159-160).

Regarding *in vitro* and *ex vivo* studies it was discovered that IL-21 promotes expansion and differentiation of NK cells from bone marrow (135), an increase lytic action by up-regulating IFN-γ and Granzyme-B expression (161) and an augmented anti-tumor immunity action Perforin dependent (162).

1.4.5 IL-21 and viral infections

All functions mentioned in precedence are characteristics that contribute to the control of acute and chronic infectious diseases. IL-21 has shown the capabilities of favoring innate and adaptive immune responses. In evaluations on Lymphocytic Choriomeningitis Virus (LCMV) infection, where generally there are high viremia and progressive reductions in the functional CD8+ T cells antiviral capacity, the impact of this IL-21 in infection response was found to correlate with augmented production of IFN-γ, TNF-α and IL-2 and activation of anti-viral activity of CD8+ T. IL-21 was fundamental for the

survival of activated CD8+ T cells and long-lived memory cells generation (141).

Mice with a lack of IL-21 showed impaired polyfunctional effector CD8+ T cell responses during the initial phases of LCMV infection, different from functional quality of antiviral CD4+ and CD8+ T cell responses versus +/+ IL-21 mice. It was suggested that IL-21 has a fundamental contribution to ensure the successful control of infection (163). Furthermore, IL-21 has important capacities in inflammation, during the activation and development of non-lymphoid cells, neutrophils, and mononuclear cells, as well as to induce the functional maturation of NKs (164), all characteristics that contribute to the antiviral response.

In observations of chronic infection of HIV, HBV or HCV, IL-21 provides alternative mechanisms that sustain effector activity and help resolve persistent infections, contributing to non- cellular exhaustion or the cooperation between different subsets cells. As an example, IL-21 stimulates the expression of Basic leucine zipper Transcription factor (**BATF**) by CD4+ cells that is required to maintain antiviral function of CD8+ T cells, which contributes to the long-term response against chronical infections (165-166).

In the field of HIV infections, IL-21 has been positively correlated to the control of HIV-1 infection. Not for nothing, CD4+ T cells are the major producers of IL-21 and also the main targets of HIV 1 infection. Studies of different groups of patients with HIV 1 infection have shown that the amount of IL-21 production correlates with viral load in the early stage of infection, but IL-21 plasma levels are considerably diminished when the viral load reaches 20,000 copies per mL, at which point peripheral blood IL-21+ CD4+ T cells are not detectable (167). Conversely, elite controllers of HIV 1 infection, patients who do not progress to AIDS despite the absence of

antiretroviral therapy, can maintain normal levels of IL-21+ CD4+ T cells in opposition with HIV-infected viremic patients or patients with progressive disease where IL-21-producing CD4+ T cells are decreased (141-142)(168). Stimulation of HIV 1 specific CD4+ T cells in the presence of IL-21 leads to enhanced degranulation and cytotoxic effector function (142).

Moreover, the role of IL-21 in controlling the equilibrium of different CD4+ T cell subsets during HIV and SIV infections could be essential in the regulation of immune responses during HIV infection, for example, it stimulates the proliferation of TH17 population, insomuch as TH17 cells are critical for gut mucosal integrity during HIV infection and are associated with reduced microbial translocation, systemic inflammation, and morbidity (141) (169).

1.4.6 IL-21 and autoimmune diseases

IL-21 is a cytokine “double-edged sword” so in the same way that it has the capacity to help in the control of viral infections and to enhance the mechanisms previously mentioned, it has also been related to a pathogenic role in diverse autoimmune diseases. Not only is the aberrant production of this cytokine related to disturbances in the immune response, also its receptor, IL-21R.

The fact that IL-21 participates in the B cells proliferation and differentiation of plasma cells suggested that it could be related with autoimmune regulation and allergies. In this context, some investigations suggest that IL-21 and IL-21R may be associated with increased immunoglobulin, auto-antibody production, and hyperactivity of B-lymphocyte in autoimmune disease (170-171) an overproduction of IL-21 in some chronic inflammatory disorders, as

well as Sjogren's syndrome, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel diseases, type I diabetes and psoriasis .

Some studies in Sjogren's Syndrome (SS) have been correlated with higher levels of IL-21 in subjects affected with this pathology are positively correlated with gamma-globulin levels. Specifically, the increased serum IL-21 levels were connected with augmented levels of IgG1 globulin, supporting the hypothesis that IL-21 may promote autoantibody production by inducing IgG class switch recombination in B cells (172). In addition, lower levels of IL21R could be correlated with SS too (173).

In Systemic Lupus Erythematosus (SLE) there is an elevated IL-21 serum levels that were correlated with the severity of the disease and SLE has been associated with two single nucleotide polymorphisms of the IL-21 gene, rs907715 and rs2221903 (174) Also, levels of BAFF in serum are increased in these patients (175).

In Rheumatoid Arthritis (RA), alterations of the IL-2R structure have been described; and the percentage of IL-21R was observed significantly higher in the blood and synovial fluid. In this pathology, IL-21 probably acts to upregulate the proinflammatory cytokines secretion and T cells activation that may contribute to disease progression (176-177).

Several studies of Genome-Wide Association (GWA) have provided clues about association between IL-21 genes and some chronic inflammatory disorders like inflammatory bowel disease and psoriasis, suggesting the importance of deep analysis in the context of autoimmune disorders and the IL-21 and/or Il-21R balance (178).

1.4.7 IL-21 and clinical uses

The different actions that IL-21 has on different cell types, mainly on CD4+ T cells, NKs and on the cytotoxicity of CD8+ T cells, could have a great impact to consider IL-21 as a probable immunotherapeutic agent against cancer. In fact, several *in vitro* and preclinical and clinical studies supported the antitumor activity of this cytokine. In the same way, the possible uses as adjuvant in viral diseases, in cancer or in vaccines reveal a potential clinical application of IL-21 in novels treatment strategies.

Numerous evidences indicate effects on modulation of immune activation that may elicit effective immune responses, encourages and improves current therapy and enhances the treatment response. It is important to discover all molecular mechanisms and how these affect the control of the tumour cell. For example, in one research IL-21 inhibited FOXP3 in naive CD4+ T cells, whose expression is induced by tumours secreting higher levels of TGFβ1 (179).

The expansion of CD8+ T cells and induction of cytotoxic capacities have important roles against tumours. The capacity of stimulate cluster differentiation on this subset in murine mammary carcinoma (180) and the increment in yield of CD8+ T central memory cells against B16 melanoma (181) demonstrated these direct effects. In combination with anti-CD4+ monoclonal antibodies, recombinant IL-21 in a syngeneic model of disseminated neuroblastoma resulted in tumour eradication and long-lasting immunity due to induction of CD8+ cytotoxic T lymphocyte response (182).

In vitro investigations with multi gene-armed oncolytic adenoviruses -vectors for gene therapy designed to generate specific antitumor immune responses-, applied in concomitance with IL-21 encouraged for oncolytic effects and activation of tumour specific cytotoxicity of CTL,

confirms that the cytolytic activity of IL-21 could contribute to control tumor cells (183). Moreover, the capability of IL-21 to promote a specific CD8+ subset, Memory Stem T CD8+, presents higher efficiency to mediate powerful antitumor responses by expression of T-bet via Jak3 (184).

About NKs, PD-1 and Tim-3 blockade with IL-21 showed that the interleukin enhanced the antitumor effect inducing CXCR3-dependent infiltration of NK cells into tumour sites (185). An *in vitro* study with cetuximab-coated pancreatic tumour cells, which are used as model treatment tests for this kind of cancer, displayed that IL-21 enhances NKs cells activation and effector functions secreting higher levels of IFN-γ and chemokines via ERK and STAT1 pathway activation, resulting in significant inhibition of tumour growth (186).

In hematological malignancies such as multiple myeloma, Hodgkin lymphoma and Burkitt lymphoma, neoplastic B-cells that express IL-21R have been observed, potentially useful in lymphoma therapy with IL-21 for an activation of specific cytolytic activities by NKs cells and T-cells subsets (187).In B cell lymphoma, an *in vitro* study with Mesenchymal Stem cells (MSCs) -specific target tool for cancer treatment- genetically modified to express high levels of IL-21 (IL-21/MSCs), showed delayed tumor incidence as well as improved survival in mice. These effects were associated with high levels of IL-21 delivered to the liver, which prevented the formation of tumour nodules, besides the induction of NKs and effector T cells (188). Another study in same pathology also showed that IL-21 arrests the cell-cycle and actives caspase-dependent apoptosis in these kind of sickened cells (189), all these evidence adding other useful components to consider in new therapeutic strategies.

On an experimental melanoma model, an attenuated *Salmonella*

typhimurium strain VNP20009 was employed as a delivery tool carrying antiangiogenic or proapoptotic genes, demonstrating a useful use together with IL-21 for promotion of better antitumor immune responses (190).

The IL-21 adjuvant properties have been observed in Phase 1 and 2 clinical studies on metastatic renal cell carcinoma in combination with sorafenib, a Vascular Endothelial Growth Factor (VEGFR) tyrosine kinase inhibitor. Patients showed a better disease control compared with monotherapy, increasing the efficacy of therapy and improving patient outcomes an 82% (191). In hematopoietic stem cell transplantation administration of IL-21R, immune reconstitution by triggering the proliferation of bone marrow subsets and recovery of thymocytes and stromal cells and production of regulatory B cells (B10) was improved, as it restored thymic function (192).

Furthermore, IL-21 has a potent adjutant effects in whole-cell vaccines, when combined with other interleukins, the immune response was incremented (193).

In HIV/SIV infections, the frequencies of HIV-1-specific cytokine-producing CD4+ T and CD4+ TEM cells were significantly increased by administrating IL-21 also showing a significant increase of NKs cells (194). The effect of recombinant IL-21-IgFc treatment, given at the time of SIV infection diminished immune activation and preserved effective antiviral responses by CD8+ T cells (195). In SIV-infected rhesus macaques, IL-21 administration in association with ARVs showed improved restoration of intestinal Th17 and Th22 cells and a reduction of immune activation in blood and intestinal mucosa, correlated with minor levels of SIV RNA in plasma (196). The supplementation of ARVs therapy with probiotic and IL-21 were associated with an increase of Th17 expansion and reduction of microbial

translocation markers and dysbiosis compared with infected controls treated only with ARVs (197).

Likewise, the possibility of re-establishing the balance of IL-21 or Il21R present in some autoimmune disorders could be key to restore the normal function and avoid the high-self-reaction of the immune system. In the case of type 1 diabetes models, the combination therapy strategy to suppress the autoimmune response against pancreatic beta cells using anti-IL-21 together with liraglutide, Glucagon-Like Peptide-1 Receptor (GLP-1R) agonist, showed that this treatment was effective in reverse established disease in mouse model compared to either monotherapy, suggesting the importance of in-depth studies about this combinations to improve the therapy replay and be able to get even more efficiencies responses (198).

1.4.8 IL-21 cytokine network

In addition to the effects already mentioned on the different cell types and on the negative or positive regulation that may have in the production of interleukins, there are other cytokines that are directly interconnected with the functions and effects on the immune system that IL-21 has, among these the most important to discard are Interleukin-6 (IL-6), Interleukin-17 (IL-17) and Interleukin-10 (IL-10).

1.4.7.a. Interleukin-6

Interleukin-6 (**IL-6**) is a pleiotropic cytokine involved in numerous physiologic process of the cell: proliferation, differentiation, survival, and apoptosis, responses to viral, bacterial and parasitic infections, tissue injuries and stimulation of acute phase responses in natural progression of the inflammation pathway. It also participates in pathologic processes, in the maintenance of chronic inflammation in various disorders and in autoimmune

diseases including rheumatoid arthritis, plasmacytomas, Castleman's disease, multiple sclerosis, amyloidosis and systemic lupus erythematosus. Generally, this cytokine is considered pro-inflammatory but was recognized as anti-inflammatory in characteristic, depending on the in vivo environmental circumstances (199-201). In humans IL-6 is located on chromosome 7p21 producing a polypeptide of 212 amino acids, including a 28-amino-acid signal peptide (202).

IL-6 is expressed by Antigen Presenting Cells (APCs) such as macrophages, dendritic cells, B cells, T lymphocytes. Also secreted by different non-hematopoietic cells including osteoblasts, smooth muscle cells, keratinocytes, fibroblasts, astrocytes and epithelial cells (199-200).

The IL-6 receptor exists in two forms, one of them is bound to the lipid membrane (**IL-6R**) and activates the IL-6 classic signaling, which is only expressed on hepatocytes and some subpopulations of leukocytes. The other receptor is soluble (**sIL-6**), activates IL-6 trans-signaling. Transduction of classical signal is mediated by the Beta- receptor glycoprotein 130 (gp130). The pro-inflammatory effect of IL-6 have been ascribed to the trans-signaling pathway, whereas anti-inflammatory and regenerative properties are mediated by IL-6 classic signaling (203).

IL-6R expression is on hepatocytes, megakaryocytes and some leukocytes (monocytes, macrophages, B cells) and subtypes of T cells. To its activation, the formation of complex IL-6–IL-6R–gp130 is fundamental for development, hematopoiesis, cell survival and growth. The intracellular pathway involves the Jak family (Jak1, Jak2 and Tyk2) and transcription factors of the STAT family (STAT1, STAT3, STAT5) (199) MAPK and PI3K pathways (203) induce similar ways as the IL-21. These cascades trigger the regulation of various genes, including acute phase proteins and the SOCS1 and SOCS3. In late

steps of these pathways, SOCS1 binds to JAK and SOCS3 binds to gp130 acting as a negative feedback loop to block IL-6 signaling (204).

