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**EVALUATION OF IMMUNE MECHANISMS INVOLVED IN THE LACK  
OF CD4<sup>+</sup> T CELL RECOVERY IN HIV-INFECTED PATIENTS NON  
RESPONDER TO ANTIRETROVIRAL THERAPY**

Settore MED/04 - Patologia Generale

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## LIST OF ABBREVIATIONS

ACTB: Actin beta  
ADCC: Antibody Dependent Cell-mediated Cytotoxicity  
AICD: Activation Induced Cell Death  
Apaf1: Apoptosis Activating Factor-1  
APC: Antigen Presenting Cell  
BCR: B Cell Receptor  
cAMP: Cyclic Adenosine MonoPhosphate  
CCL2: Chemokine (C-C motif) Ligand 2  
CDC: center for disease control  
CHUK: Conserved Helix-loop-helix Ubiquitous Kinase  
CLEC4E: C-type Lectin domain family 4, member E  
CNS: central nervous system  
CSF2: Colony Stimulating Factor 2  
CSF3: Colony Stimulating Factor 3  
C<sub>T</sub>: Threshold Cycle  
CTLA-4: Cytotoxic T-Lymphocyte Antigen-4  
CXCL10: Chemokine (C-X-C motif) Ligand 10  
CQ: Chloroquine  
DC: Dendritic Cell  
DDN: Didanosine  
ECSIT: ECSIT homolog (Drosophila)  
EIF2AK2: Eukaryotic translation Initiation Factor 2-Alpha Kinase 2  
FasL: Fas ligand  
FI: fusion inhibitor  
FR: Full Responder  
FS: Forward Scatter  
FSC: Forward Scatter Channel  
GALT: Gut-Associated Lymphoid Tissue  
GAPDH: GlycerAldehyde-3-Phosphate DeHydrogenase  
GITR: Glucocorticoid-Induced TNF Receptor family-related protein  
HBV: Hepatitis B Virus  
HCM: Hydroxycarbamide  
HCQ: Hydroxychloroquine  
HCV: Hepatitis C Virus  
HRAS: v-Ha-ras Harvey Rat Sarcoma viral oncogene homolog  
HSPA1A: Heat Shock 70kDa Protein 1A  
HSPD1: Heat Shock 60kDa Protein 1 (chaperonin)  
HU: Hydroxyurea  
IDO: Indoleamine 2,3-DiOxygenase  
IFN: Interferon  
IKKB: Inhibitor of Kappa light polypeptide gene enhancer in B-cells, Kinase Beta  
IKK: IKappaB Kinase  
INR: Immunological Non Responder  
INSTI: INtegrase Strand Transfer Inhibitor  
IP: Protease Inhibitor  
IRAK: Interleukin-1 Receptor-Associated Kinase  
IRF1: Interferon Regulatory Factor 1  
IRF3: Interferon Regulatory Factor 3  
iTreg: Inducible Treg  
JUN: Jun proto-oncogene  
LAG3: Lymphocyte Activation Gene 3  
LES: Lupus erythematosus  
LN: Lymph Node  
LPS: LipoPolySaccharide  
LTA: LymphoToxin Alpha (TNF superfamily, member 1)  
LTR: Long Terminal Repeat sequences  
mAb: monoclonal Antibody  
MALT: Mucosa-Associated Lymphoid Tissue  
MAP2K3: Mitogen-Activated Protein Kinase Kinase 3  
MAP3K1: Mitogen-Activated Protein Kinase Kinase Kinase 1

MAP3K7: Mitogen-Activated Protein Kinase Kinase Kinase 7  
MAP3K7IP1: Mitogen-Activated Protein Kinase 7 Interacting Protein 1  
MAP4K4: Mitogen-Activated Protein Kinase Kinase Kinase 4  
MAPK8: Mitogen-Activated Protein Kinase 8  
MAPK8IP3: Mitogen-Activated Protein Kinase 8 Interacting Protein 3  
mDC: Mieloid Dendritic Cell  
MFI: Mean Fluoresce Intensity  
MHC I: Major Hystocompatibility Complex type I  
MHC II: Major Hystocompatibility Complex type II  
MI: Maturation Inhibitor  
M-MLV: Moloney Murine Leukemia Virus  
MRP1: Multidrug Resistance Protein-1  
MYD88: Myeloid Differentiation primary response gene (88)  
NFKB: Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells  
NFKBIA: Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells Inhibitor, Alpha  
NFKBIL1: Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells Inhibitor-Like 1  
NKT: NK T cells  
NNRTI: Non Nucleotide Inhibitor of the Reverse Transcriptase  
NR2C2: Nuclear Receptor subfamily 2, group C, member 2  
NRTI: nucleotide inhibitor of the reverse transcriptase  
nTreg: Natural occurring Treg  
PAMP: Pathogen-Associated Molecular Patterns  
PD1: Programme Death receptor 1  
pDC: Plasmacitoid Dendritic Cells  
PDL: PD1 Ligand  
PEL1: Pellino homolog 1 (Drosophila)  
PI: Protease Inhibitor  
PKR: dsRNA-binding protein kinase  
PMT: PhotoMultiplier Tube  
PRKRA: Protein kinase, interferon-inducible double stranded RNA dependent Activator  
PRR: Pattern Recognition Receptor  
PTGS2: Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)  
REL: v-rel Reticulo Endotheliosis viral oncogene homolog  
RELA: v-rel reticuloendotheliosis viral oncogene homolog A  
RIPK2: Receptor-Interacting serine-threonine kinase 2  
RT: Reverse Transcriptase  
SARM1: Sterile Alpha and TIR motif containing 1  
SIGIRR: Single Immunoglobulin and toll-interleukin 1 receptor (TIR) domain  
SIV: Simian Immunodeficiency Virus  
SS: Side Scatter  
SSC: Side Scatter Channel  
ssRNA: single stranded RNA  
TAK1: Transforming growth factor- $\beta$ -Activated protein Kinase 1  
TBK1: TANK-Binding Kinase 1  
TCR: T Cell Receptor  
TICAM2: Toll-like receptor Adaptor Molecule 2  
TIR: Toll/Interleukin-1 Receptor  
TLR: Toll Like Receptor  
TNF: Tumor Necrosis Factor  
TREC: Tcr Rearrangement Excision Circle  
Tr1: Type 1 regulatory T cells  
TRAF6: Tumor necrosis factor Receptor-Associated Factor 6  
Treg: T regulatory cells  
TRIF: TIR-domain containing adaptor inducing IFN $\beta$   
ZDV: Zidavudine

***Abstract***



## RIASSUNTO

### INTRODUZIONE

L'infezione da HIV è caratterizzata da marcati difetti nella funzionalità delle cellule T CD4<sup>+</sup> con uno sbilanciamento verso le cellule Th2 durante la progressione ad AIDS. La terapia antiretrovirale HAART mira a sopprimere la replicazione virale e ad indurre il recupero quantitativo dei linfociti T CD4<sup>+</sup>. Il 15-30% dei pazienti trattati risulta essere immunologicamente non responsivo alla terapia. Diversi sono i fattori coinvolti in questo mancato recupero e il nostro interesse si è concentrato sulle cause alla base di un'eccessiva distruzione dei linfociti T helper. Recente è stato evidenziato come anche l'idrossiclorochina (HCQ), farmaco antimalarico, presenti effetti antivirali e immunomodulanti. Lo scopo di questo studio è stato quello di identificare i meccanismi immuni coinvolti nel mancato recupero dei linfociti T CD4<sup>+</sup> osservato in pazienti immunologici non responder alla terapia e verificare se HCQ può ridurre lo stato di immunoattivazione caratterizzante l'infezione da HIV e quindi portare all'aumento delle cellule T CD4<sup>+</sup>.

### MATERIALI E METODI

Per la prima parte dello studio sono stati arruolati 67 pazienti divisi in due gruppi in base alla conta di linfociti T CD4<sup>+</sup> (superiore o inferiore alle 500 cellule/ $\mu$ l) mentre per la seconda parte, le analisi sono state condotte su 20 pazienti non responsivi alla HAART caratterizzati da una conta CD4<sup>+</sup> inferiore alle 200 cellule/ $\mu$ l nell'ultimo anno di terapia. I pazienti sono stati trattati con HCQ per sei mesi con dose somministrata pari a 400mg/die. Le analisi sono state condotte al momento dell'arruolamento, al termine del trattamento e due mesi dopo la sospensione.

I parametri di immunosoppressione, immunoattivazione e apoptosi sono stati valutati in citometria a flusso. Per identificare la presenza di traslocazione microbica è stato dosato l'LPS plasmatico mentre il pathway di signaling dei TLR è stato analizzato in Real-Time PCR. Le analisi sono state condotte in condizione basale e stimolata in modo HIV-, CMV-, LPS-, ssRNA-specifico in base ai diversi parametri presi in considerazione.