The characteristic function of IL-6 is the activation of inflammation process. When there is a local reaction to any injury, IL-6 is synthesized starting the first phase of inflammation, then reaches the liver through the bloodstream, it is then recognized by hepatocytes followed by the rapid induction of an extensive range of acute phase proteins such as C-reactive protein, α1-antichymotrypsin, fibrinogen, serum amyloid A and haptoglobin (205).

The principal hematopoietic cellular effects of IL-6 are analogous to IL-21, stimulates proliferation and maturation of B cells into antibody-secreting cells, the survival, maintenance of long-lived plasma cell and immunoglobulin class switching, induces the differentiation of CD8+ T cells into cytotoxic T cells and prompts T-follicular helper-cell differentiation and production of IL-21 (199)(206).In combination, IL-21 and IL-6 cooperate with transforming growth factor-β to regulate T-cell differentiation, having a complementary role in regulating CD4+ T-cell differentiation, principally to TH 17 and TFH (147), (207-208). Moreover, IL-6 is known as a principal inducer of IL-21 in CD4+ T cells (147).

In previous studies conducted in our cohort, we have observed an increase in this cytokine, which indicates that the profile of resistance to infection may be related in some way with IL-6 production increment (209).

1.4.8.b. Interleukin-17

Interleukin 17 (IL-17), also nominated **IL-17A**, belongs to a family member of six cytokines, IL-17A-F. IL-17A is expressed by lymphoid cells, including TH17, CD8+ T cells (Tc17), and γδ T cells, Group 3 I Innate

lymphoid cells (ILC3), and neutrophils. It is a pro-inflammatory cytokine that plays a crucial role in the defense of the host, inducing the secretion of cytokines and chemokines (TNF, IL-1β, IL-6, Granulocyte Colony-Stimulating Factor -GCSF-, CCL2, CCL7, CCL20, CXCL1 and CXCL2) as well as matrix metalloproteases (MMPs), recruiting neutrophils, activating T cells and B cells, producing anti-microbial peptides to kill pathogens (such as β-defensins and S100A8/A9) and acting on macrophages to promote their recruitment and survival. Further, IL-7A promotes proliferation of conventional T cells and TREG cells and enhances the capacity of human CD4+ T cells to produce IL-2 (210-211).

IL-17 receptors are heteromeric compounds of two subunits IL-17RA and IL-17RC, both are part of SEFIR protein family (Similar Expression to Fibroblast growth factor genes, IL-17 Receptors and Toll–IL-1R), so it contains a cytoplasmic SEFIR domain. This domain recruits the Act 1 protein (transcription factor NF-κB activator 1), resulting in the ulterior recruitment of others proteins (TAK1 and TRAF6), which intervenes in the activation of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) transcription factor (210)(212).

IL-17 participates in immune responses against infections of extracellular bacteria, intracellular bacteria, fungi, parasites and viral particles. In these last ones, this interleukin plays a dual role: protective and detrimental. It helps to control the infection but contemporaneously could enhance tissue destruction by inflammation, depending on optimal amounts and regulation of these events Moreover, IL -17 is important for the maintenance of oral mucosa and intestinal barrier integrity whose deficiency may result in the appearance of inflammatory bowel diseases. Besides this, IL-17 is also implicated in various inflammatory autoimmune disorders such as

rheumatoid arthritis, inflammatory bowel disease and experimental autoimmune encephalomyelitis (212).

As mentioned above, one of the cellular subtypes that produces IL-17 is Th17 cells, this subset is characterized by the production of this cytokine. IL-21 promotes the differentiation of Th17 cells and induces IL-17 production. Other determinant factors for the generation of Th17 cells from naïve CD4+ cells T are IL-6 and TGF- β1, but there are other cytokines that can influence the Th17 phenotype such as IL-1β, IL-21, IL-10 and IFN-γ (213).

Different viruses may also prompt IL-17 production, such as rotavirus, Human Leukaemia Virus type 1 (HTLV-1), Respiratory Syncytial Virus (RSV) and Herpes Simplex Virus (HSV). Similarly, in HIV infection, an increase of IL-17 production by peripheral T cells compared to healthy controls was observed (214).

Besides, IL-17 is correlated with cancer (215), Central Nervous System (CNS) diseases (210), atherosclerosis (216) autoimmunity and different autoimmune disorders as psoriasis and multiple sclerosis among other (217-220).

1.4.9 IL-21 and miRNA

As mentioned in previous chapter, microRNAs to play important role in the regulation of different physiological responses by transcriptional or post-transcriptional proteins modulations, among these, the immune response against virus. Up or down regulation of specifics miRNAs suggested the presence of a certain profile depending of conditions of response to different stimulus, in this case HIV-1 infection. This miRNA response stems further replay on specific cellular pathways exercising a coordinated feedback.

One of the most representative miRNAs detected up regulated in studies

carried out in different HIV-important groups, such as Elite Controllers and HESNs are miRNA belonging to the human family 29 microRNAs which has three mature members: miR-29a, miR-29b, and miR-29c. Principally miR-29a and miR-29b as previously specified (132-133), as well as a negative correlations in HIV-1 viremic individuals (224).

This miRNAs family are expressed in human peripheral blood mononuclear cells (230) and have been described that these miRNAs have HIV regulatory roles. MiR-29 family has seed complementarity in the HIV-1 3′-UTR and in encoded protein *Nef* site (226) Some studies relieved that miR-29a and 29b inhibit *Nef* expression preventing viral replications and influencing disease progression (226). Accordingly, reporter assay by transfection of miR-29 as well as ectopically expressed pri-miRNA resulted in a strong reduction of p24 levels, while knockdown of endogenous miR-29a/b by anti-miR transfection led to enhanced HIV-1 infection (225). Another study observed that miR-29a enhances HIV-1 mRNA interactions with epitope tagged Argo 2 of complex RISC to accumulated viral mRNA in P-bodies for translational suppression (227). The Figure 6 below suggests a model on how the miR-29 is able to modulate the cell-virus interaction. Likewise, miR-29b mediates the targeting of Cyclin T1 mRNA to the RISC down regulating this protein in resting $CD4^+$ T cells, therefore affect Tat-mediated transcription (129) Cyclin T1 is one of the three regulatory subunits of p-TEFβ (Positive Transcription Elongation Factor β) binding to Tat protein and leading to an efficient viral transcription (129). All these evidences suggested that miR-29 family might contribute to a successful defense against HIV replication.

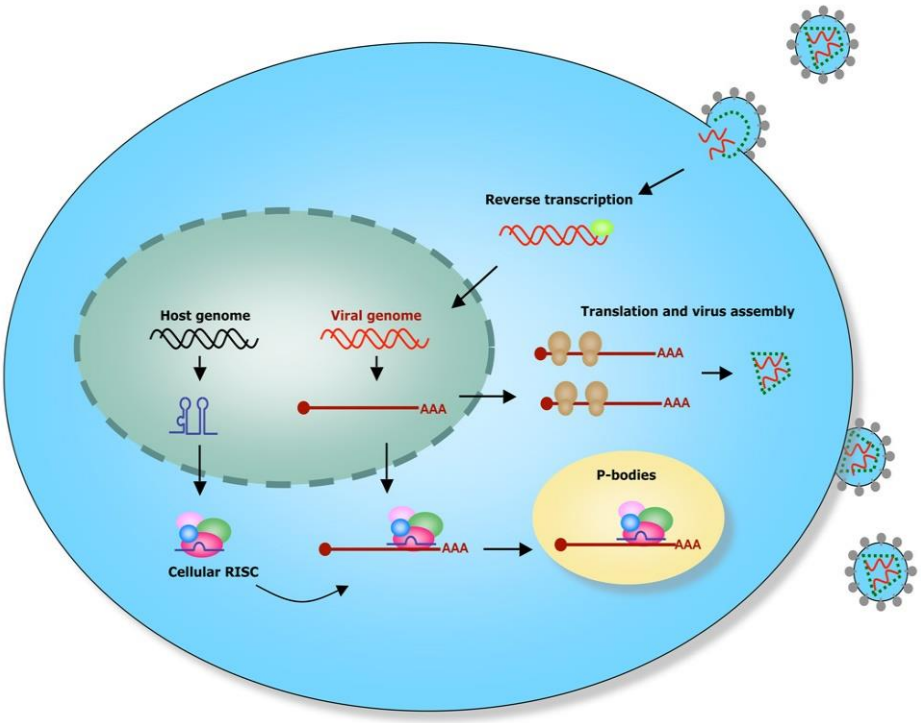

Figure 6. Model of miRNAs-29a modulating host-virus interaction. HIV-1 virus invades to human cells and integrates its DNA into the host genome. This infection promotes expression of specific miRNAs patterns, including miR-29a. HIV-1 mRNA is transcribed, exported from the nucleus, and translated into viral proteins. Cellular RISC containing specific miRNAs, such as miR-29a, targets HIV-1 mRNA and sequesters the ribonucleoprotein complex in P-bodies. Depending upon cellular stimuli or viral pathogenesis cues, HIV-1 mRNA could be stored in P-bodies and released for subsequent translation of viral proteins. Alternatively, viral mRNA could be degraded in P-bodies (232).

It is worth noting that this miRNA family has been correlated with cancer since it has the capacity to downregulate oncogenes and/or upregulate tumor suppressors, promotes apoptosis in cancer cells and blocks proliferation and invasiveness of human cancer cells. Although there are data that also contributes to the metastasis in some cancer of digestive system (228).

In last years, Adoro *et al.* correlated miR-29 with IL-21, where reported

an innovative antiviral activity of IL-21 that is mediated by miR-29 and results in suppressed HIV-1 infection in primary lymphoid CD4+ T cells In particular, the abrogation of IL-21-mediated miR-29 induction on STAT3 blockade, coupled with enriched STAT3 binding to putative regulatory sites within miRNA-29 genes and supports STAT3 as a positive regulator of miRNA-29 expression in CD4+ T cells during HIV-1 infection (57).This study links the antiviral properties of IL-21 with the direct effect of miR-29 against HIV-1 virus, connecting both elements and associating the axis IL-21/miR-29 with the control of HIV-1-infection.

2. AIM

Interleukin 21 (IL-21) is a pleiotropic cytokine produced primarily by Natural Killer T (NKT) cells, T Follicular Helper (TFH) cells and Th17 cells. Functional IL 21R is broadly expressed on lympho-hematopoietic populations. IL-21 exerts its effects on a wide range of cell types. IL-21 promotes the functional differentiation of several CD4+ T cell subsets. In particular, IL-21 production by Th17 cells can further stabilize and enlarge this cellular population through the induction of Retinoic acid receptor-related Orphan Receptor-γt (RORγt), a transcription factor that works as a master regulator of the Th17 phenotype. Finally, IL 21 promotes CD8+ T cell proliferation and functional responses.

An innovative antiviral activity of IL-21 mediated by miR-29 was recently reported. In particular, the abrogation of IL-21-mediated miR-29 induction on STAT3 blockade, coupled with enriched STAT3 binding to putative regulatory sites within miR-29 genes and supports STAT3 as a positive regulator of miR-29 expression in CD4+ T cells during HIV-1 infection. The plausible mechanisms by which the increased expression of this miRNA could exert a protective role in HIV-1 infection are more than a few.

Based on these data the existence of an IL-21/miR-29 axis which in several ways contributes to the control of HIV-1 replication and that could be exploited in the setting up of new therapeutic strategies was proposed.

We recently reported that miR-29 is highly expressed in plasma and PBMCs (Peripheral blood mononuclear cell) from HIV-1 exposed seronegative individuals (HESN), subjects who despite repeated exposure to the virus do not become infected, indicating a key role for this miRNA in controlling the

establishment of overt infection.

Based on these premises this project aims to deepen the role played by the IL-21/ miR-29 axis in the natural resistance to HIV-1 infection in different HESN cohorts. In particular, we aimed:

- To analyze IL-21 and miR-29 level expression in unstimulated conditions and following *in vitro* HIV-1 infection in HESNs,
- To evaluate IL-21 and IL-17 production by CD4+ T cells of HESNs compared to HCs,
- To analyze IL-6 and IL-17A expression levels in unstimulated condition and following *in vitro* HIV-1 infection in HESNs,
- To analyze CTL responses in unstimulated condition and following *in vitro* HIV-1 infection in HESNs,
- To deeply analyze the pathway and target genes directly or indirectly controlled by miR-29 and IL-21 following *in vitro* HIV-1 infection in HESNs.

3. MATERIALS and METHODS

3.1 Sample population

Italian Cohort

Blood samples were collected from 15 HESN and 15 HIV-1 positive individuals, who are part of a serodiscordant cohort of heterosexual couples. Such cohort, recruited at the S. Maria Annunziata Hospital in Florence (Italy), has been followed since 1997 (Mazzoli, 1997). Fourteen Healthy Controls (HC), without known risk factor for HIV infection, were also included in the study. All the subjects enrolled in the study are of Caucasian origin. Inclusion criteria for HESN were a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrolment, with at least 3 episodes of at-risk intercourse within 4 months prior to study entry. The average of reported unprotected sexual contacts is 30 (range, 18 to >100) per year. All individuals (HESN and HIV-1 infected) have been longitudinally followed for >4 years before the study by the Department of Obstetrics and Gynecology of the S. M. Annunziata Hospital, which allowed us to exclude HESN and HIV-1 infected patients with sexually transmitted diseases or other reported pathologies during the time of study.

The HESN, HIV-infected, and HC individuals have similar demographic characteristics as reported in Table 1. HC subjects were matched by sex and age with HESN individuals.

ITALIAN COHORT

HESN (15)	
Female Gender	46.6%
Age (y)	44.5 (33-71)
N° Sexual Intercourses per Month	3.1 (1-4)
Lenght of Relationship (y)	12.73 (3-21)
HIV+ (15)	
Age	43.3 (31-76)
Plasma HIV-RNA (Copies/ml)	Undetectable*
CD4 count (cells/ml)	617.3 (224-1007)
Antiretroviral therapy (mo)	95 (8-156)
Length of HIV infection since diagnosis (y)	10.5 (4-18)

Table 1. Clinical and demographic characteristic HESN and HIV+ individuals of Italian Cohort. (*Only one HIV+ individual has 44 copies/ml).

The presence of any chronic disease or other pathologies at the enrolment was an exclusion criterion.

Colombian Cohort

Blood samples were collected from 7 HESN and 4 HIV-1 positive individuals who are part of a serodiscordant cohort of heterosexual couples recruited from the HIV-1 care program HERES in Santa Marta, Colombia. We also included 7 HCs, without any known risk factor for HIV infection and matched by sex and age with HESN individuals. The subjects enrolled in the study are of mixed origin, matched for demographic characteristics, which are reported in Table 2.

Inclusion criteria for HESN: seronegative at the time of inclusion and after 3 and 6 months of follow-up. History of ongoing unprotected sexual intercourse in the past 3 months, with 5 episodes of unprotected sexual intercourse in the past 3 months or with ≥1 episode in the 4 weeks prior to study entry. The

respective HIV- 1-infected partner with a plasma VL>3,000 copies/ml. HC subjects were matched by sex and age with HESN individuals.

COLOMBIAN COHORT

HESN (7)	
Female Gender	83%
Age (y)	36.6 (18-50)
N° Sexual Intercourses per Month	13.28 (6-24)
Lenght of Relationship (y)	6.9 (2-11.5)
HIV+ (4)	
Female Gender	25%
Age	27.5 (20-40)
Plasma HIV-RNA (Copies/ml)	1200 (U-180790)
CD4 count (cells/ml)	344 (134-804)
Antiretroviral therapy (mo)	80 (12-145)
Length of HIV infection since diagnosis	9.5 (3.6-14.4)

Table 2. Clinical and demographic characteristic HESN and HIV+ individuals of Colombian Cohort. (U= Undetectable).

All the HIV positive individuals were undergoing antiretroviral (ARV) treatment at the time of the study.

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the participating units. All subjects provided written informed consent to participate in this study.

3.2 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were separated by whole blood on lymphocyte separation medium (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA). Briefly, whole blood was centrifuged for 25 minutes at 2300 RPM, without brake, on a Ficoll discontinuous density gradient.

The PBMC layer was carefully removed from the tube and transferred to a new conical tube. The PBMCs were then washed with phosphate buffered saline (PBS) and cell number and vitality were determined.

3.3 Cell count.

The number and viability of the cells were determined by an automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). ADAM-MC automatic cell counter measures total cell numbers and viabilities by cutting edge detection technologies. Two sensitive fluorescence dye staining solutions, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium IodideI/PBS) are used in ADAM instead of tryphan blue staining which can lead to inaccurate data. AccuStain Solution T allows plasma membrane disruption and nucleus staining for measurement of total cell concentration. AccuStain Solution N allows staining of non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

3.4 Isolation of CD4+ T cells

This procedure was performed on PBMCs of the Italian cohort only.

CD4+ T cells were isolated in basal conditions as well as at the end of

the HIV-1 infection *in vitro* assay. To isolate CD4+ T cells CD4 microbeads were used (Miltenyi Biotech®). First, the CD4+ T cells are magnetically labeled with CD4 MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD4+ T cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD4+ T cells. After removing the column from the magnetic field, the magnetically retained CD4+ T cells can be eluted as the positively selected cell fraction, according to the manufacturer's protocol.

3.5 HIV-1 strains

Italian Cohort:

The R5 tropic HIV-1_{BaL} (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program) was used to perform *in vitro* HIV-1 infections. The virus was provided through the EU programm EVA center for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK).

Colombian Cohort:

The X4 tropic HIV-1 p24 virus was obtained from supernatants of the cell line H9-HTLV-IIIB, provided by ATCC ®, code ATCC-CRL-8543.

3.6 PBMC in vitro HIV-1 infection

Italian Cohort:

$4x10^6$ PBMCs isolated from HESNs and HCs were cultured in RPMI 1640

containing 20% FBS, with or without 0.5 ng/1x10^6 cells HIV-1_{Ba-L} virus and incubated for 24 hours at 37°C and 5% CO_2. Cells were then washed and resuspended in medium containing IL-2 (15ng/ml) (R&D systems, Minneapolis, Minnesota, USA) and PHA (1µg/ml) (Sigma–Aldrich, Saint Louis, Missouri, USA). Two days later cells were washed, resuspended in complete medium with IL-2, plated in 24-well tissue culture plates and incubated at 37°C and 5% CO2. One and 7 days post-infection 1x10^6 PBMC were analyzed for gene expression while p24 antigen ELISA was performed on 7-days post infection supernatants.

Colombian Cohort:

1.5x10^6 PBMCs isolated from HESNs and HCs were cultured in RPMI 1640 medium supplemented with 10% FBS. To active and promote cell proliferation these cells were treated with 8 µg/mL phytohemagglutinin (PHA) and 100 IU/mL IL-2 (Sigma-Aldrich) for 72 hours. The viral input used was 50 ng/mL of X4- tropic HIV-1 p24 with 10 mg/mL polybrene (Sigma-Aldrich) for 3 hours, following the spinoculation protocol to improve the efficiency of infection (229).

3.7 p24 measurament

For the Italian cohort, p24 quantification was performed by enzyme-linked immunosorbent assay (ELISA) using HIV-1 p24 Elisa assay kit (XpressBio, Frederick, MD, USA) in supernatants of 7-days HIV-1 infected PBMC, according to the manufacturer's protocol.

Microtitration wells were pre-coated with murine high specificity and affinity anti-HIV-1 P24 capture antibody. Five serial dilution (1:50 dilution) were prepared from a positive control to generate a linear standard curve. Controls and specimens were seeded together with a lyses buffer and incubated at

37°C for 60 minutes. All controls and samples were tested in duplicate.

After washing the plate with a wash buffer (Tris buffered saline containing 0.05% Tween 20) a detector antibody (anti-HIV p24 conjugated to biotin) was added into each well and incubated at 37°C for 60 minutes. Subsequently, Streptavidin HRP conjugate (Streptavidin conjugated to horseradish peroxidase enzyme containing 0.01% Bromonitrodioxane) was added into wells and incubated for 30 minutes at room temperature (18-25°C). Substrate solution (Tetramethlbenzidine) was added into the plate and incubated at dark and room temperature (18-25°C) for 30 minutes. Reaction was stopped by adding a stop solution (1 N H_2SO_4) to all wells. The assay limit of detection was 1.7 pg/mL.

For the Colombian cohort, p24 quantification was performed by ELISA using the "QuickTiter™ Lentivirus-associated p24" ELISA kit (Cell Biolabs, San Diego, CA) in supernatants after 7-days HIV-1 infection, according to the manufacturer's protocol.

Previously, to concentrate and to inactive the virus ViraBind™ Lentivirus Reagent A and ViraBind™ Lentivirus Reagent B were used. Then, 100 µL of the inactivated sample or p24 antigen standard were added to anti-p24 antibody-coated plate and incubated at 4ºC overnight. The wells were washed 3 times with 250 µL 1X Wash Buffer and 100 µL of the diluted FITC-Conjugated Anti-p24 Monoclonal Antibody were added and incubated at room temperature for 1 hour on an orbital shaker. After washing the plates as previously described, 100 µL of the diluted HRP-Conjugated Anti-FITC Monoclonal Antibody were added and the plates were incubated at room temperature for 1 hour on an orbital shaker. Then, the wells were washed following the steps above and 100 µL of Substrate Solution were added and incubated at room temperature on an orbital shaker until the color changed

(from 2-30 minutes). To stop the enzyme reaction 100 µL of Stop Solution was added into each well. The kit has a detection sensitivity limit of 1 ng/mL HIV p24.

The colorimetric reaction was analysed reading absorbance at 450 nm, using the IMark microplate reader equipped with Microplate Manager® 6 software (both from Biorad, Hercules, CA, USA). The absorbance of each microplate well was calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cut-off factor were considered initially reactive and were retested in duplicate to determine whether the reactivity was reproducible.

3.8 Gene expression analysis

RNA was extracted from basal and HIV-1 *in vitro* infected CD4+ T cells using the acid guanidium thiocyanate–phenol–chloroform method. The RNA was dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA). One µg of RNA was reverse transcribed into first-strand cDNA in a 20 µl final volume containing 1 µM random hexanucleotide primers, 1 µM oligo dT and 200 U reverse transcriptase (Promega, Madison, Wisconsin, USA).

In Italian cohort, cDNA quantification was performed by real-time PCR on a real-time on a CFX ConnectTM Real time PCR system (BIO RAD, Hercules, CA, USA) using a SYBR Green PCR mix (BIO RAD).

The expression of IL-21 and IL-17A using specific primers provided by BIORAD ®. To others targets the following primers sequence were used to amplify them:

GAPDH:

Forward: 5'-CGGATTTGGTCGTATTGG-3'

Revers: 5’-GCTTCCCGTTCTCAGCCTTG-3

IL-6:

Forward: 5’-GGTGTTCCCTGCTGCCTTC-3’

Revers: 5’-GCCAGTGCCTCTTGCTGCT-3’

Perforin:

Forward: 5’-TGCCGTAGTTGGAGATAAGCC-3’

Revers: 5’- CCCAGAAGACCCACCAGGAC-3’

Granzyme:

Forward: 5’-GGGTCGGCTCCTGTTCTTTG-3’

Revers: 5’- CGGTGGCTTCCTGATACGAGA-3’

For the Colombian cohort, cDNA expression was performed by real time PCR on a CFX ConnectTM Real time PCR system (BIO RAD, Hercules, CA, USA) using 1X Maxima SYBR green Qpcr master mix kit (Thermo Scientific) with following specific primers:

B-actin:

Forward: 5’-CTTTGCCGATCCGCCGC -3’

Revers: 5’-ATCACGCCCTGGTGCCTGG -3’

IL-21:

Forward: 5’-TATGTGAATGACTTGGTCCCTAG-3’

Revers: 5’-AGGAAAAAGCTGACCACTCACAG-3’

IL-6:

Forward: 5’-ATTCGGTACATCCTCGAC-3’

Revers: 5’-GGGGTGGTTATTGCATC-3’

Perforin:

Forward: 5’- CCGCTTCTACAGTTTCCATGT-3’
Revers: 5’- GTGCCGTAGTTGGAGATAAGC-3’

Granzyme:

Forward: 5’-CACTGTTGGGGAAGCTCCAT-3’
Revers: 5’-TGGGGGATGGGTCTTTTCAC -3’

All reactions of both cohorts were performed according to the following thermal profile: an initial 95°C for 15 minutes (denaturation) followed by 40 cycles of 15 sec at 95°C (denaturation), 1 min at 60°C (annealing) and 20 seconds at 72°C (extension). Melting curve analysis was also performed for amplicon identification. Ct values of 35 or higher were excluded from the analyses.

Results were calculated by 2-ΔΔCt equation as ratios between the target and GAPDH and Beta-actin (B-actin) in Italian cohort and B-actin in Colombian cohort used as housekeeping genes.

3.9 Interleukin-17 protein concentration in supernatants

This analysis was performed on supernantants of the Italian cohort only.

The human IL-17A High Sensitivity ELISA Kit (eBioscience™ San Diego, CA) was used to measure Interleukin 17A in 7-days post HIV-1 infection supernatant, according to manufacturer’s protocol. The limit of detection of the assay was 0.01 pg/mL.

An anti-human IL-17A coating antibody was adsorbed onto microwells. Human IL-17A present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin- conjugated anti-human IL-17A antibody was added and bond to human IL-17A captured by the first antibody.

Following incubation at room temperature (18° to 25°C) over night, unbound biotin- conjugated anti-human IL-17A antibody was removed during a wash step. Streptavidin-HRP was added and bond to the biotin-conjugated anti-human IL-17A antibody. Following 1 hour of incubation on a microplate shaker in the dark at room temperature (18° to 25°C), unbound Streptavidin-HRP was removed during a wash step, and amplification reagent I (Biotinyl Tyramide) was added to the wells.

Following 15 minutes of incubation, unbound amplification reagent I was removed during a wash step and amplification reagent II was added and incubate at room temperature (18° to 25°C) for 30 minutes on a microplate shaker in the dark.

After a wash step, the TMB Substrate Solution was added and the microwell strips were incubated at room temperature (18° to 25°C) for about 10-20 minutes in the dark.

A colored product is formed in proportion to the amount of human IL-17A present in the sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from 7 human IL- 17A standard dilutions and human IL-17A sample concentration was determined.