### RISULTATI E DISCUSSIONE

Confrontando i pazienti con conta CD4<sup>+</sup> inferiore alle 500 cellule/ $\mu$ l con i pazienti con conta CD4<sup>+</sup> maggiore di 500 cellule/ $\mu$ l emerge che i pazienti con conta dei linfociti T CD4<sup>+</sup> inferiore alle 500 cellule/ $\mu$ l mostrano: 1) maggiore percentuale di linfociti T regolatori, 2) incremento dell'apoptosi, 3) maggior percentuale di linfociti T CD4<sup>+</sup> produttori citochine immunosoppressive quali IL10 e TGF $\beta$ , 4) maggiore percentuale di T CD4<sup>+</sup> esprimenti il K167 sia in condizione basale che stimolata; 5) maggior percentuale di Treg HIV-specifici esprimenti TLR2 e TLR4; 6) maggiore concentrazione di LPS plasmatico. Questi dati indicano che il mancato recupero dei linfociti T CD4<sup>+</sup> è associato alla presenza di un basso nadir, alla traslocazione microbica, all'immunoattivazione, alla maggior presenza e attività dei linfociti T regolatori, ed aumentata suscettibilità all'apoptosi. Nella seconda parte dello studio, è stato visto come nei pazienti non responsivi alla terapia antiretrovirale, caratterizzati da una conta linfocitaria inferiore alle 200 cellule/ $\mu$ l e trattati con idrossiclorochina si osservi la riduzione dell'immunoattivazione sia dei linfociti T CD4<sup>+</sup> che T CD8<sup>+</sup>, nonché dei monociti. Alla riduzione dell'immunoattivazione si accompagna anche la riduzione del LPS plasmatico durante tutto lo studio, decremento che permane anche nei due mesi di sospensione. I risultati mostrano che l'idrossiclorochina è in grado di controllare l'immunoattivazione e promuovere la riduzione della citochina infiammatoria IL6, come dimostrato dalle indagini citofluorimetriche e dai dosaggi immunoenzimatici condotti. Si è inoltre osservato il controllo dell'infezione da parte delle cellule dendritiche plasmacitoidi (pDC). La presenza di uno stato immunosoppressorio è stata accertata tramite la valutazione della percentuale di linfociti Treg naïve e attivati e di Treg esprimenti il TLR2 e TLR4: entrambe le popolazioni di Treg incrementa in percentuale dopo sei mesi di terapia, l'immunoattivazione e l'immunosoppressione sono quindi processi strettamente correlati e interagenti l'una con l'altra. In letteratura è stato sottolineato il ruolo dell'idrossiclorochina sul pathway di signaling dei TLR e questi ultimi, a loro volta, sullo stato di immunoattivazione. Dati citofluorimetrici mostrano la riduzione dell'espressione dei TLR da parte dei monociti anche in presenza di agonisti dei TLR4 (LPS) e TLR7/8 (ssRNA). Lo studio del pathway di signaling dei TLR è stato analizzato in presenza e assenza di agonisti dei TLR: già con sei mesi di terapia si osserva una down regolazione dei geni coinvolti nel signaling intracellulare.

### CONCLUSIONI

Si può affermare che il mancato recupero dei linfociti T CD4<sup>+</sup> caratterizzante i pazienti immunologicamente non responsivi alla HAART è associato ad un basso nadir dei CD4 pre-terapia, all'immunoattivazione, dovuta anche ad un aumento nella traslocazione microbica, e all'immunosoppressione operata dai linfociti Treg sia tramite induzione dell'apoptosi nelle cellule target sia con la promozione di un ambiente citochinico immunosoppressorio. L'incremento della popolazione di Treg potrebbe giocare in prima linea un ruolo fondamentale nel ridurre l'immunoattivazione e l'incremento di Treg esprimenti TLR supporta ancora di più come una forte attività immunosoppressoria sia in grado di controllare lo stato di immunoattivazione. L'idrossiclorochina, per i risultati preliminari ottenuti, mostra un effetto positivo sul recupero dei linfociti T CD4<sup>+</sup> nei pazienti immunologicamente non responsivi alla HAART e sul controllo dell'immunoattivazione: si può pensare ad una possibile applicazione di questo farmaco nella terapia dell'infezione da HIV come immunomodulante.

## ABSTRACT

### INTRODUCTION

HIV infection is characterized by a profound impairment of CD4<sup>+</sup> T cell functionality with an immunological unbalance toward Th2 response during progression to AIDS. Antiretroviral therapy HAART suppresses viral replication and leads to the recovery of CD4<sup>+</sup> T lymphocytes. 15-30% of HIV-infected HAART-treated patients are immunological non responder (INR) to therapy. Several factors are involved in this lack in CD4<sup>+</sup> T cell recovery, our attention was focused on the excessive depletion of CD4<sup>+</sup> T cells. Recently, hydroxychloroquine (HCQ), an anti-malarian drug, demonstrates to have antiviral and immune modulating effects. Aim of this study was to identify immune mechanisms involved in the lack of CD4<sup>+</sup> T cell recovery observed in INR patients and to verify if HCQ might reduce immune activation typical of HIV infection thus leading to a recovery in CD4<sup>+</sup> T cells.

### MATERIAL AND METHODS

In the first part of this study, 67 HAART-treated HIV-infected patients were enrolled and stratified into two groups of the basis of CD4<sup>+</sup> T cell counts (<500cells/ $\mu$ l and >500cells/ $\mu$ l) while in the second part of this study, analyses were conducted on 20 HAART-treated HIV-infected patients with an absolute CD4 count less than 200 cells/ $\mu$ l during the last 12 months of therapy. Patients were treated with 400mg/die of HCQ and analyses were conducted at baseline, 6 months of therapy and 2 months after HCQ suspension. Immune suppression, immune activation and apoptosis were evaluated in flow cytometry. To identify microbial translocation, plasma LPS was measured with immunoenzymatic assay and TLR signaling pathway was analyzed in Real-Time PCR.

Analyses were conducted in both unstimulated and stimulated (HIV-, CMV-, LPS-, ssRNA-specific) conditions.

### RESULTS AND DISCUSSION

Patients characterized by CD4<sup>+</sup> cell counts less than 500cells/ $\mu$ l compared with individuals characterized by CD4<sup>+</sup> cell counts more than 500cells/ $\mu$ l showed: 1) increase percentage of Treg cells subpopulations and 2) apoptosis, 3) higher percentage of immune suppressive cytokine-expressing CD4<sup>+</sup> T cells, 4) a higher percentage of KI67<sup>+</sup> CD4<sup>+</sup> T cells in both unstimulated and stimulated conditions. 5) an increased percentage of TLR expression on Treg cells and 6) a higher concentrations in plasma LPS. Data herein indicate that defective CD4<sup>+</sup> cell counts recovery observed in patients characterized by CD4<sup>+</sup> cell counts less than 500cells/ $\mu$ l is associated with lower CD4<sup>+</sup> nadir, gut microbial translocation and immune activation, augmented percentage and activity of Treg cells, and higher susceptibility to apoptosis.

In the second part of the study, data showed a reduction in immune activation of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and monocytes. which was coupled with a reduction in plasma LPS concentration and of IL6 pro-inflammatory cytokines production. Moreover it has been shown the pivotal role played by plasmacytoid dendritic cells in the control of infection. Immune activation and immune suppression are strictly correlated, presence of immune suppressor state was well studied thanks to the analyses of both naïve and activated Treg lymphocytes, and TLR-expressing Treg cells: these populations were increased in percentage after 6 months of therapy. Several studies put in evidence HCQ effectiveness on TLR signaling pathway which influences immune activation. Flow cytometric analyses elucidate the reduction in TLR expressions by monocytes even in the presence of TLR4 and TLR7/8 agonists (LPS and ssRNA respectively).

TLR signaling pathway was studied following PBMC stimulation with specific TLR agonists by Real-Time PCR: 6 months of HCQ treatment resulted in a reduction of TLR responsiveness and the effect was maintained also 2 months after HCQ suspension.

### CONCLUSIONS

The lack in the recovery of CD4<sup>+</sup> T lymphocytes characterizing immunological non responder patients to HAART is associated with a low CD4<sup>+</sup> nadir, with immune activation due to an increase in microbial translocation and with immune suppression conducted by Treg cells through the induction of apoptosis mechanism and immune suppressor environment. The augment in Treg population might play a pivotal role in reducing immune activation and the increase of TLR-expressing Treg cells supports the effectiveness of a strong immune suppressive activity in controlling immune activation.

Preliminary data suggest that HCQ plays a positive effect on CD4<sup>+</sup> T cell recovery in immunological non responder patients to HAART and a control of immune activation, sustaining an application of this drug as immune modulating agent, in the treatment of HIV infection.

***Introduction***

## 1. HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND INFECTION

Human immunodeficiency virus (HIV) belongs to the Retroviridae family, Lentiviridae gender.

HIV is the etiological agent provoking the human immunodeficiency syndrome, characterized by variable clinical manifestations, and in particular by a state of severe immunodeficiency determined by the reduction in the number of CD4<sup>+</sup> T cells that accompanies the onset of opportunistic infections and cancers.

Two different types of HIV have been identified: HIV-1 is more pathogenic and mainly spread in America and Europe, HIV-2 is less pathogenic and common in West Africa.

### 1.1. HIV virus structure and genome

HIV has a spherical form with an external lipoproteic envelope and an internal nucleocapsid called core. The envelope is formed by a double phospholipid layer of host-cell origin in which the viral glycoproteins gp120 and gp41 are embedded. Gp120 and gp41 originate from the proteolytic cleavage of the gp160 precursor encoded by the env gene, they are exposed on the envelope in a trimeric form and control viral tropism by mediating the fusion of the virus membrane with the target cell. The core is made by p24 proteins and it encloses HIV genome, and virus-specific enzymes like reverse transcriptase, integrase and protease. The integrity of virion particle is ensured by the viral protein p17, which forms the matrix and surrounds the internal core (fig.1).

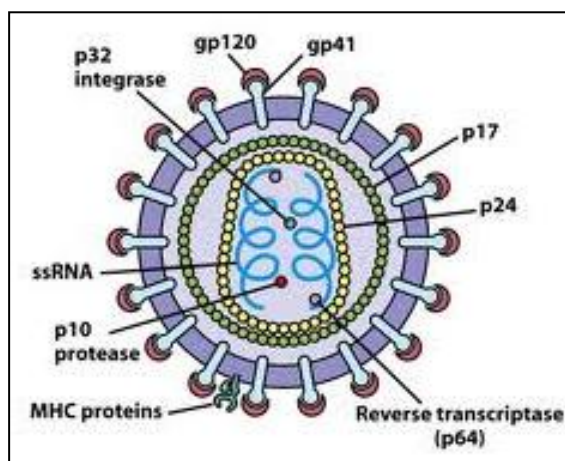


Figure 1. Structure of Human Immunodeficiency Virus type-1.

HIV genome (fig.2) is composed by two identical copies of single-strand RNA, 9.2kb long, associated with two nucleocapsid proteins (p7 and p9). HIV genome shows the typical genomic organization of virus belonging to Retroviridae family. It encodes for structural genes (gag, pol and env), regulator genes (tat, rev, nef, vpr, vpu and vif) and regulator sequences.

Gag encodes for nucleocapsid and matrix proteins, pol for reverse transcriptase, integrase and protease while env for gp160 glycoprotein, this is a precursor from which gp120 and gp41 proteins derive. [1]

A peculiar characteristic of HIV consists in its capability to create overlapping transcripts, to operate alternative splicing and to produce polyproteins which are subsequently splitted into different functional proteins.

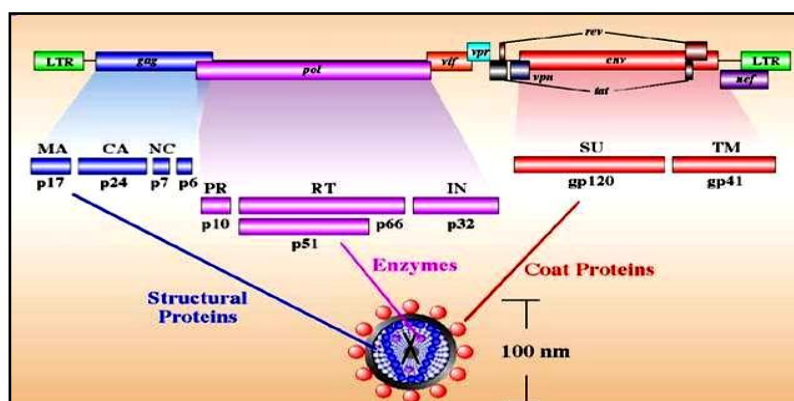


Figure 2. Genome organization of Human Immunodeficiency Virus type-1.

## 1.2. Replicative cycle of HIV-1

HIV begins its life cycle when the viral glycoproteins gp120/gp41 (ENV complex) bind to a CD4 receptor and one of two co-receptors on the surface of a target cell (fig.3). Physiologically HIV-1 co-receptors belong to chemokine receptor family the most important of which are CCR5 and CXCR4. CCR5 is typically expressed on macrophages therefore viral strains that exploit it are named M-tropic strains, conversely CXCR4 is mainly expressed on T cells and virus strains that use it are known as T-tropic strain.

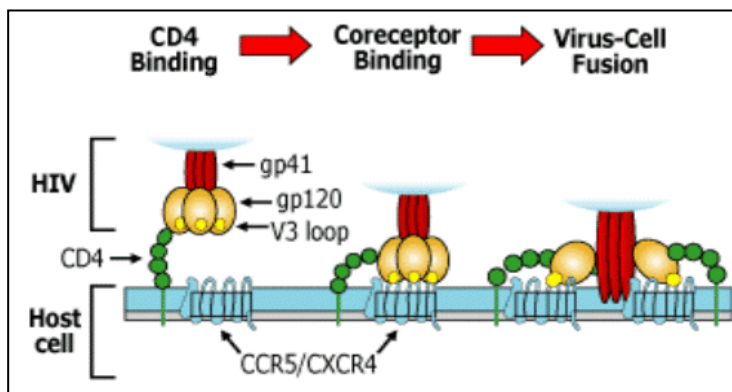


Figure 3. Schematic representation of HIV entry mechanism in a target cell.

The Env complex mediates a multistep fusion process. Gp120 binds to CD4 receptor and subsequently to the chemokine coreceptor, allowing gp41 to change its conformation and to expose a hydrophobic region called fusion peptide; this peptide inserts into the cell membrane and allows the fusion between the target cell membrane and the viral membrane.

After the fusion, the nucleoprotein core is disrupted, reverse transcriptase transcribes genomic RNA in double strand DNA which enters into the nucleus together with the integrase enzyme. Integrase promotes the integration of viral DNA in the cellular genome. The integrated viral DNA is called provirus and it can be latently inactive for long periods of time without being transcribed.

Transcription of the provirus genes is regulated by the LTR sequences. LTR are regulator sequences at the ends of HIV genome which form during retrotranscription of RNA in proviral DNA; they are important because they contain polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF- $\kappa$ B and AP1 which regulate the provirus transcription following the interaction with viral and host factors. Initiation of HIV gene transcription in T-cells can be initiated only after T-cell activation induced by antigen presentation or cytokine action.

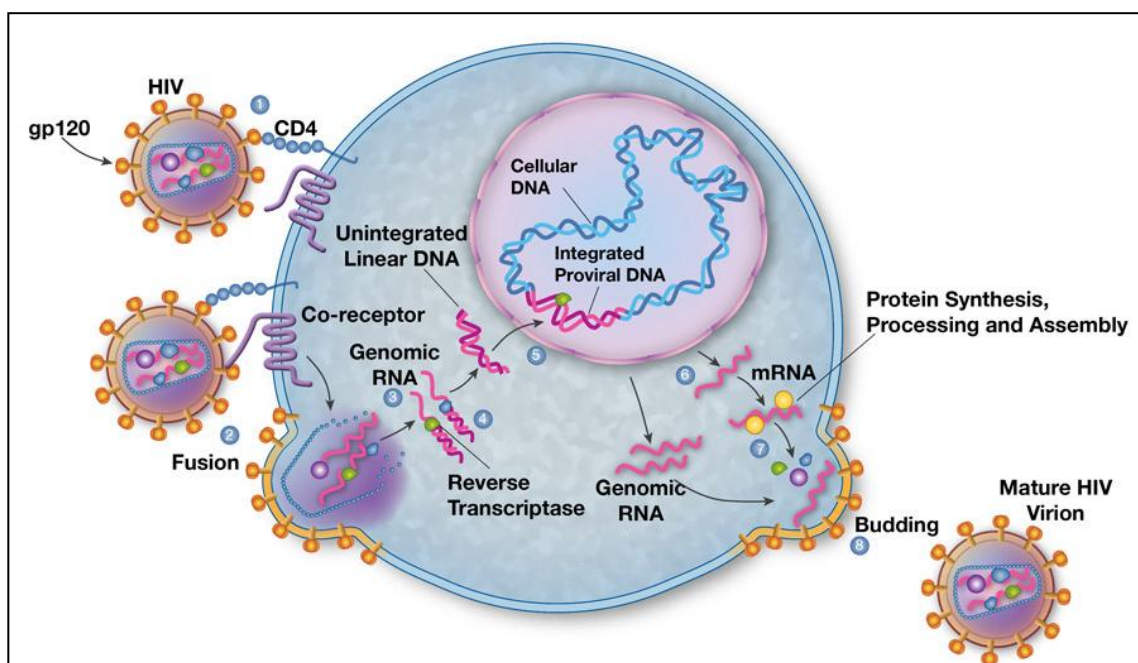


Figure 4. Mechanism of action of HIV-1 virus.