3.10 Flow cytometry

This analysis was performed on PBMCs of the Italian cohort only.

Flow cytometric analyses were performed on 7 days post HIV-1 *in vitro* infection PBMCs. $0.5x10^6$ PBMCs were stained with anti-human CD4 labeled with PE-Cyanine7 (eBioscience), followed by fixation, permeabilization and incubation with anti-human IL-21 labeled with APC (Biolegend - San Diego, CA) and anti-human IL-17 labeled with FITC (eBioscience). At least 200,000

events were acquired in the gate of CD4+ cells, using a FC500 flow cytometer (Beckman-Coulter CA, USA).

3.11 MicroRNAs Reverse transcription and Real Time PCR array analysis

This analysis was performed for the Italian cohort only.

One microgram of RNA was reverse transcribed into first-strand cDNA in a 20µl final volume at 37°C for 60min using miScript II RT Kit (Qiagen, Venlo, The Netherlands) in accordance with the manufacturer's protocol.

3.12 MicroRNA-29a, b, c reverse transcription and Real Time PCR

This analysis was performed for the Italian cohort only.

One microgram of RNA isolated from HIV-1 *in vitro* infection CD4+ T cells was reverse transcribed into first-strand cDNA in a 20µl final volume at 37°C for 60min using miScript II RT Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Samples were amplified using the miScript SYBR Green PCR Kit with the same running protocol used for array analyses. The primers (Qiagen) were: hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-29c-3p. RNAU6 was used as endogenous control to normalize the relative miRNA expression as previously described by others (132)(234-235). The relative expression levels of miRNAs and target genes were calculated using the comparative ΔΔCt. The fold changes were calculated by the equation 2-ΔΔCt

3.13 Statistical analysis

Data were analyzed using Student's T or ANOVA test by GRAPHPAD PRISM version 7 (Graphpad software, La Jolla, Ca, USA), and *p*-values of 0.05 or less were considered to be significant.

4. RESULTS

Susceptibility to in vitro HIV-1 infection in both HESN cohorts:

We evaluated the susceptibility of PBMCs isolated from both the Italian and Colombian cohorts to R5 tropic HIV-1_{Ba-L} and X4 tropic HIV-1, respectively, by measuring p24 levels in cell culture supernatants seven days after *in vitro* HIV-1 infection. Results confirmed a significant reduced susceptibility to HIV-1_{Ba-L} infection in HESNs compared to HCs from the Italian cohort (mean ± SD of 52682 pg/ml and 92969 pg/ml, $p< 0.05$) (Figure 7). Likewise, HESNs from the Colombian cohort were less susceptible to *in vitro* X4 tropic HIV-1infection compared to HCs (mean ± SD 430502 pg/ml and 267394 pg/ml, $p< 0.05$) (Figure 8).

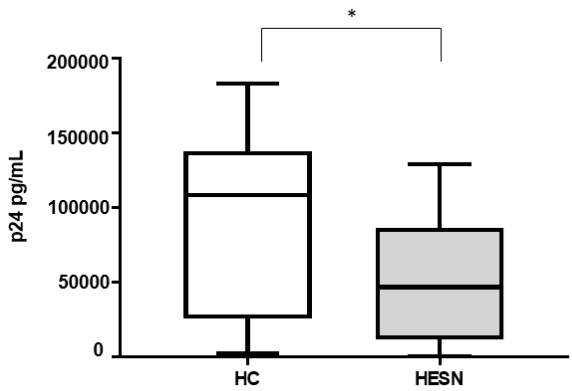

Figure 7. **Italian HESN susceptibility to *in vitro* HIV-1 infection**. Italian HESN PBMCs were significantly less susceptible to *in vitro* R5 tropic HIV-1_{Ba-L} infection compared to HCs (Mean values and S.E. are shown * = $p<0.05$).

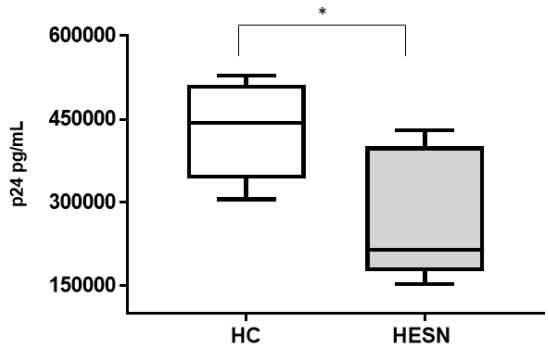

Figure 8. **Colombian HESN susceptibility to *in vitro* HIV-1 infection**. HESN PBMCs were significantly less susceptible to *in vitro* X4 tropic HIV-1 infection compared to HCs (Mean values and S.E. are shown * = p<0.05).

IL-21 expression at baseline and 7 days after in vitro HIV-1-infected CD4+ T cells or PBMCs from HESN cohorts

To verify if the reduced susceptibility to HIV-1 infection observed in HESNs is at least in part dependent on an increased production of the antiviral cytokine IL-21, expression levels of this cytokine were evaluated in CD4+ T cells and PBMCs in the Italian and Colombian cohort respectively, both at baseline and 7 days post *in vitro* HIV-1 infection.

In the Italian cohort at basal level, IL-21 mRNA expression by CD4+ T cells was comparable in HESNs, HCs and HIV-infected patients (Figure 8a). Notably, 7 days post *in vitro* HIV-1 infection, IL-21 expression was not modulated in HC CD4+ T cells but in the HESN cohort it was twice as high than the uninfected condition; these differences were statistically significant (p<0.05) (Figure 8b). This result was confirmed by cytofluorimetric analyses showing an increased, percentage of IL-21 producing CD4+ T cells in HESNs compared with HCs7-days post *in vitro* HIV-1 infection, although these differences approached but did not reach statistical significance (Figure 8c).

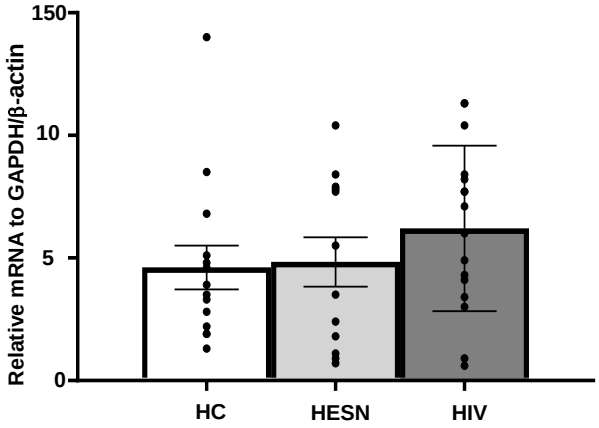

Figure 8a. **IL-21 mRNA expression in CD4+ T cells isolated from the Italian cohort at baseline**. CD4+ T cells from HC, HESN and HIV+ individuals expressed similar levels of IL-21 mRNA in basal condition. (Mean values and S.E are shown).

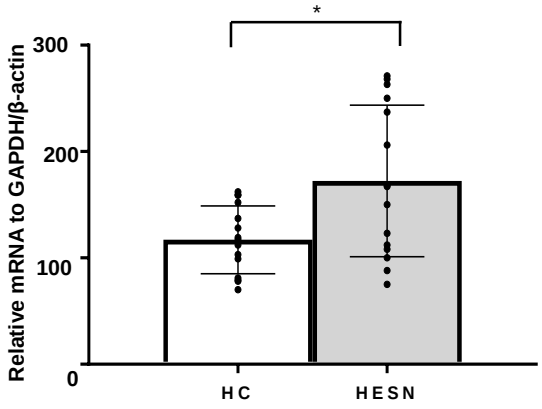

Figure 8b. **IL-21 mRNA expression by CD4+ T cells isolated from the Italian cohort at 7 days post *in vitro* HIV-1 infection.** 7 days post *in vitro* HIV-1 infection CD4+ T cells isolated from Italian HESN individuals expressed significantly higher levels of IL-21 mRNA compared with HCs. (Mean values and S.E. are shown * = $p<0.05$).

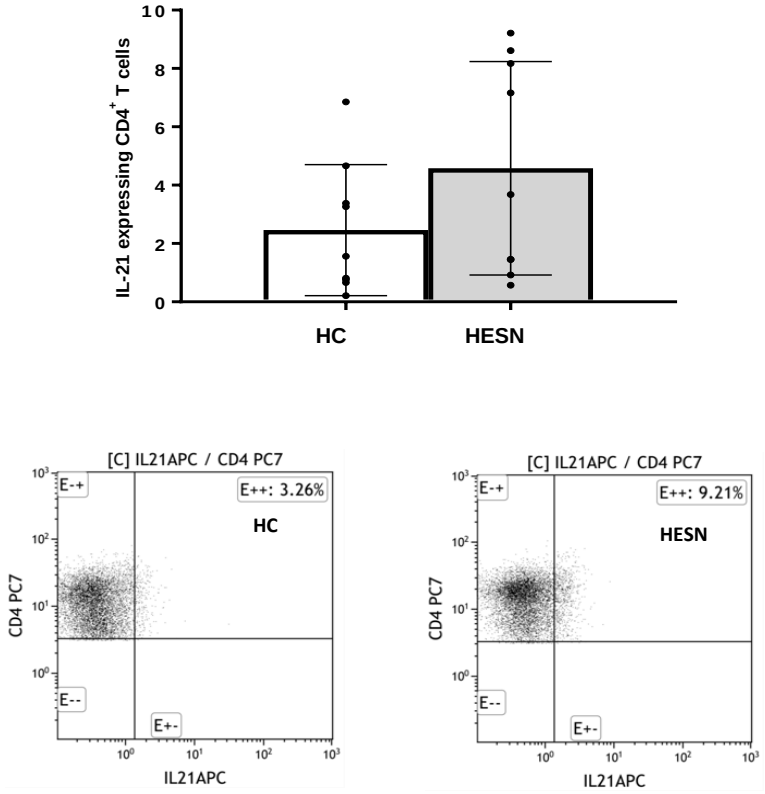

Figure 8c. **IL-21 expressing CD4+T cells in the Italian cohort at baseline.** The percentage of IL-21-expressing CD4+ T cells was increased in HESNs compared to HCs 7-days post *in vitro* HIV-1 infection. (Mean values and S.E. are shown). A representative cytofluorimetric analysis is shown in the lower panel.

In the Colombian cohort at baseline, IL-21 mRNA expression levels were significantly augmented in PBMCs from HESN and HIV+ individuals in comparison with HCs (*p<0.05, ** p<0.005, respectively) (Figure 9a). Seven days post *in vitro* HIV-1 infection, the increased IL-21 mRNA expression in HESN versus HC subjects was maintained (*p<0.05) (Figure 9b).

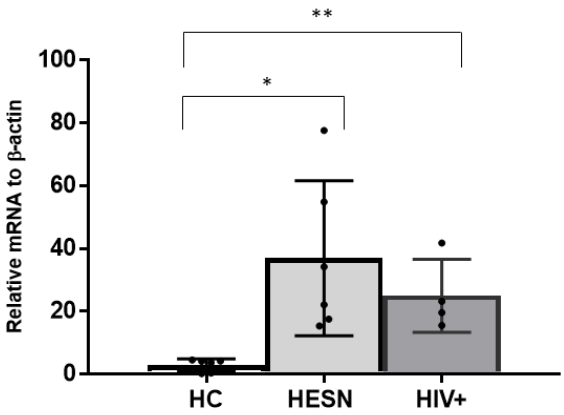

Figure 9a. **IL-21 mRNA expression in PBMCs isolated from the Colombian cohort at baseline**. PBMC IL-21 mRNA expression in basal condition was approximately 12-fold higher in HESNs compared to HCs. (Mean values and S.E are shown. * = p<0.05, ** p<0.005).

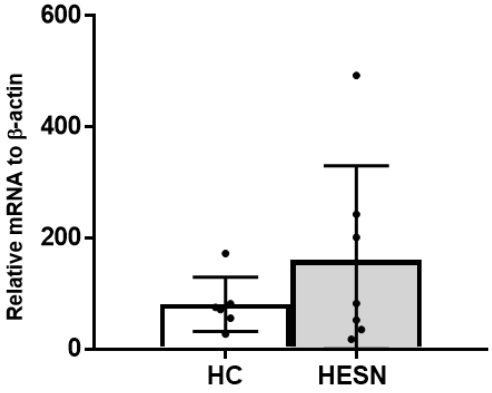

Figure 9b. **IL-21 mRNA expression by PBMCs isolated from the Colombian cohort 7 days post *in vitro* HIV-1 infection.** HESN individuals expressed approximately 2-fold higher levels of IL-21 mRNA at 7 days post *in vitro* HIV-1 infection compared to HCs (Mean values and S.E. are shown * = p<0.05).

IL-6 expression in basal and in vitro HIV-1-infected CD4+ T cells and PBMCs from the HESN cohorts

As the antiviral effects of IL-21 are at least partially mediated by the activation of the transcription factor STAT3 (237), we investigated the expression level of other STAT3-inducing factors. We first took into consideration IL-6, a pro-inflammatory cytokine whose expression level was shown to increase in HESN individuals following Toll-like receptor stimulation (238).

In the Italian cohort at basal level, IL-6 expression was similar in CD4+ T cells of HESN and HIV-1 individuals and was significantly higher compared to HCs (p<0.05 in both comparisons) (Figure 10a). These differences were maintained even after *in vitro* HIV-1 infection as IL-6 expression was significantly higher in HESNs compared to HCs (p<0.05) (Figure 10b).