HIV genes expression can be divided in an early and late phase. Regulator genes (*tat*, *rev*, *nef*) are transcribed in the early phase of HIV life cycle, in a full-length transcript which is spliced and translocated from nucleus to cytoplasm to be translated in proteins.

Rev promotes nuclear export of incompletely spliced and unspliced viral mRNAs. Tat is required for elongation of viral transcripts by direct binding to mRNA and by increasing the processivity of host RNA-polymerase. Moreover Tat can be secreted by infected cells, as a matter of fact, its concentration is consistent in sera of HIV-infected and extracellular Tat can act as a viral toxin triggering T cell apoptosis and the secretion of many cytokines by monocytes contributing to immune dysfunction and progression of HIV disease.

Nef plays a pivotal role in accelerating endocytosis and degradation of CD4 and MHC I host cell molecules other than enhancing the release of virus from infected cells. The role played by Vif, Vpr and Vpu in viral replication is not completely clarified. Vif enhances infectivity of viral particles, Vpr promotes nuclear import of viral DNA and Vpu plays a similar role to that played by nef and also promotes maturation and trafficking of gp160 protein.

The switch from early gene to late gene is regulated by Rev, which allows the translocation of unspliced late genes to the cytoplasm.

As previously mentioned, late genes (*pol*, *gag* and *env*), are synthesized as precursor protein and are subsequently cleaved. Pol gene encodes for reverse transcriptase, protease, ribonuclease and integrase protein. Gag encodes for a precursor protein that it's subsequently cleaved in p24, p17 and p15 by pol protease, these are core proteins, necessary for assembly of viral particles. Env gene encodes for gp160, precursor of gp120 and gp41.

Assembly of viral particles begins by packaging full-length RNA transcripts of the proviral genome with proteins encoded by gag genes and pol enzymes. Viral particles are released from the target cell through a process of budding and in this passage they acquire the phospholipid layer and expose gp120 and gp41 proteins on viral particles surface (fig.4). [2]

### 1.3. Pathogenesis of HIV disease

HIV infection can be transmitted through three main different route: contact with HIV infected blood, unprotected sexual contact and vertical transmission between mother to child. Thus, groups at risk of HIV infection include: homosexual, heterosexual partner of HIV infected subjects, intravenous drug users, babies born from HIV infected mother; healthcare workers accidentally exposed to the virus. [3]

The chance of HIV-1 transmission is directly associated with the biologic properties of the virus, its concentration in the exposed body fluid and the intrinsic susceptibility of the host.

The evolution of HIV infection can be subdivided in three stages (fig.5):

- 1) Primary infection or acute phase
- 2) Clinically asymptomatic period
- 3) AIDS

The course of HIV infection can be followed quantifying the viral load and the blood CD4<sup>+</sup> T cell count. The different stages of HIV-1 infection are commonly classified using the CDC system. The system combines clinical data and the number of CD4<sup>+</sup> T lymphocytes for HIV-1 infection classification [4].

HIV infection begins with the acute phase, which lasts few weeks (2-3 weeks) and whose manifestation is commonly similar to a short flu-like illness. Typical symptoms include: fever, headaches, sore throat with pharyngitis, generalized lymphadenopathy and rashes.

This stage is characterized by an intense viral replication at mucosal sites thus it's possible to find up to 10<sup>7</sup> viral copies per ml in the plasma. Usually after this viral spread, viral load stabilizes around 10<sup>2</sup>-10<sup>5</sup> RNA copies per ml. This reduction of viral replication is due to the activation of a CTL-mediated immune response which allows a partial control of HIV-infection and it's followed by the production of HIV specific antibodies, which signs the seroconversion of the patients.

HIV preferentially infects activated T cells, DCs and macrophages while naïve T cells are not susceptible of HIV infection because they express the active form of a cytosine deaminase called APOBEC3G which is able to inhibit HIV replication by introducing mutations within single-stranded DNA during reverse transcription.

Recent studies suggest that a higher expression of this enzyme within cell is associated with lower viral replication during acute infection [5] while others suggested that its antiviral activity depends on the interference exerted in primer tRNA annealing, progression, removal and DNA elongation [6].

During the acute phase [7] DCs in the lamina propria capture the virus and migrate to lymphnodes where they transfer HIV to CD4<sup>+</sup> T cells HIV begins to replicate. Thus in a few day it's possible to detect it in the lymphoid tissue. This replication leads to viremia, during which high numbers of HIV particles are present in the patient's blood, accompanied by an acute HIV syndrome that includes a variety of non specific signs and



symptoms typical of many viral diseases. The viremia allows the virus to disseminate throughout the body and to infect helper T cells, macrophages and dendritic cells in the peripheral lymphoid tissues. As the HIV infection spreads, the adaptive immune system mounts both humoral and cell-mediated immune responses directed at viral antigens. These immune responses partially control the infection and viral production, and such control is reflected by a drop in viremia to low but detectable levels by 12 weeks after the primary exposure.

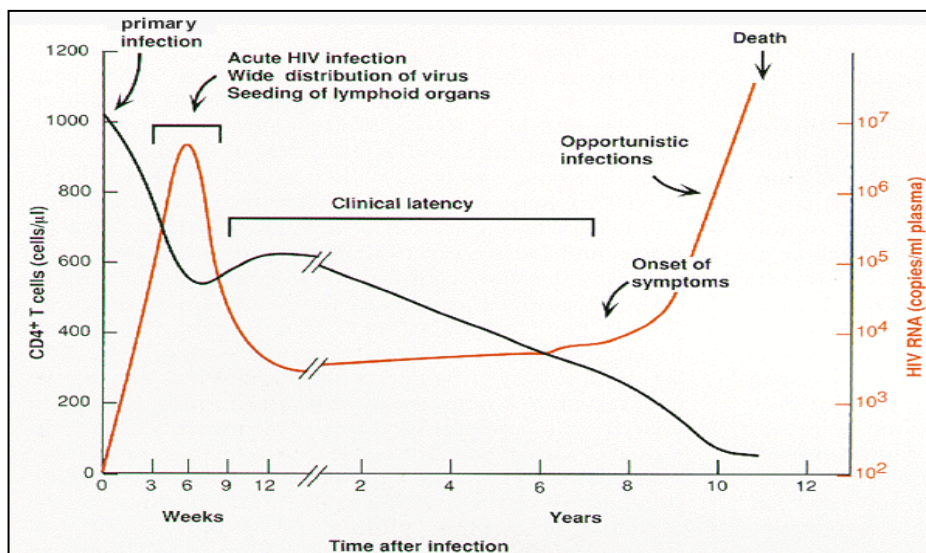


Figure 5. Course of HIV infection.

The acute infection, is followed by a clinically asymptomatic period characterized by persistent HIV replication in the lymph nodes and spleen without clinical manifestations. During this period, the immune system is still capable of handling most opportunistic infections but it's possible to observe the reduction in the number of CD4<sup>+</sup> T may be due to continuous virus infection, T cell death and other viral co-infection. As the immune system fails, symptoms develop; initially they are mild but as the immune system deteriorates the symptoms worsen. Overall this transition period between acute infection and progression to AIDS can last 7-10 years, in presence of an antiretroviral therapy it could be longer. When HIV disease progresses to AIDS, there's the complete destruction of peripheral lymphoid tissue and reduction of CD4<sup>+</sup> T lymphocytes level (below 200cells/mm<sup>3</sup>); the increase of plasmatic viremia is due to the inability of immune system to control any kind of infection. AIDS patients suffer from combinations of opportunistic infections, neoplasms, cachexia, kidney failure and CNS degeneration. Because CD4<sup>+</sup> T helper cells are essential for both cell-mediated and humoral immune responses to various microbes, the loss of these lymphocytes is the main reason that patients with AIDS become susceptible to many different types of infections.

#### 1.4. Immune response to HIV

Following HIV infection the immune system responds to the virus through the production of HIV-specific antibodies and CD8<sup>+</sup> T lymphocytes. Neutralizing antibodies appear 2-3 weeks after primary infection, they explicate their antiviral activity binding to viral particles and mediating their incorporation and destruction by phagocytes. However because of the high rate of HIV mutation, their antiviral action is easily overcome by immune escaping HIV-1 variants. [4, 8]

CD8<sup>+</sup> T lymphocytes control virus replication by killing infected cells and producing cytokines and chemokines which affect viral replication. CTLs induce cell-death in infected cell through the release of perforins and granzymes after binding a target cells expressing viral antigens in association with MHC I molecules on cell surface. Target cells can be also induced to apoptosis through the interaction between FasL and Fas receptor expressed on CTL surface on infected cells respectively. Moreover CTLs are able to produce and release antiviral factors like IFN $\alpha$ , MIP1 $\alpha$ , MIP1 $\beta$  and RANTES. [4, 9]

## 2. CD4<sup>+</sup> T CELLS DEPLETION IN HIV INFECTION

Many factors can be involved in CD4<sup>+</sup> T lymphocytes loss and immune dysfunction and according to their origin they can be classified in virologic (direct cytopathic) or immunopathologic (indirect cytopathic) [10].

### Direct cytopathic mechanisms

Direct cytopathic effects may depend on viral replication. HIV replication can influence cell homeostasis in several ways:

- When HIV replicates, it exploits cellular ribosomes and enzymes to synthesize its own proteins so host cells are unable to produce their own proteins and to survive.
- Cell death may be secondary to early genes (nef, vpr and tat) mechanism of action.
- Intracellular accumulation of viral genome may be cytotoxic.
- Budding of new virions from infected cells may induce osmotic lysis.

### Indirect cytopathic mechanism

These mechanisms are independent from viral replication and are secondary to immune response and alteration in presentation of viral peptides:

- Defects in antigen presentation could depend on altered expression levels of receptors involved in antigen recognition/presentation (TCR or MHCII molecules)
- Reduction of TCR surface expression inhibits proper T cell activation
- Down-regulation of MHCII molecules causes a reduction in antigen presentation and therefore CD4<sup>+</sup> T lymphocytes priming.
- ADCC, antibody dependent cell death, secondary to antibodies specific for soluble gp120 bound to CD4 molecules expressed by non infected lymphocytes that induce cell lyses in these cells.
- CTL responses against infected cells that expose viral antigens on their surfaces.
- Inhibition of T helper activation due to cross reaction between gp120/gp41 and HLA molecules.

### 2.1. Apoptosis in HIV infection

Apoptosis is a mechanism of programmed cell death which plays a key role in the development of immune system and in controlling the immune responses [11].

Since 90's, apoptosis has been described as a mechanism responsible for T cell depletion in HIV-infected patients. There are two main apoptotic pathways: the extrinsic one activated by death signals and the intrinsic one induced by cellular stress signals (fig.6).

Apoptosis results in the activation of aspartate-specific cysteine proteases known as caspase family.

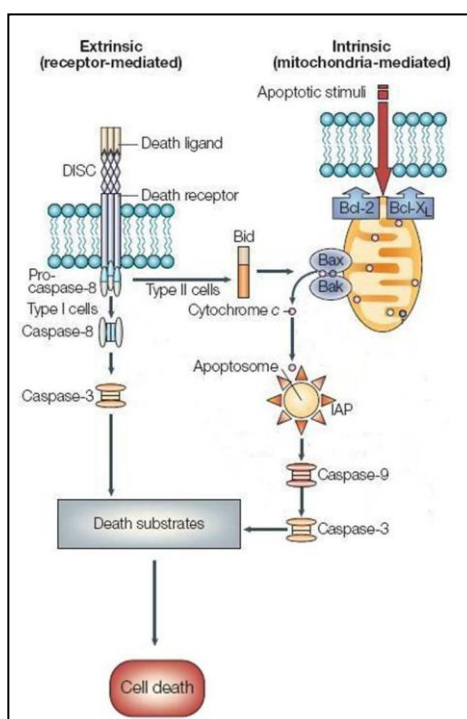


Figure 6. Extrinsic and intrinsic apoptotic pathways.



The extrinsic apoptotic pathway is initiated by the interaction between death ligands and death receptors expressed on cell surface. The death receptors mainly involved in this process are members of the tumor necrosis factor receptor superfamily and include Fas, TNFR1 and TRAIL. At intracellular level these receptors show death domains and when activated by binding with their specific ligands, they recruit adaptor proteins that lead to the activation of caspase 8 and the priming of apoptosis execution phase.

The intrinsic apoptosis is independent by death receptors and it is induced by cellular stress signals acting on mitochondria membrane permeability. Mitochondria permeability is controlled by proteins belonging to the Bcl2 family, their alteration leads to the release of cytochrome c, a pro-apoptotic protein, from intermembrane space of mitochondria. In the cytoplasm, cytochrome c works as a cofactor of Apaf-1. Cytochrome c associates with Apaf-1 and procaspase 9 (inactive form) is converted into caspase 9, the active form. Both the intrinsic and extrinsic pathway culminate in the activation of the effector caspase 3. Cellular alterations induced by the apoptotic process include chromatin condensation, DNA fragmentation and bubble-like blebs on cellular membrane. Cells in apoptosis are splitted in little fragments known as apoptotic bodies. [2]

HIV infection provokes apoptosis in both infected cells and uninfected cells through several mechanism: 1) direct infection of T lymphocytes, 2) direct role of HIV proteins, 3) AICD and 4) deregulation of cytokine production.

### 2.1.1. Direct infection of T lymphocytes

HIV can induce lymphocytes death during the infection stage through direct cytophatic mechanisms as previously described in paragraph 2.

### 2.1.2. Direct role of HIV proteins

Most of the proteins encoded by HIV genome show pro-apoptotic and anti-apoptotic properties.

A key role is played by gp120, which can mediate apoptosis in both infected and uninfected cells. Gp120 is expressed in two isoforms, soluble and membrane bound, both are able to induce the expression of death receptors on target cells following CD4 or CXCR4/CCR5 binding.

Gp120-CD4 interaction can lead to the activation of apoptosis through the upregulation of Fas/FasL (extrinsic pathway) or the alteration of Bcl-2 expression (intrinsic pathway). Gp120-CXCR4 interaction activates the intrinsic apoptosis pathway while CCR5 binding mediates the upregulation of Fas/FasL expression.

CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells from HIV infected patients show an upregulation in the expression of Fas and of FasL on APC and of TRAIL on CD4<sup>+</sup> T lymphocytes. It has also been reported that in resting CD4<sup>+</sup> T lymphocytes following the signaling through the homing receptor CD62L, Fas/FasL expression is upregulated. [12]

The complex gp120/gp41 can induced syncytia formation. Syncytia derives from cell membranes fusion to form large cells with many nuclei. In HIV infection it happens when infected cells expose gp120/gp41 complex on their membrane and they interact with CD4 receptor expressed by an uninfected cell. Syncytia have short lifespan and are more often seen in advance stage of infection. Different studies suggest that nuclear apoptosis of syncytia is caspase-dependent, the key events include the activation of p53 that induces the transcription of pro-apoptotic proteins (Bax, PUMA), the translocation of Bax to mitochondria where it induces the release of cytochrome c and the activation of the intrinsic apoptosis. [13]

Nef plays a chief role in inducing viral replication. Studies conducted on infected T cells have confirmed the role of nef in upregulating Fas/FasL and PD1 expression, and in downregulating anti-apoptotic factors as bcl-2 and bcl-xl. [14, 15]

Tat is secreted by infected cells and taken up by uninfected cells in which it interacts with microtubules preventing their depolymerization leading to the alteration of microtubules dynamics. Furthermore tat is responsible for inducing BimL release. BimL is a pro-apoptotic factor, belonging to Bcl-2 family, involved in apoptotic signal transduction as a consequence of microtubule perturbation. Interestingly, HIV-infected cells are resistant to tat-mediated apoptosis and probably this represents a viral strategy to allow HIV survival. [16]

As previously said, HIV uses its protease to obtain several proteins from precursor ones. Nie et al. [17] have shown that viral protease cleaves procaspase 8 to generate a fragment of 41kDa (Casp8p41) which is able to induce apoptosis in infected memory CD4<sup>+</sup> T cells. Moreover Sainski et al have demonstrated that Casp8p41 doesn't contain a catalytic site of caspase 8 and it induces death of infected cells through bax/bad mitochondrial depolarization and caspase 9 activation. Bax/bad are pro-apoptotic factors at mitochondrial level, Casp8p41 is unable to bind them directly but probably it induces other factors (BH3-only proteins) that bind to anti-apoptotic proteins (bcl-xl or bcl-2) allowing bax/bad activation. Once bax/bad are activated they oligomerize and create pores in mitochondrial membrane, cytochrome c is released and intrinsic apoptosis pathway begins. [18, 19]