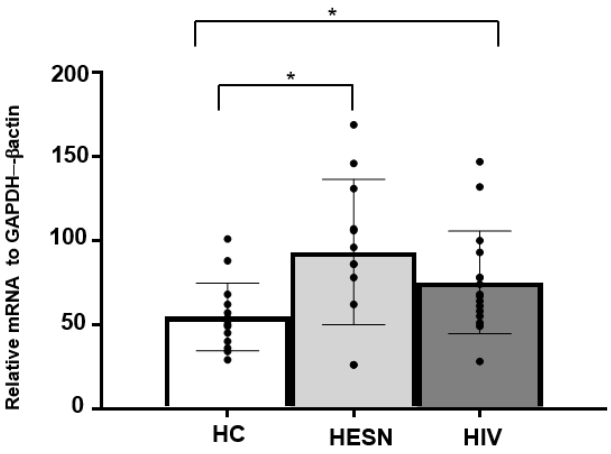

Figure 10a. **IL-6 mRNA expression by basal CD4+ T cells isolated from the Italian cohort.** CD4+ T cells isolated from HESN and HIV+ individuals expressed significantly higher IL-6 mRNA levels compared with HCs at baseline. (Mean values and S.E. are shown * = p<0.05).

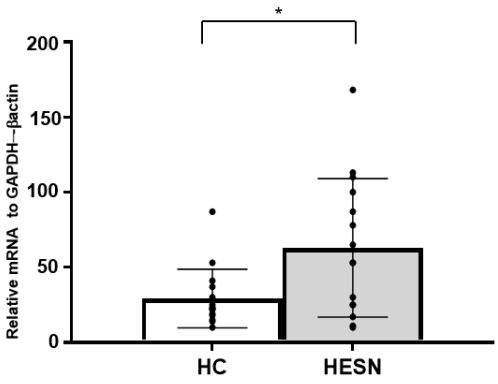

Figure 10b. IL-6 mRNA expression by magnetically isolated CD4+ T cells isolated from the Italian cohort 7 days post *in vitro* HIV-1 infection. CD4+T cells isolated from HESNs expressed significantly higher IL-6 mRNA levels 7 days post *in vitro* HIV-1 infection compared to HC individuals. (Mean values and S.E. are shown; * = p<0.05).

In the Colombian cohort IL-6 expression by PBMCs at baseline was 136-fold augmented in HESN individuals compared to HCs (p<0.005). Likewise, IL-6 mRNA levels were 4-fold higher in PBMCs from HIV-1-infected individuals compared to HCs (p<0.05) (Figure 11a). These differences were maintained even 7-days after *in vitro* HIV-1 infection (p<0.05) (Figure 11b).

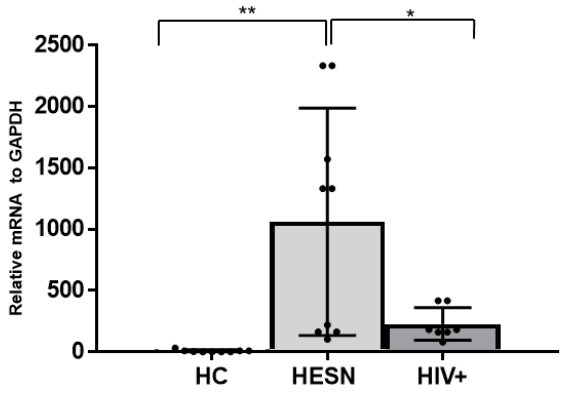

Figure 11a. **IL-6 mRNA expression in PBMCs isolated from the Colombian cohort at baseline.** PBMCs of HESNs expressed significantly higher levels of IL-6 mRNA compared with HC and HIV-1-infected individuals in basal condition. (Mean values and S.E. are shown * = p<0.05, **= p<0.005).

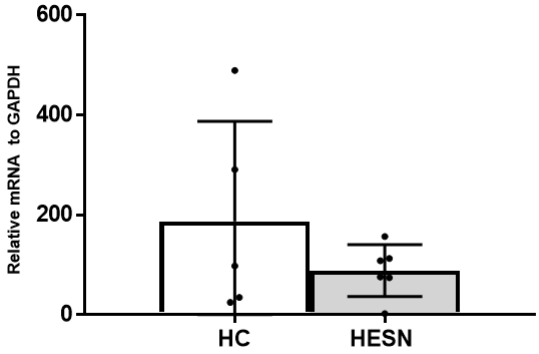

Figure 11b. **IL-6 mRNA expression in the Colombian cohort at 7 days post *in vitro* HIV-1 infection.** No significant differences in IL-6 mRNA expression was observed in PBMCs isolated from HESNs and HCs at 7 days post infection. (Mean values and S.E. are shown).

IL-17 expression in CD4+ T cells from Italian HESNs following in vitro HIV-1 infection

IL-21 production by TH17 cells stabilizes and enlarges this cellular population, we therefore analyzed whether the observed increases in IL-21 production in *in vitro* HIV-1-infected HESN CD4+ T cells could result in an augmented production of other Th17-produced cytokines. Results showed this to be the case as IL-17 mRNA and protein expression were increased in HESN compared to HC from the Italian cohort, although these differences did not reach statistical significance (Figure 12a and b). Notably, even the percentage of IL-17-producing CD4+ T cells was significantly increased in HESNs compared to HCs following HIV-1 infection *in vitro* (HESN vs HC: $p < 0.05$) (Figure 12c).

IL-17 expression and production were not evaluated in the Colombian cohort.

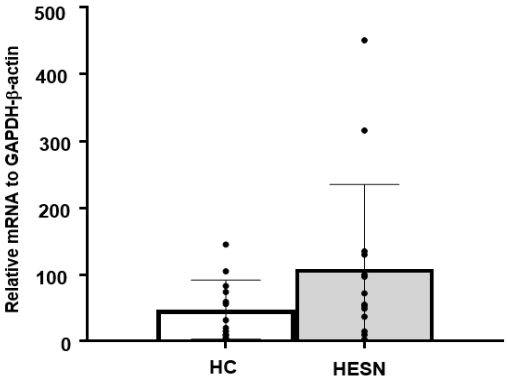

Figure 12a. **IL-17 mRNA expression by magnetically isolated CD4+ T cells isolated from the Italian cohort at baseline.** At baseline IL-17A mRNA expression by CD4+ T cells was higher in HESNs compared to HCs although these differences did not reach statistical significance. (Mean values and S.E. are shown).

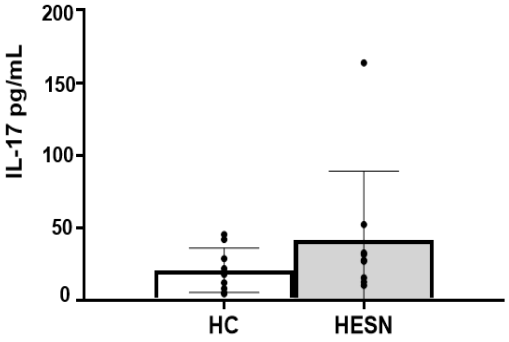

Figure 12b. **IL-17 protein expression detected in supernatant from 7-day post *in vitro* HIV-1 infected PBMCs from the Italian cohort.** IL-17 production was increased in *in vitro* HIV-1 infected CD4+ T cells from HESN compared to HCs although these differences did not reach statistical significance. (Mean values and S.E. are shown).

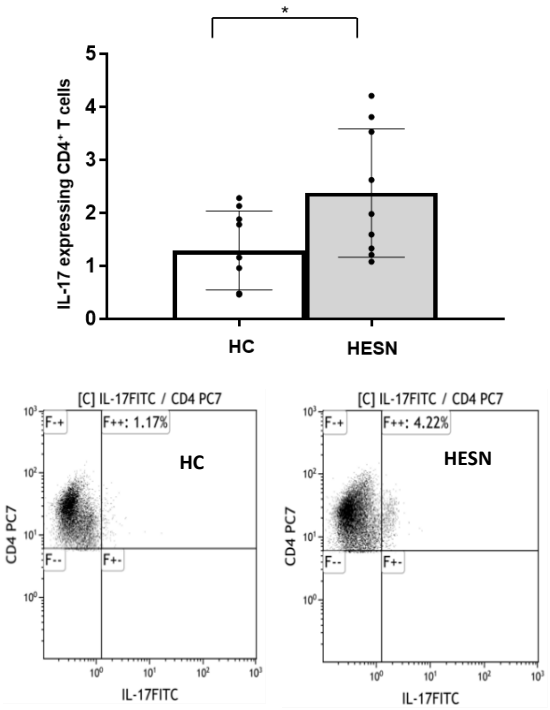

Figure 12c. **Percentage of IL-17 expressing CD4+ T cells in the Italian cohort 7 days post *in vitro* HIV-1 infection.** The percentage of IL-17A expressing CD4+ T cells was significantly higher in HESNs than in HCs at 7 days post *in vitro* HIV-1 infection (Mean values and S.E. are shown, *= $p<0.05$). A representative cytofluorimetric plot is shown in the lower panel.

MiRNA-29 (miR-29) family expression in CD4+ T cells from Italian HESNs following in vitro HIV-1 infection

IL-21 induces the expression of the miR-29 family members to limit HIV-1 replication. We, therefore, verified if the early anti-HIV-1 response promoted by IL-21 in HESNs, is mediated by the up-regulation of the HIV-restricted miR-29 family. Interestingly, 7-days post HIV-1 infection *in vitro*, the expression of all the miR-29 members was significantly increased in CD4+ T cells from HESNs compared to HCs (miR-29a, miR-29b, and miR-29c: $p<0.05$) (Figure 13).

MicroRNA-29 family expression was not evaluated in the Colombian cohort.

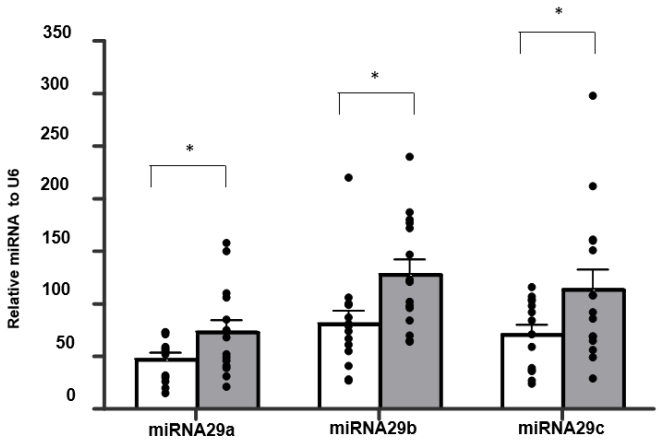

Figure 13. **miR-29 family expression by CD4+ T cells isolated from the Italian cohort 7 days post *in vitro* HIV-1 infection.** CD4+ T cells isolated from Italian HESNs expressed significantly higher levels of miR-29a, b, c 7 days post *in vitro* HIV-1 infection compared to HC individuals. (Mean values and S.E. are shown; * = $p<0.05$)

Perforin and Granzyme mRNA expression by PBMCs at baseline and by in vitro HIV-1 infected PBMCs.

Ex vivo assays suggested, that IL-21 can stimulate perforin and granzyme expression in HIV-1- specific cytotoxic T cells. Therefore, we quantified perforin (Figure 14a and 14b) and granzyme (Figures16a and 16b) expression in both basal condition and 7-days post *in vitro* HIV-1 infection by PBMCs. No differences were observed analyzing PBMCs isolated from the Italian cohort. Conversely, in the Colombian cohort we observed an augmented expression in perforin (Figures 15a and 15b) and granzyme expression (Figures17a and 17b) in both unstimulated and 7-days post *in vitro* HIV-1 infection. Nevertheless, these differences were not statistically significant.

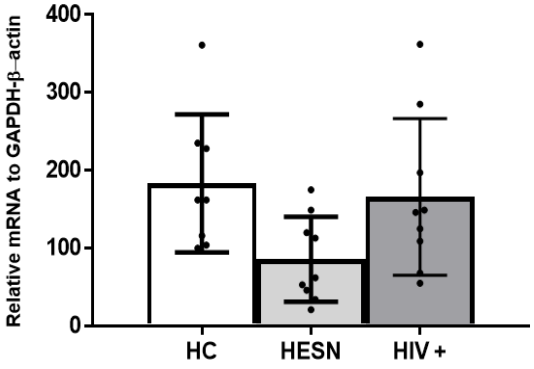

Figure 14a. **Perforin mRNA expression by PBMCs isolated from the Italian cohort at baseline.** No significant differences in perforin mRNA expression was observed in PBMCs isolated from HESNs, HCs and HIV+ in basal condition. (Mean values and S.E. are shown).

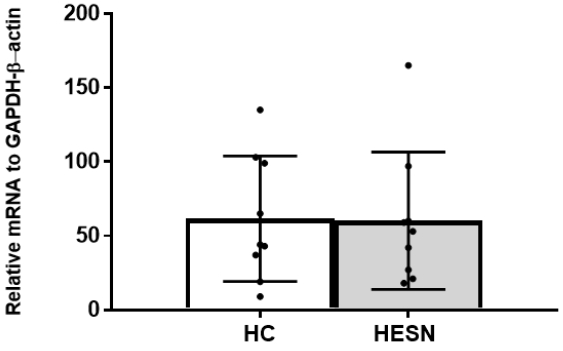

Figure 14b. **Perforin production by *in vitro* HIV-1 infected PBMCs isolated from the Italian cohort 7 days post HIV-1 infection.** No differences in perforin mRNA expression was observed *in vitro* HIV-1 infected PBMCs isolated from HESNs, HCs and HIV+ 7 days post infection *in vitro* (Mean values and S.E. are shown).