### 2.1.3. AICD

Activated induced cell death (AICD) is a mechanism of programmed death induced in persistent activated lymphocytes in the absence of the appropriate co-stimulation signal. Several studies have demonstrated that during chronic activation of immune system by HIV virus, expression of death receptors and death ligands is upregulated, in particular the Fas/FasL pathway is altered leading to an increased susceptibility of lymphocytes to apoptosis. Fas is a membrane protein expressed on the surface of several cellular types and FasL is a homotrimer expressed on T lymphocytes cellular membrane after activation induced by antigen presentation and IL-2 action. Activated lymphocytes coexpress Fas and FasL, inducing apoptotic process in both autocrine and paracrine ways. Several studies underline that as a consequence of the Fas/FasL pathway impairment, PBMC of HIV-infected patients are more susceptible to apoptosis than PBMC of healthy subjects. [20]

### 2.1.4. Disregulation of cytokine production

Lymphocyte survival is guaranteed by survival signals, as the ones furnished by IL2. IL2 is a factor involved in growing, surviving and differentiation of T cells, it works in autocrine and paracrine ways and it plays an important role in regulation of T-dependent pathway. IL2 is fundamental for cell survival because it induces the expression of the anti-apoptotic protein Bcl-2. In the absence of IL2, Bcl-2 expression is downregulated while the pro-apoptotic Bax protein is upregulated. Bax induces an increase in mitochondrial membrane permeability, cytochrome c is released and the intrinsic apoptotic pathway is activated. Bcl-2 is an anti-apoptotic factor because it blocks the mitochondrial membrane permeability, the release of cytochrome c and of Apaf-1 inhibiting caspase 9 activation.

The balance between bcl-2 and bax monomers is key to establish an apoptosis or survival status.

The positive effect of IL2 on cell survival is well documented. In particular it has been demonstrated that low levels of bcl-2 associated with IL2 absence and CD28 downregulation leading to apoptosis in CD4<sup>+</sup> T lymphocytes. Therefore it has been hypothesized the use of a combined therapy based on antiviral drugs and cytokines with immunomodulating properties able to prevent apoptosis. [21, 22]

In conclusion apoptosis is both physiologic and "pathologic" mechanism, it determines the correct development of immune system and self-tolerance but following viral infection, like HIV one, it's used by the virus to control, overcome and destroy immune response and cells. In particular HIV virus acts through different mechanisms that culminate in lymphocyte depletion usually observed during the infection.

### 3. HIV AND IMMUNE ACTIVATION

Persistent immune activation plays a central role in driving immune pathogenesis and progression to AIDS. Immune activation is characterized by increased frequencies of T-cells and B-cells with an activated phenotype, accelerated lymphocyte turnover with abnormalities in cell cycle regulation and high serum levels of pro-inflammatory cytokines and chemokines. Typical markers of immune activation are CD38 and Ki67. CD38 expression on CD8<sup>+</sup> T lymphocytes increases all along the progression of the disease that's why it's considered the most reliable marker of immune activation, disease progression to AIDS and death. Ki67 is expressed in the nucleus of cycling cells.

Considering that HIV replicates better in activated cells than in resting ones, immune activation favors HIV replication cycle as well as the spread and maintenance of infection because of the continuous recruitment of activated lymphocytes to lymphoid tissue.

CD38 has been correlated with CD4<sup>+</sup> T cells decline. Several works established a correlation between immune activation and CD4<sup>+</sup> T cells decline and clinical progression independently from viral replication. It has been supposed that CD38 might be a useful set-point of immune activation to take in consideration at the time of introduction of antiretroviral therapy: pre-therapy T cell activation set-point may be predictive of subsequent T cell recovery concomitantly of a therapy.

A study by Deeks and colleagues [23] asserts that immune activation set-point is a steady state level of T-cell activation which characterizes untreated HIV-infected patients even in early HIV infection. Furthermore it has been suggested that immune activation set-point is negatively correlated with viral load set-point and that the level of viremia and of T-cell activation provide important prognostic information for the introduction of antiretroviral treatment in a HIV subject naïve to therapy. [24, 25, 26]

Hazenberg and colleagues demonstrated that persistent immune activation is associated with progression to AIDS. They established that the size of the CD4<sup>+</sup> T cell pool and the level of immune activation before HIV-1 infection were key factors for subsequent disease progression. Furthermore, patients characterized by a small naïve T cell pool have a more rapid progression of the disease because T cell depletion induced by persistent immune activation provokes continuous recruitment of naïve T cells. [27]

The degree of immune activation is a better predictor of disease progression than plasma viral load.

An important consequence of HIV immune activation is its impact on immune tissue, persistent immune activation leads to the dysregulation of the architecture of tissues like bone marrow, thymus and lymph node, that are important for T cell regeneration and function.

To date it's well known that the main mechanisms promoting immune activation are: microbial translocation and HIV RNA that binding TLR in plasmacytoid dendritic cells (pDC) directly stimulates IFN $\alpha$  production.

#### 3.1. *Mucosal immune dysfunction and microbial translocation*

The mucosa-associated lymphoid tissue (MALT) plays a central role in protecting the organism from the invasion of different pathogens like HIV. MALT is mainly localized in the gastrointestinal tract (gut-associated lymphoid tissue – GALT) where it plays a pivotal role in maintaining the immunologic tolerance toward the commensal and non commensal flora.

GALT is organized in inductive and effector sites, the inductive sites comprehend mesenteric lymph node, Peyer's patch and isolated lymphoid follicles while the effector ones are represented by epithelium and epithelial lamina propria. Within the inductive sites the antigens of mucosal lumen are collected and immune responses are induced, while within the effector sites effector cells are activated, differentiated and they exert their immune effector functions, either cellular response mediated by T-cells or humoral responses mediated by B-cells and plasma cells. Anatomically, the lymphocytic populations can be divided into the one inside the epithelium, counting intraepithelial lymphocytes mainly CD8<sup>+</sup> T cells, and the one inside the underlying lamina propria, containing CD4<sup>+</sup> T helper cells, CD8<sup>+</sup> T cells, natural killer cells and B-cells.

It's essential to maintain the integrity of the mucosal barrier and this can be realized by several factors like phagocytic activity, immunoglobulin A secretion and the presence of a thick mucosal layer [28]. A damage in the mucosal barrier allows pathogens to penetrate in the sub-mucosal layer and to lead to the activation of the immune system.

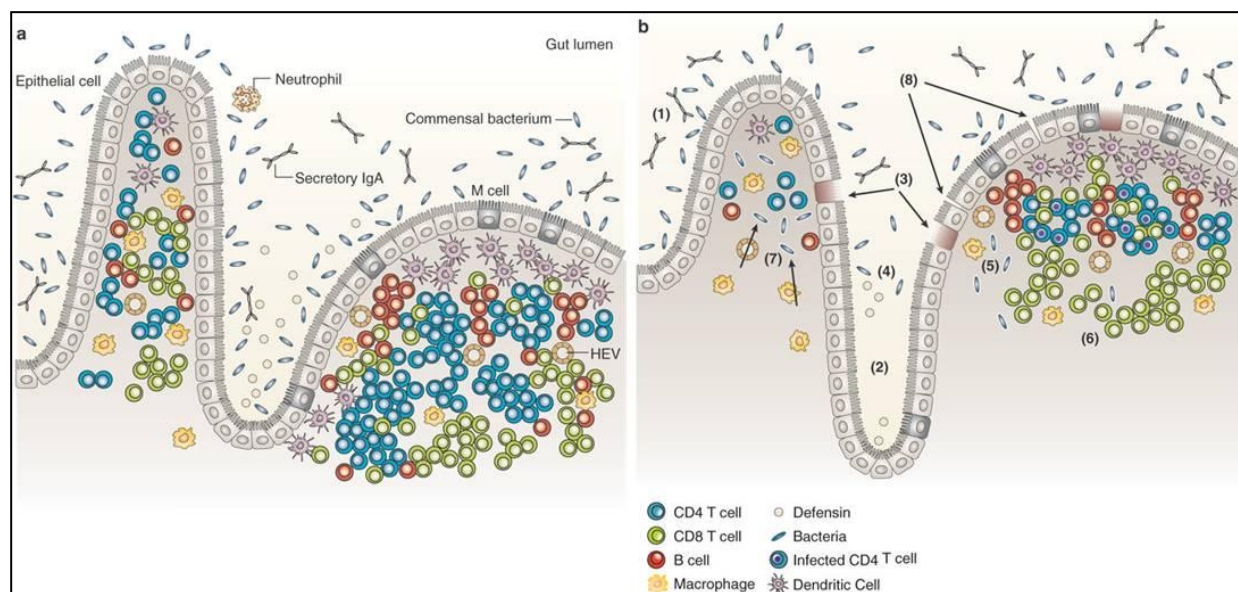
Considering that HIV spreads mainly through mucosal surfaces, several studies have focused their attention on the immunologic consequences of HIV infection in the mucosal tissue.