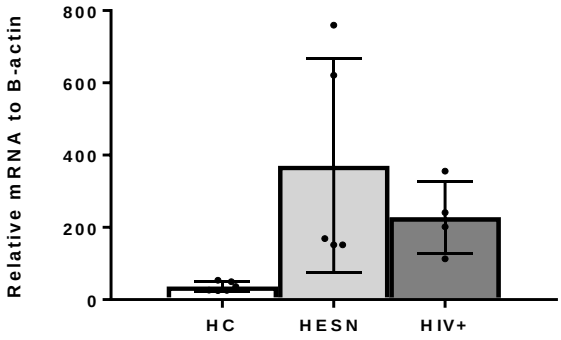

Figure 15a. **Perforin mRNA expression by PBMCs isolated from the Colombian cohort at baseline.** Perforin mRNA expression was higher in PBMCs from HESNs compared to HC and HIV+ individuals at baseline, but these differences did not reach statistically significative level (Mean values and S.E. are shown).

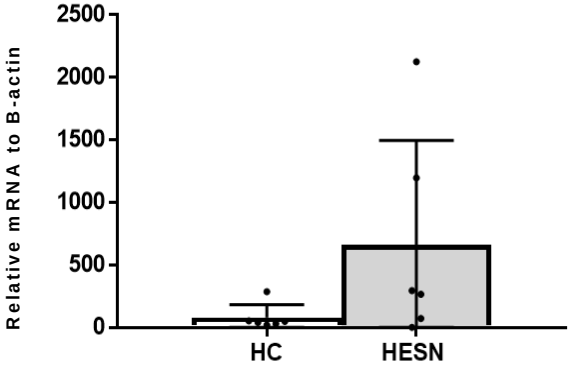

Figure 15b. **Perforin mRNA expression by *in vitro* HIV-1 infected PBMCs isolated from the Colombian cohort 7 days post infection.** Augmented perforin mRNA expression was observed in PBMCs from HESNs compared to HCs at 7 days HIV-1 post *in vitro* infection but these differences did not reach statistical significative level (Mean values and S.E. are shown).

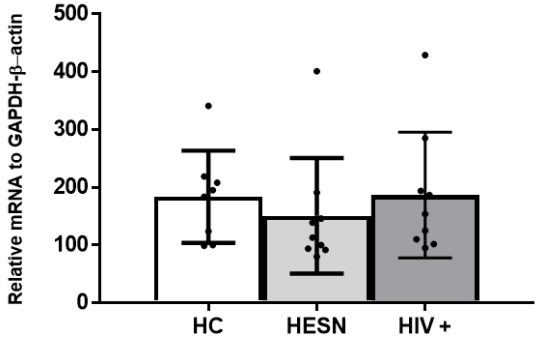

Figure 16a. **Granzyme mRNA expression by PBMCs isolated from the Italian cohort at baseline.** No significant differences in granzyme mRNA expression was observed in PBMCs isolated from HESNs, HCs and HIV+ at basal level. (Mean values and S.E. are shown).

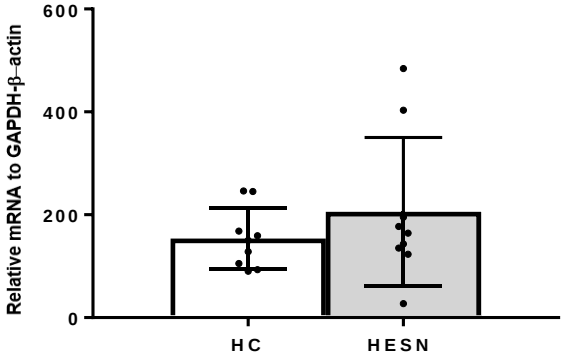

Figure 16b. **Granzyme mRNA expression by *in vitro* HIV-1 infected PBMCs isolated from the Italian cohort 7 days post infection.** No significant differences in granzyme mRNA expression was observed in *in vitro* HIV-1 infected PBMCs isolated from HESNs, HCs and HIV+ 7 days post HIV-1 infection (Mean values and S.E. are shown).

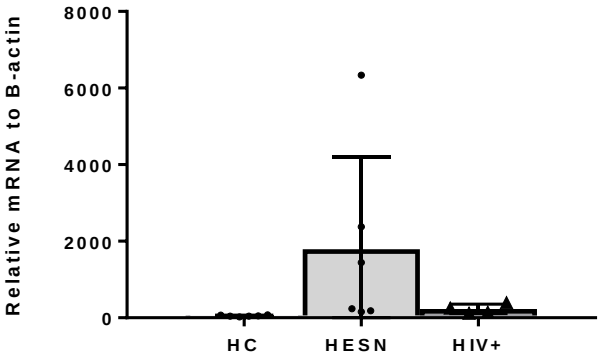

Figure 17a. **Granzyme mRNA expression by PBMCs isolated from the Colombian cohort at baseline.** Granzyme mRNA expression was higher in PBMCs from HESNs compared to HC and HIV+ individuals at baseline, but these differences did not reach significance (Mean values and S.E. are shown).

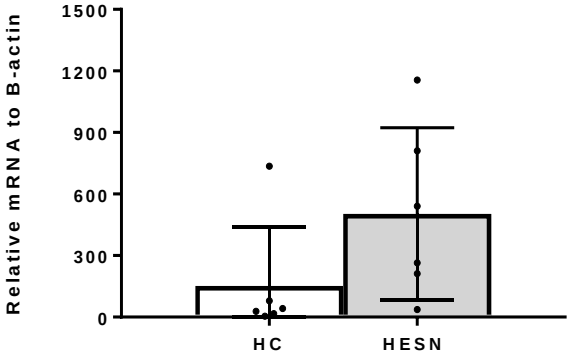

Figure 17b. **Granzyme mRNA expression by *in vitro* HIV-1 infected PBMCs isolated from the Colombian cohort 7 days post infection.** Augmented granzyme mRNA expression was observed in PBMCs from HESNs compared to HCs 7 days post HIV-1 *in vitro* infection but these differences did not reach significance (Mean values and S.E. are shown).

5. DISCUSSION and CONCLUSIONS

The study of natural resistance to HIV-1 infection in HESNs offers an opportunity to identify mechanisms of 'innate' protection that could be exploited in the setting up of preventive or therapeutic strategies. IL-21 is a potent immune modulator functionally linking innate and adaptive immunity. In the field of HIV-1-infection an association between IL-21 and progression of infection in Elite Controllers has already been established (142)(167), and serum IL-21 levels are associated to both progression of HIV-1 infection and the response to antiretroviral therapy (231). Nonetheless, thus far its role in the field of natural resistance to HIV-1 infection has not been investigated. In the attempt to address this issue, we analyzed the expression of this cytokine in two cohorts of well-characterized HESNs both in basal condition and in HIV-1-infected *in vitro* cells.

As a mixed cell culture more accurately reflects what might happen *in vivo*, we performed the HIV-1-infection assays on PBMCs isolated from HESNs and HCs but on Italian cohort cytokine expression was analyzed only on CD4+ T cells isolated from these cultures for two main reasons: they are the main producers of IL-21 as well as the main target of HIV-1. Conversely, in the Colombian cohort these analyses were performed on PBMCs because the number of cells used in the infection assays were insufficient to allow CD4+ T cell isolation.

Results showed that IL-21 expression in unstimulated condition were significantly higher in the Colombian HESN cohort but not in the Italian one. Several possible explanations could account for such discrepancy including

the different type of cell used, PBMCs *vs* CD4+ cells respectively, as well as genetic or environmental variances.

Notwithstanding, IL-21 expression was significantly increased following HIV-1 exposure and was accompanied by a substantial reduced viral replication in both cohorts.

The molecular mechanisms responsible for IL-21 antiviral activity are numerous. First, IL-21 induces the expression of the cytotoxic molecules granzyme B and perforin in CD8+ T cells and NK cells of mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (154)(166)(234).

Second, IL-21 triggers antiviral humoral response by enhancing B cell functions in both chronically SIV-infected rhesus macaques (224) and humans (158, 235-237). Third, IL-21 elicits the STAT3-dependent expression of miR-29 (57), a miRNA family which plays a crucial role in thwarting HIV-1 replication (225) and whose expression is significantly augmented in HESN PBMCs. In our experimental setting, no significant differences were detected in perforin and granzyme expression in any of the analyzed conditions in both the Italian and the Colombian cohort. Conversely, our findings show that the expression of all the miR-29 family members was significantly increased in HIV-1-infected CD4+ T cells of Italian HESNs, thus strengthening the association between IL-21 and miR-29.

Nevertheless, the mechanism responsible for IL-21 up-regulation in HESNs remains elusive and an in-depth analysis of the molecular scheme through which IL-21/miR-29 axis could control susceptibility to HIV-1 infection is mandatory.

As IL-21 serves as an autocrine factor secreted by Th17 cells that promotes and sustains Th17 lineage commitment (239), we verified if IL-21 increase was paired with the release of other cytokines by this cellular subset. Notably,

the percentage of IL-17-expressing CD4+ T cells was significantly increased in the Italian HESN cohort and a similar trend was observed for both IL-17 mRNA and protein expression in supernatant from 7-days post HIV-1 infected PBMCs. Based on the recent acquisition of Th17 role in HIV-1 replication (239-240) Wacleche and colleagues reported an extremely detailed phenotyping of Th17 cells in ART-treated HIV-1-infected patients. In particular, they identified a CCR6 (+) subset lacking CXCR3 and CCR4 that shares Th17 features and is a major source of IL-21 (241). The analyses of this and other Th17 cellular subsets would be essential to gain new insights in the understanding of IL-21 role in controlling susceptibility to HIV-1 infection.

Concerning IL-6 expression, results from both cohorts suggest that IL-6 expression is elevated in HESNs in basal condition even in the absence of an overt HIV-1 infection and this could be a key element in the natural resistance to HIV-1 infection. Nevertheless, after *in vitro* HIV-1 infection there are discrepancies comparing the Italian and Colombian cohorts.

One of the plausible explanation for this discrepancy is that IL-6 analyses were performed on different cellular subsets in the two cohorts of HESNs.

These observations are interesting as IL-6 induces IL-21 production in CD4+ T cells (246-248). It is thus possible to speculate that the high production of IL-21 and, in turn, the augmented expression of miR-29 seen in HESNs, could be ascribable to this cytokine. It is important to underline that, while IL-6 expression was significantly increased in Italian HESN CD4+ T cells both in unstimulated conditions and upon HIV-1 infection, many studies report a reduced expression of this cytokine as well as of IL-17 (243-245) in other HESN cohorts (246-248). Once more, these contrasting results recall the unresolved issue of the role played by immune-activation and immune-

quiescence in the HESN phenomenon.

Actually, the presence of higher levels of pro-inflammatory cytokines, such as IL-6, in HESNs is apparently difficult to justify because different reports suggest that T cell activation facilitates the spreading of HIV-1 infection. Nevertheless, the systemic immune activation that in our hands associates with the HESN phenotype even in basal condition is biologically interesting.

Overall the increased expression of IL-6 and IL-17 described herein support the concept that an inflammatory response is critical to contain exposure to (limited) amounts of HIV-1, hampering viral replication and dissemination, thus lowering the likelihood of infection, as previously reported (248-250). Additionally, an inflammatory profile could boost the activation of a more vigorous adaptive antiviral immune response and might result in a virus exposure-prompted natural immune defensive phenotype against HIV-1. Though this idea is confirmed by several findings this is just one of the theories currently being investigated. A possible way to reconcile these discrepant results is the difference in the rate of HIV-1-exposure in the analyzed cohorts.

Sporadic contacts with the virus, as is the case of our HESN cohorts, would result in an immune activation profile; recurrent contact with HIV-1 would trigger immune quiescence, as observed in the sex workers from the Nairobi cohort (252).

Certainly, this study presents some limitations, such as the reduced number of individuals enrolled in both cohorts, the evaluation of different cytokines in PBMCs instead of CD4+ T cells, the use of viruses with different tropisms and other variables which could be modify the robustness of the data obtained, but even so we have observed many similarities in the results. However, the observation that miR-29 expression is increased in HIV-1-

infected CD4+ T cells confirms previous findings on PBMCs from our HESN cohort and corroborate the possibility that these miRNAs play a role in limiting HIV-1-replication.

Furthermore, the results obtained by Paiardini's group (253) suggest that exogenous IL-21 administration, during early SIV infection, improved the maintenance of Th17 cells and the integrity of the intestinal mucosal barrier, thus reducing the onset of immune dysfunctions and chronic activation. These results support our findings and provide a plausible explanation on how IL-21 could exert a protective effect in the very first phases of HIV-1 exposure.

The similar results obtained in two independent cohorts with different demographic origin and with different levels of exposure make us think that IL-21 expression play a key role in resistance of HIV-1 infection which is at least partially dependent on miR-29 family activation.

Taken together these findings suggest that the antiviral IL-21/miR-29 axis is one of the antiviral host-factors able to modulate susceptibility to HIV-1 infection in HESNs. This being the case, approaches that exogenously increase IL-21 or prompt pre-existing cellular reservoir of IL-21 might be useful in stimulating immune resistance against initial HIV-1 infection.

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Scientific publications

1. Poster PD23

ICAR 2017 9° Italian Conference on AIDS and Antiviral Research, 12-14 June 2017, Siena, Italia

"The interleukin 21 (IL 21)/ microRNA-29 (miR-29) in natural resistance to HIV-1 infection"

Paula Andrea Serna Ortega, Irma Saulle, Vincenzo Mercurio, Elisa Maria Lori, Claudio Fenizia, Daria Trabattoni, Sergio Lo Caputo, Francesca Vichi, Francesco Mazzotta, Mario Clerici, Mara Biasin.

2. Poster TUPEA0155

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"The interleukin 21 (IL 21)/ microRNA-29 (miR-29) in natural resistance to HIV-1 infection"

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"The interleukin 21 (IL 21)/ microRNA-29 (miR-29) axis is associated with natural resistance to HIV-1 infection"

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THE INTERLEUKIN 21 (IL 21)/ microRNA-29 (mir29) AXIS IS ASSOCIATED WITH NATURAL RESISTANCE TO HIV-1 INFECTION

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Short Title: IL-21/Mir29 axis in HESN

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ABSTRACT

Background: Interleukin-21 (IL-21) modulates HIV-1 infection through the elicitation of different antiviral mechanisms, including Th17 lineage commitment and induction of microRNA (miR)29, a miRNA endowed with anti-HIV activity. As IL-21 expression is significantly increased in HIV-exposed seronegative individuals (HESN), we investigated its role in the natural control of HIV-1 infection.

Methods: PBMCs isolated from 15 Italian sexually-exposed HESN and 15 HIV-unexposed healthy controls (HC) were in vitro infected with an R5-tropic HIV-1Ba-L strain. Seven days post infection we evaluated: 1) p24 production (ELISA); 2) CD4+ /IL-21+ and CD4+ /IL-17+ T lymphocytes (FACS); 3) IL-17 concentration in supernatants (ELISA); and 4) IL-6, IL-17, IL-21, perforin and granzyme as well as mir29a,b,c expression by CD4+ T lymphocytes (qPCR). The same analyses were performed on the 15 HIV+ partners of HESNs.

Results: At baseline IL-6 expression alone was increased in HESN compared to HC. Seven days after in vitro HIV-1 infection, nevertheless, differences emerged. Thus, CD4+ /IL21+ and CD4+ /IL17+ T lymphocytes, as well as IL-21 and IL-17 expression and production were significantly augmented in HESN compared to HC. Interestingly, IL-21 upregulation correlated with a significantly increased expression of miR29 a,b,c and a reduced susceptibility to in vitro HIV-1 infection in HESN alone. No differences were observed in perforin and granzyme expression.

Conclusions: The IL-21–miR-29 axis is upregulated by HIV infection in HESN; this axis could play an important role in the natural resistance to infection seen in HESN. Approaches that exogenously increase IL21 production or prompt pre-existing cellular IL-21 reservoir could confine the magnitude of the initial HIV-1 infection

Introduction

Interleukin 21 (IL-21) is a pleiotropic cytokine composed of four α helical bundles and produced by natural killer T (NKT) cells, CD4+ , T lymphocytes, T follicular helper (TFH) cells and TH17 lymphocytes, with lower levels of production by several other populations of lymphohaematopoietic cells [1,2]. The functional IL-21 receptor (IL-21R) is broadly expressed on lympho-haematopoietic populations [1]. Accordingly, IL-21 exerts its effects on a wide range of cell types. Indeed, in B cells, IL-21 stimulates immunoglobulin responses and increases the number of plasma cells [3], but it can also potently induce B cell apoptosis, a mechanism that could determine the elimination of improperly activated autoreactive B lymphocytes [4]. IL-21 also promotes the functional differentiation of several CD4+ T cell subsets and stimulates CD8+ T cell proliferation and functional responses, including the autocrine production of granzyme B [8]. In particular, IL-21 production by Th17 cells stabilizes and enlarges this cellular population [5] through the induction of retinoic acid receptorrelated orphan receptor-γt (RORγt), a transcription factor that works as a master regulator of Th17 cells [6]. This effect results in the potentiation of IL-17 production, a proinflammatory cytokine which effectively mediates immune response against microorganisms [7]. IL-21 has been convincingly correlated with the control of HIV-1 infection. Thus, analyses performed in different groups of HIV+ patients have shown that, whereas IL-21 production negatively correlates with viral load in the early stages of infection [9], IL-21 plasma levels are considerably diminished when the viral load reaches 20,000 copies per ml, at which point peripheral blood IL-21-secreting CD4+ T cells are not detectable [10]. Conversely, HIV-1 infection elite controllers who do not progress to AIDS despite the absence of antiretroviral therapy, maintain normal levels of IL-21-producing CD4+ T cells [11], [12]. Notably, stimulation of HIV-1 specific CD8+ T cells in the presence of IL-21 leads to enhanced degranulation and cytotoxic effector function [12]. Moreover, CD8+ T cells in HIV-infected patients produce IL-21, and the frequencies of these cells are closely associated with viral control [13,14]. Recently, Adoro et al. reported a novel antiviral activity for IL-21 that is mediated by microRNA (miRNA or miR)-29 and results in

suppressed HIV-1 infection in primary lymphoid CD4+ T cells [15]. MiRNAs are small non-coding RNA sequences (about 22 nucleotides), which negatively regulate gene expression at the post-transcriptional level and repress the target messenger RNA through binding to their 3' untranslated region (UTR) [16]. In particular, by blocking STAT3, the ability of IL21 to induce miR29 is suppressed. It's therefore plausible that STAT3 binds a putative regulatory sites within MIR29 genes, thus acting as a positive regulator of miR-29 expression in CD4+ T cells during HIV-1 infection [15]. The human miRNA-29 family of microRNAs is composed by miR-29a, miR-29b, and miR-29c[17]. MiR-29a binds its seed region to the 3' UTR of HIV-1 mRNA and redirects it to cellular P bodies, resulting in viral mRNA degradation and suppression of translation. MiR-29 also down-regulates HIV Nef transcripts and Nef protein expression, which decreases HIV-1 replication [18]. Moreover, miR-29b inhibits cyclin T1 expression in resting CD4+ T cells, which regulates the positive transcription elongation factor b (p-TEFb), necessary for Tat-dependent transactivation of viral gene expression [19]. Accordingly, reporter assay by transfection of miR-29 as well as ectopically expressed pre-miRNA results in a strong reduction of p24 levels, while knockdown of endogenous miR-29a/b led to enhanced HIV-1 infection [20]. These data suggest that the IL-21/miR-29 axis contributes to the control of HIV-1 replication and could be exploited in the design of new therapeutic strategies. Consistently with this observation, we recently showed that miR-29 is highly expressed in plasma and peripheral blood mononuclear cells (PBMCs) of HIV-1 exposed seronegative individuals (HESN), suggesting a role of this miRNA in the natural resistance to this infection [21]. Based on these premises we performed an in-depth analysis of the possible role played by the IL-21-miR29 axis in the natural resistance to HIV-1 infection observed in HESN.

Materials and methods

Study population: Blood samples were collected from 15 HESN and 15 HIV-1 positive individuals who are part of a serodiscordant cohort of heterosexual couples recruited at the S. Maria Annunziata Hospital in Florence, Italy, that has been followed since 1997 [22]. Fifteen HCs, without known risk factor for HIV infection, were also included in the study. All these individuals are Italian of Caucasian origin. Inclusion criteria for HESN were a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrolment, with at least 3 episodes of at-risk intercourse within 4 months prior to study entry, and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. All individuals (HESN and HIV-1 infected) have been longitudinally followed for >4 years before the study by the Department of Obstetrics and Gynaecology of the S. M. Annunziata Hospital, which allowed us to exclude HESN and HIV-1 infected patients with sexually transmitted diseases or other reported pathologies during the time of study. The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the participating units. All subjects provided written informed consent to participate in this study.

Isolation of PBMCs and cell count: Whole blood was collected by venepuncture in Vacutainer tubes containing EDTA (Ethylene diamine tetra acetic acid) (BD Vacutainer, San Diego, CA). Peripheral Blood Mononuclear Cells (PBMC) were isolated from whole blood by density gradient centrifugation on Ficoll (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and washed twice in phosphate-buffered saline (PBS) (PBI, Milan, Italy). Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Korea). Isolation of CD4+ T cells PBMCs were cultured for two hours in treated plates at 37°C to allow the adhesion of CD4- expressing monocytes. CD4+ T cells were isolated in basal conditions as well as at the end of the in vitro HIV-1 infection assay. CD4+ T cell purity was assessed by flow cytometry and ranged between 92% and 97%.

HIV-1 strain: The R5 tropic HIV-1BaL (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program) was used to perform in vitro HIV-1 infection. The virus was provided through the EU program EVA Centre for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK).
In vitro PBMCs HIV-1 infection: Forty x10^6PBMCs isolated from HESN and HC were cultured in RPMI 1640 containing 20% fetal bovine serum, with or without 0.5ng/1x10^6 cells HIV-1Ba-L virus with a cellular density of 2x10^6 /mL, and incubated for 24 hours at 37°C and 5% CO2. Cells were then washed and resuspended in medium containing IL-2 (15ng/ml) (R&D systems, Minneapolis, Minnesota, USA) and PHA (1µg/ml)(Sigma–Aldrich, Saint Louis, Missouri, USA). Two days later, cells were washed, resuspended in complete medium with IL-2, plated in 6-well tissue culture plates and incubated at 37°C and 5% CO2. One and 7 days post-infection 1x10^6 PBMCs were analyzed for gene expression while ELISA and flow cytometry analyses were performed on 7-days post infection cultures.

p24 ELISA: An HIV-1 p24 Elisa assay kit (XpressBio, Frederick, MD, USA) was used to measure viral p24 in supernatant after 7-days infection, according to the manufacturer's protocol. Plates were read at 450 nm, using the IMark microplate reader equipped with Microplate Manager® 6 software (both from Biorad, Hercules, CA, USA). The absorbance of each microplate well was calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cut-off factor were considered initially reactive and were retested in duplicate to determine whether the reactivity was reproducible. The assay limit of detection was 1.7 pg/mL. Interleukin-17 protein concentration in supernatant The human IL-17A High Sensitivity ELISA Kit (eBioscience™ San Diego, CA) was used to measure Interleukin 17A in 7-days post infection supernatant, according to the manufacturer's protocol. The limit of detection of the assay was 0.01 pg/mL.

Flow cytometry: Flow cytometric analyses were performed on unstimulated cells aas well as on cells 7 days post in vitro HIV-1 infection. 0.5x106 PBMCs were stained with anti-human CD4 labeled with PE-Cyanine7 (eBioscience), followed by fixation, permeabilization and incubation with anti-human IL-21 labeled with APC (Biolegend - San Diego, CA) and anti-human IL-17 labeled with FITC (eBioscience). At least 200,000 events were acquired in the gate of CD4+ cells, using a FC500 flow cytometer (Beckman-Coulter CA, USA).

Gene expression analysis: RNA was extracted from basal and in vitro HIV-1 infected CD4+ T cells and PBMC using the acid guanidium thiocyanate–phenol–chloroform method. RNA was dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega, Madison, Wisconsin, USA). One µg of RNA was reverse transcribed into first-strand cDNA in a 20 µl final volume containing 1 µM random hexanucleotide primers, 1 µM oligo dT and 200 U reverse transcriptase (Promega).
cDNA quantification for IL-6, IL-21 and IL-17, perforin and granzyme was performed by real-time PCRusing a CFX ConnectTM Real time PCR system (BIO RAD, Hercules, CA, USA) and a SYBR Green PCR mix (BIO RAD).
Results were calculated by the 2 -ΔΔCt equation as ratios between the target and GAPDH plus Beta-actin housekeeping genes (n-fold). Reactions were performed according to the following thermal profile: an initial 95°C for 15 minutes (denaturation) followed by 40 cycles of 15 sec at 95°C (denaturation), 1 min at 60°C (annealing) and 20 seconds at 72°C (extension).
Melting curve analysis was also performed for amplicon identification. Ct values of 35 or higher were excluded from the analyses.

MicroRNA29a,b,c reverse transcription and Real Time PCR array analysis: One microgram of RNA was reverse transcribed into first-strand cDNA in a 20µl final volume at 37°C for 60min using miScript II RT Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Samples were amplified using the miScript SYBR Green PCR Kit with the same running protocol used for array analyses.

The primers (Qiagen) were: hsa-miR-29a-3p, hsamiR-29b-3p, hsa-miR-29c-3p. Endogenous controls used to normalize the relative miRNA expression were: small RNAU6 for PBMC, miR-425 and miR-93 for plasma and cell culture medium as used by others [23-25]. Synthetic cel-miRNA-39 was also used as quality control of miRNA extraction for the measurement of extracellular miRNA [24].

Statistical analysis:The relative expression levels of miRNAs and target genes were calculated using the comparative ΔΔCt. The fold changes were calculated by the equation 2-ΔΔCt. Data were analyzed using Student's T or ANOVA test by GRAPHPAD PRISM version 5 (Graphpad software, La Jolla, Ca, USA), and pvalues of 0.05 or less were considered to be significant.