Definitely mucosal immune dysfunction contributes to HIV-associated chronic immune activation through the massive CD4<sup>+</sup> T depletion in the early stage of infection (fig.7). The first cells to be depleted in the GALT are CCR5<sup>+</sup> CD4<sup>+</sup> T and mucosal T lymphocytes consist predominantly of memory cells, expressing CCR5 and markers of activation that transform them in the ideal target of HIV virus.

In the chronic stage of infection, the massive depletion of CD4<sup>+</sup> T lymphocytes causes the alteration of immune control that favors the breakdown of physical and biological mucosal barrier.

Experiments conducted in SIV (Simian Immunodeficiency Virus)-infected rhesus macaques showed that the early stages of infection are characterized by a massive depletion of mucosal CD4<sup>+</sup> T cells, and monkeys

that are able to partially reconstitute this cellular population progress to AIDS at a slower rate than those that don't display T cell recovery. As a consequence of the impaired balance in immune response CD8<sup>+</sup> T cell are activated, pro-inflammatory cytokines are released leading to chronic immune activation and mucosal damage. [28, 29]



*Figure 7. HIV-associated damage to GI tract. Schematic representation of a healthy GI tract (a) and of a chronically HIV-infected GI tract (b), blunted villi (1), crypt hyperplasia (2), epithelial barrier compromise (3), decrease antimicrobial agents (4), massive CD4<sup>+</sup> T cell depletion (5-6), microbial translocation (7) and increased permeability (8) are indicated.*

Based on these observations it has been formulated a pathogenetic model in which selective depletion of mucosal memory CD4<sup>+</sup> T cells, during acute HIV infection, is primary in determining the progression of the disease. According to this model the depletion of CD4<sup>+</sup> T cells in the MALT induces an impairment in mucosal immunity that leads to a series of pathogenic sequelae distinctive of the chronic phase of the disease [30]

Microbial translocation is the first event following the disruption of mucosal barrier. Several factors participate to promote microbial translocation such as enterocytes apoptosis and loss of gastrointestinal CD4<sup>+</sup> T cells. Studies conducted in SIV-infected rhesus macaques demonstrated that enterocytes apoptosis might be the cause of increased intestinal permeability that allows microbial translocation. HIV virus could have a main role in enterocytes apoptosis. Moreover HIV infection is associated with the presence of a pro-inflammatory environment. [31]

Breakdown of intestinal barrier allows the microbial translocation process, pathogens are able to colonize sub-epithelial layer and microbial products activate the immune system via their binding to TLR.

As previously said, persistent CD4<sup>+</sup> T cell loss characterizes the early phase of infection while immune activation is typical of the chronic stage of the disease. Brenchley and Douek proposed a device to connect these two processes.

The increased translocation of microbial products like lipopolysaccharide (LPS) or flagellin from the intestinal lumen to the systemic circulation, may cause the persistent activation of immune system through TLR triggering. LPS is a component of Gram negative bacteria cell walls that can be quantitatively assayed in plasma.

Brenchley and Douek conducted several studies on immune activation and mucosal immunity, in both SIV-infected animal models and HIV-infected patients. They proposed that LPS might be the factor responsible for the persistent loss of MALT CD4<sup>+</sup> T cells and immune activation. They found that HIV-infected patients, in chronic stage of the disease, were characterized by high quantities of plasma LPS, persistent activation of monocytes and by a pro-inflammatory environment. All of these factors suggest that increased microbial translocation directly contributes to systemic immune activation in the chronic phase of HIV infection and so they may determine the rate of progression to AIDS. It has been found a positive correlation between LPS levels and frequency of activated memory CD8<sup>+</sup> T cells and pro-inflammatory cytokines. Maybe plasmatic LPS is detectable only during the chronic phase because during the acute phase microbial products are localized mainly within tissue macrophages (fig. 8). Moreover they compared LPS level in HIV-infected patients before and after initiating antiretroviral therapy, the results obtained showed a reduction in plasmatic

LPS level during HAART therapy suggesting that HIV plays a direct role in maintaining microbial translocation and in the disruption of mucosal barrier. [26, 31, 32, 33, 34]

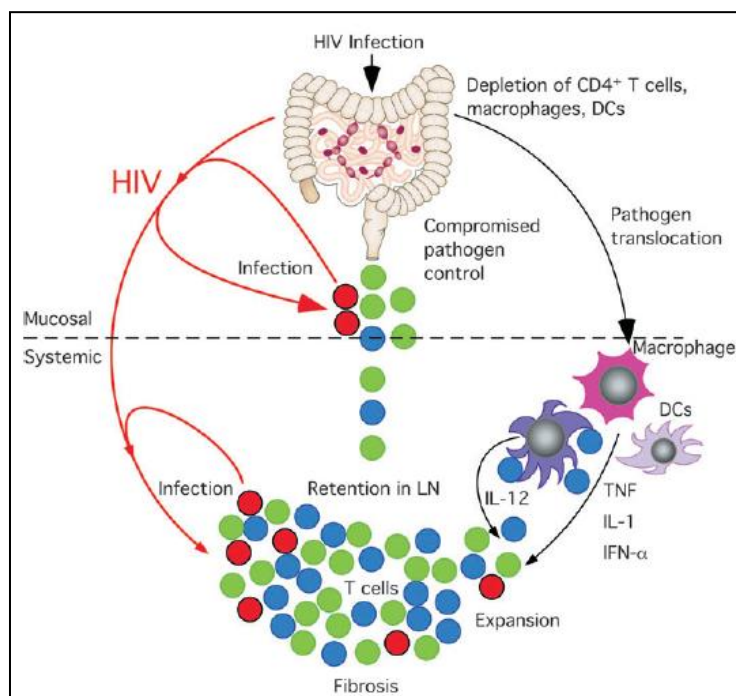


Figure 8. Immune activation hypothesis.

HIV can induce immune activation through both direct and indirect mechanisms. HIV gene products can induce directly the activation of lymphocytes and macrophages, the production of pro-inflammatory cytokines and chemokines. Gp120, binding CD4, induces cellular activation even in the absence of direct infection leading to increased calcium concentrations in enterocytes and an impairment in intracellular ionic balances. Nef shows pleiotropic effects, it's able to promote the synthesis of paracrine factors by infected macrophages resulting in the activation of bystander T cells and it targets cell membranes of enterocytes eliciting organelle formation, cytoskeletal rearrangement and increases intestinal epithelial tight junctions permeability. It has been demonstrated that nef could be secreted from infected cells in association with small membrane-bound vesicles and encompassed by enterocytes, moreover in vitro model showed that nef might prolong cell survival allowing accumulation of viral particles before inducing cell destruction of intestinal cells and virus release. Nef upsets the IFN $\gamma$ -induced impairment of intestinal epithelial cells because it interferes with IFN $\gamma$  driven pathways. [35]

Furthermore, Tat provokes intestinal dysfunction because it influences the uptake of glucose by enterocytes and causes microtubule depolymerization.

HIV may also act through indirect mechanism by inducing the synthesis of inflammatory cytokines (TNF $\alpha$ , IL1, IL6). High level of TNF $\alpha$  is thought to cause apoptosis of enterocytes while high levels of IL1 and IL6 have been found in the lamina propria of the colon of HIV-infected individuals. [36]

To date it has been described how HIV is responsible for the gastrointestinal CD4<sup>+</sup> T cell depletion and how it compromises the integrity of mucosal barrier. Considering that HAART is capable of restoring CD4<sup>+</sup> T cell count and suppress viral load, several studies are investigating whether antiretroviral therapy might control mucosal CD4<sup>+</sup> T cells depletion and microbial translocation. HAART effects on immune activation will be discussed in paragraph 5.1.1.

### 3.2. TLR in the immune system

TLRs play an important role in innate immune recognition, they are the first line of innate defense against microorganisms. TLRs belong to the family of pattern recognition receptors (PRR) due to their ability to recognize pathogen conserved structures, known as pathogen-associated molecular patterns (PAMPs). PRRs are expressed mainly by macrophages, dendritic cells, endothelial cells mucosal epithelial cells and lymphocytes, they can be localized at intracellular level or on the surface of these cellular populations. Other receptors are involved in innate recognition but TLRs have been studied most extensively, to date 13 receptors (TLR1-TLR13) have been described. They are distinct on the base of their cellular localization and their specific ability in PAMPs recognition.