Results

p24 ELISA Assay: We evaluated the susceptibility of PBMCs obtained from 15 HESN and from 15 HC to HIV-1Ba-L infection by measuring P24 levels in supernatants seven days after in vitro HIV-1 infection. The results confirmed a reduced susceptibility to HIV-1Ba-L infection in HESN compared to HC (mean±SD of 52682 pg/ml and 92969 pg/ml, $p< 0.05$), (Figure 1).

Interleukin-21 expression in basal and HIV-infected CD4+ T cells from HESN

To verify if the reduced susceptibility to HIV-1 infection observed in HESN is associated with an increased production of the antiviral cytokine IL-21, the expression levels of this cytokine were evaluated in CD4+ T cells, both in basal condition and 7 days post in vitro HIV-1 infection. In basal condition, IL-21 mRNA expression levels were comparable in HESN, HC and HIV+ (Figure 2A). Notably, 7 days post in vitro HIV-1 infection IL-21 expression was not modulated in HC CD4+ T cells but in the HESN cohort it was twice as high than the uninfected conditions; these differences were statistically significant ($p< 0.05$ in both comparisons) (Figure 2B). Similarly, 7-days post infection the percentage of IL-21 producing CD4+ T cells was higher in HESN in comparison with HC, although these differences approached but did not reach statistical significance (Figure 2C).

Interleukin-6 expression in basal and HIV-infected CD4 + T cells from HESN

The antiviral effects of IL-21 are at least partially mediated by the activation of the transcription factor STAT3 [12], we thus investigated the expression level of other STAT3-inducing factors. We first took into consideration IL-6, a pro-inflammatory cytokine whose expression level was shown to increase in HESN individuals following Toll-like receptor stimulation [18]. In basal condition, IL-6 expression was similar in CD4+ T cells of HESN and HIV-1 individuals and was significantly higher compared to HC ($p< 0.05$ in both comparisons) (Figure 3A). These results suggest that IL-6 expression is elevated in HESN even in the absence of an overt infection and could be a key element in the natural resistance to HIV-1. In support of this hypothesis these differences were maintained even after in vitro HIV-1 infection as IL-6 expression was significantly higher in HESN compared to HC ($p< 0.05$) (Figure 3 B).

IL-17 expression in CD4+ T cells from HESN following in vitro HIV-1 infection

IL-21 production by TH17 cells stabilizes and enlarges this cellular population, we therefore next analyzed whether the observed increases in IL-21 production in HIV-1-infected HESN CD4+ T cells could result in an augmented secretion of other Th17-produced cytokines. Results showed this to be the case as IL-17 mRNA and protein expression were increased in HESN compared to the values seen in HC (Figure 4 A and B). Notably, even IL-17-producing CD4+ T cells were significantly increased in HESN compared to HC following in vitro HIV-1 infection (HESN vs HC: $p< 0.04$).

MiRNA-29 (miR-29) family expression in CD4+ T cells from HESN following in vitro HIV-1 infection

IL-21 induces the expression of the miR-29 family members to limit HIV-1 replication. We, therefore, verified if the early anti-HIV-1 response promoted by IL-21 in HESN, is mediated by the up-regulation of the HIV-restricted miR-29 family. Interestingly, 7-days post in vitro HIV-1 infection, the expression of all the miR-29 members was significantly increased in CD4+ T cells from HESN compared to HC (miR29a, miR29b, and miR29c: $p< 0.05$ 8 (Figure 5).

Perforin and Granzyme expression in HIV-1 infected PBMCs

Ex vivo assays suggested that IL-21 can stimulate perforin and granzyme expression in HIV-specific cytotoxic T cells. Therefore, we quantified perforin and granzyme expression in both basal conditions and 7-days post in vitro HIV-1 infection. However, no differences were observed either in unstimulated (Supplementary figure 1A) or in HIV-infected PBMC (Supplementary figure 1B).

Discussion

The study of natural resistance to HIV infection in HESN offers an opportunity to identify mechanisms of 'innate' protection that could be exploited in the setting up of preventive or therapeutic strategies. IL-21 is a potent immune modulator functionally linking innate and adaptive immunity. In the field of HIV-infection an association between IL-21 and progression of infection in elite controllers has already been established [12,26], and serum IL-21 levels are associated to both progression of HIV infection and the response to antiretroviral therapy [14]. Nonetheless, thus far its role in the field of natural resistance to HIV-1 infection has not been investigated. In the attempt to address this issue we analyzed the expression of this cytokine in a cohort of well-characterized HESN both in basal condition and in in vitro HIV-infected cells. As a mixed cell culture more accurately reflects what might happen in vivo, we performed the HIV infection assays on PBMC isolated from HESN and HC but cytokine expression was analyzed only on CD4+ T cells isolated from these cultures for two main reasons: they are the main producers of IL-21 as well as the main target of HIV-1. Results showed that IL-21 expression in HESN is significantly increased following HIV-1 exposure and is accompanied by a substantial reduced viral replication. The molecular mechanisms responsible for IL-21 antiviral activity are numerous. First, IL-21 induces the expression of the cytotoxic molecules granzyme B and perforin in vitro in CD8+ T cells and NK cells of mice chronically infected with lymphocytic choriomeningitis virus (LCMV) [9,26–29]. Second, IL-21 triggers antiviral humoral response by enhancing B cell functions in both chronically SIV-infected rhesus macaques [30] and humans [4,31–33]. Third, IL-21 elicits the STAT3-

dependent expression of miR-29 [15], a miRNA family which plays a crucial role in thwarting HIV replication [17] and whose expression is significantly augmented in HESN PBMC [21]. In our experimental setting, no significant difference were detected in perforin and granzyme expression in any of the analysed conditions. This result is consistent with the observation that protective virus-specific cellular responses promoted by IL-21 develops several weeks after HIV-1 exposure, therefore this protective mechanism would not be involved in the initial days after exposure [34]. Conversely, our findings show that the expression of all the miR29 family members was significantly increased in HIV-infected HESN CD4+ T cells, thus strengthening the association between IL-21 and miRNA29. Nevertheless, the mechanism responsible for IL-21 upregulation in HESN remains elusive and an in-depth analysis of the molecular scheme through which IL-21/miR-29 axis could control susceptibility to HIV-1 infection is mandatory. As IL-21 serves as an autocrine factor secreted by Th17 cells that promotes and sustains Th17 lineage commitment [35] we verified if IL-21 increase was paired with the release of other cytokines by this cellular subset. Notably, the percentage of IL-17-expressing CD4+ T cells was significantly increased in HESN and a similar trend was observed for both IL-17 mRNA and protein expression in supernatant from 7-days post infection PBMC. IL-6 was increased as well, even in basal conditions in HESN. This observation is interesting as IL-6 induces IL-21 production in CD4+ T cells [46–48]. It is thus possible to speculate that the high production of IL-21 and, in turn, the augmented expression miR29 seen in HESN, could be ascribable to this cytokine. It is important to underline that, while IL-6 expression was significantly increased in HESN CD4+ T cells both in unstimulated conditions and upon HIV infection, many studies report a reduced expression of this cytokine as well as of IL-17 [36–38], in other HESN cohorts [39,40]. Once more these contrasting results recall the unresolved issue of the role played by immune-activation and immune-quiescence in the HESN phenomenon. Overall the increased expression of IL-6 and IL-17 described herein support the concept that an inflammatory response is critical to contain exposure to (limited) amounts of HIV, hampering viral replication and dissemination, thus lowering the likelihood of infection, as previously reported [41–44]. Additionally, an

inflammatory profile could boost the activation of a more vigorous adaptive antiviral immune response and might result in a virus exposure-prompted natural immune defensive phenotype against HIV. Though this idea is confirmed by several findings this is just one of the theories currently being investigated. A possible way to reconcile these discrepant results is the difference in the rate of HIV-exposure in the analyzed cohorts. Sporadic contacts with the virus, as is the case of our HESN, would result in an immune activation profile; recurrent contact with HIV would trigger immune quiescence, as observed in the sex workers from the Nairobi cohort [45].

Certainly, this study presents some limitations, such as the limited number of individuals enrolled, However, the observation that miR29 expression is increased in HIV-infected CD4+ T cells confirms previous findings on PBMCs from our HESN cohort and corroborate the possibility that these miRNAs play a role in limiting HIV-replication. Taken together these results suggest that the antiviral IL-21/miR-29 axis modulates susceptibility to natural HIV-1 infection; this being the case, approaches that exogenously increase IL-21 or prompt pre-existing cellular reservoir of IL-21 might be useful in stimulating immune resistance against initial HIV-1 infection.

Figure and legends

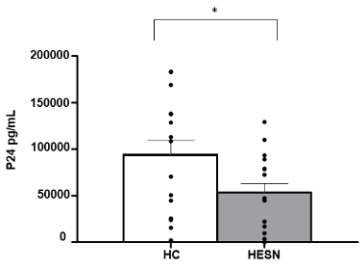


Figure 1. PBMC from were in vitro infected with HIV-1Ba-L for 7 days. P24 levels measured from HIV-1BaL-Infected cells. p24 concentration in PBMC of 15 HESN (black bar) and 15 HC (white bar) after 7 days of HIV infection. Mean values and S.E. are shown. * = $p<0.05$

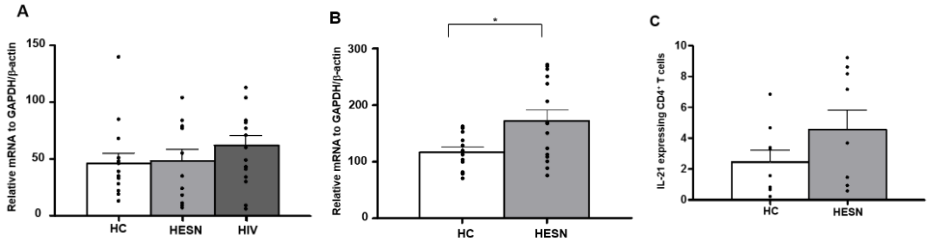


Figure 2. IL-21 expression in HC (white bars), HESN (black bars) and HIV+ (grey bars). A) IL-21 mRNA expression from unstimulated CD4+ T cells. B) IL-21 mRNA expression in 7-days post infection CD4+ T cells. C) % of IL-21-expressing CD4+ T cells. Mean values and S.E. are shown. * = $p<0.05$

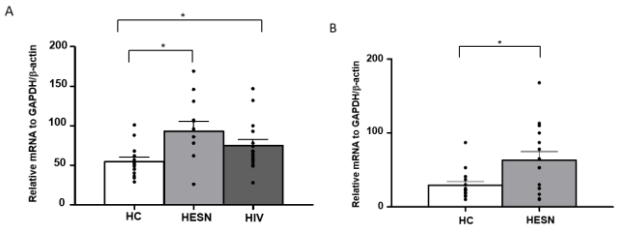


Figure 3. IL-6 mRNA expression from: A) unstimulated CD4+ T cells and B) 7-days post infection CD4+ T cells in HC (white bars), HESN (black bars) and HIV+ (grey bars). Mean values and S.E. are shown. * = $p<0.05$.

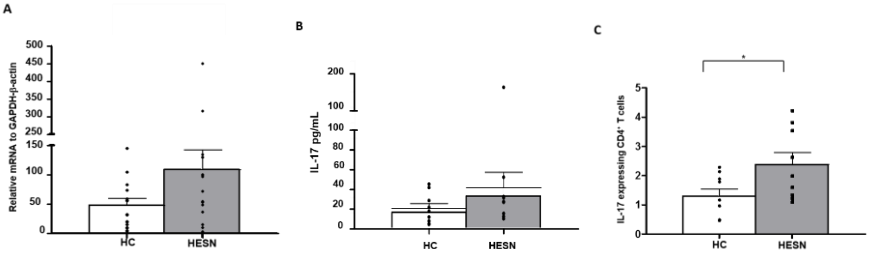


Figure 4. IL-17 expression HC (white bars), HESN (black bars) and HIV+ (grey bars). A) IL-17 mRNA expression in 7-days post infection CD4+ T cells. B) IL-17 protein expression detected in supernatant from 7-days post infection PBMC. C) % of IL-17-expressing CD4+ T cells. Mean values and S.E. are shown. * = $p<0.05$.

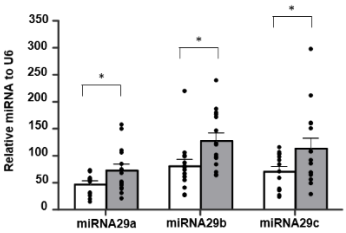
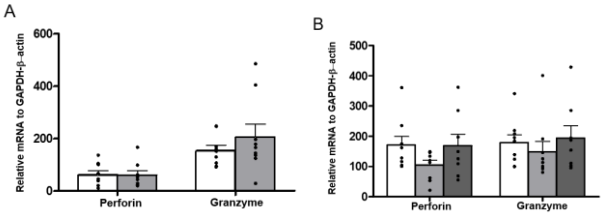


Figure 5. MiR29a, b, c expression in 7-days post infection CD4+ T cells from HC (white bars) and HESN (black bars). Mean values and S.E. are shown. * = p<0.05.



Supplementary figure 1. Perforin and granzyme mRNA expression from A) unstimulated PBMC and B) 7-days post infection PBMC from HC (white bars), HESN (black bars) and HIV+ (grey bars).

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