TLR-1, -2, -4, -5, -6 are expressed on cell surface and they are mainly involved in detecting microbial products while TLR-3, -7, -8, -9 are localized on late endosome-lysosome vesicles and do recognize nucleic acid of both viral and bacterial origin. So far, TLR-10, -11, -12, -13 are orphan receptors and less is known about them.

All the TLRs show the same structural organization, they are type I integral membrane glycoproteins characterized, at the extracellular side by leucine-rich regions responsible for the recognition of pathogens and at the transmembrane and cytoplasmic level by Toll/interleukin-1 receptor (TIR) domain. Signal transduction is activated only after TLRs dimerization because this allows the binding of adaptor proteins like MyD88 or TIR-domain containing adapter inducing IFN $\beta$  (TRIF). Recruitment of MyD88 leads to the activation of MyD88 dependent pathway while the binding of TIR domain with TRIF induces the activation of the MyD88 independent pathway

All of the TLRs, except for TLR3 and in some cases also TLR4, bind MyD88 adaptor protein.

### 3.2.1. MyD88 dependent and independent pathways

After recognition of a pathogen-specific molecular pattern, TLRs are capable of differentially activating distinct downstream signaling events via different cofactors and adaptor proteins mediating diverse immune responses (fig.9).

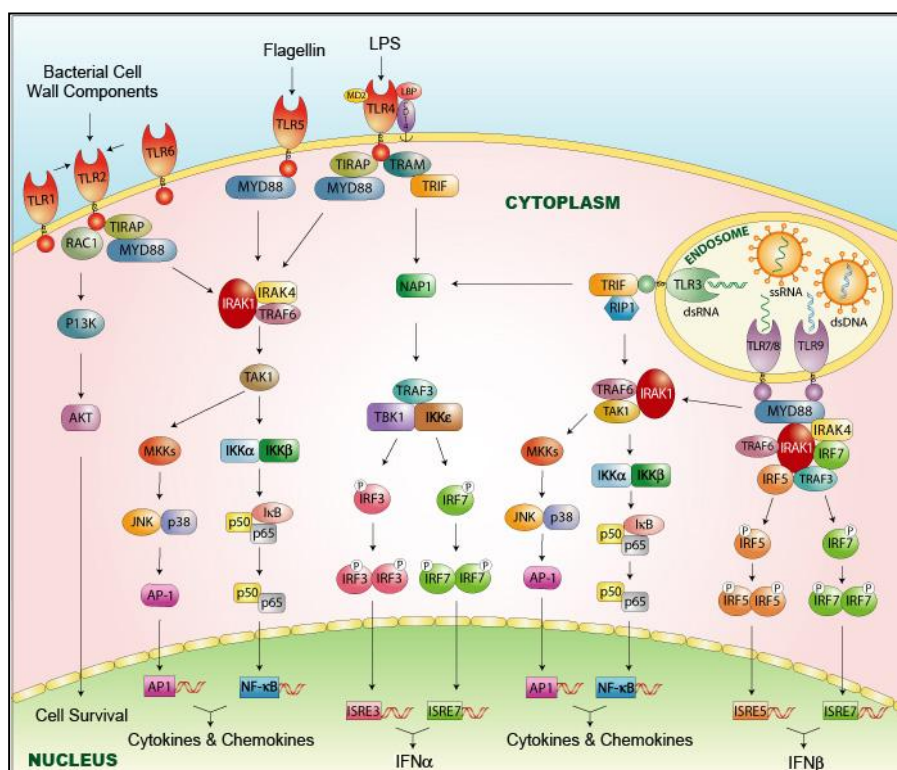


Figure 9. TLR signaling pathway. MyD88-dependent and independent pathways are illustrated.

MyD88-dependent pathway culminates in the activation of NF- $\kappa$ B and JNK transcription factors. This pathway is activated via the conserved, cytoplasmic TIR domain, which provides a scaffold for recruitment of the adaptor molecule MyD88 and serine/threonine kinases of the IL-1R-associated kinase (IRAK) family. Four IRAK have been identified: IRAK-1, IRAK-2, IRAK-4 and IRAK-M. IRAK-1 and IRAK-4 possess intrinsic serine/threonine protein kinase activities, IRAK-2 and IRAK-M lack this activity, suggesting that they negatively regulate TLR-mediated signaling. IRAK1 and IRAK4 are sequentially phosphorylated and dissociated from MyD88, which results in activation of tumor necrosis factor receptor-associated factor 6 (TRAF6). The TRAF6 adaptor protein activates transforming growth factor- $\beta$ -activated protein kinase 1 (TAK1) which in turn triggers the IKK complex that leads to the transcription factor NF- $\kappa$ B activation. TAK1 simultaneously phosphorylates two members of the MAP kinase kinase family (such as JNK) which in turn activate AP-1.

NF- $\kappa$ B and AP-1 promote the transcription of genes coding for macrophage inflammatory mediators, inducing B cell proliferation and endotoxin shock. Experiments conducted in mice elucidated that this pathway is essential for TLR-mediated inflammatory responses.

MyD88-independent pathway activation is based on IFN-regulatory factor 3 (IRF-3) activity a transcription factor that promotes IFN $\beta$  synthesis. Recently, it has been discovered that IRF-3 is associated with I $\kappa$ B

kinases (IKKs). IKKs are composed of IKK $\alpha$  and IKK $\beta$ , both of which phosphorylate I $\kappa$ B $\alpha$  thereby inducing NF- $\kappa$ B activation. In addition, there are two noncanonical IKKs, TANK-binding kinase 1 (TBK1) and IKK $\epsilon$ /IKK $\iota$ , which have distinct kinase activities compared with the canonical IKK $\alpha$  and IKK $\beta$  and both of them are able to promote the phosphorylation of IRF-3.

This signaling pathway could also be activated via the TIR-domain-containing adaptor protein (TIRAP; also designated Mal for 'MyD88-adaptor-like') and results in activation of the dsRNA-binding protein kinase PKR. This protein has been proposed to be a central downstream component of both the TIRAP- and MyD88-dependent signaling pathways and could mediate potential crosstalk between them. The MyD88-independent pathway appears to utilize both IRF3 and NF- $\kappa$ B, and results in the expression of IFN-inducible genes including IP-10.

It has been demonstrated that NF- $\kappa$ B can be induced in a later phase thanks to the activation of IRF-3. Moreover TLR4 activation might lead to the induction of both MyD88-dependent and -independent pathway, according to the adaptor protein that is recruited to the TIR domain of the receptor. Adaptor protein TIRAP promotes the activation of the MyD88-dependent pathway while TRIF is the inducer of the independent one [37, 38, 39].

Following HIV infection TLR2, TLR4, TLR7 and TLR8 are triggered. TLR2 binds to peptidoglycan and bacterial lipoprotein, TLR4 recognizes LPS, TLR7 and TLR8 bind to ssRNA.

Moreover experimental evidences show that TLRs expression is not restricted to APC but they characterize other cellular population like T regulatory cells (Tregs). Their role on Treg cells population will be discussed later in paragraph 4.2.

### 3.2.2. TLR2

TLR2 recognizes components from a variety of microorganism, as lipoproteins from Gram-negative bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria. TLR2 cooperates with TLR1 and TLR6, and its dimerization with other TLRs occurs constitutively or in response to ligand stimulation. TLR2 triggering culminates in the activation of NF- $\kappa$ B transcription factor through MyD88 dependent pathway.

### 3.2.3. TLR4

TLR4 is the first mammalian TLR identified, its ligand is LPS from Gram-negative bacteria. Recognition of LPS requires adaptor proteins, LPS firstly binds to soluble LPS-binding protein (LBP) and then to CD14, preferentially expressed on monocyte surface. Once LPS binds CD14, LBP dissociates and LPS can be recognized by TLR4. Moreover MD-2 protein is useful to increase the binding affinity of the LPS-CD14 complex with TLR4. TLR4 signaling pathway can progress through a MyD88 dependent or independent pathway.

### 3.2.4. TLR5

TLR5 binds flagellin which is the primary component of flagellar, a highly complex structure that extends out from the outer membrane of Gram- bacteria. Flagellin elicits a strong immune response.

### 3.2.5. TLR7 and TLR8

TLR7 and TLR8 are localized in the intracellular endolysosomal compartment, they are specialized in recognizing nucleic acids. TLR7 and TLR8 are activated by single stranded RNA. TLR7 is preferentially expressed on plasmacytoid dendritic cells (pDC), B cells and monocytes while TLR8 is expressed by monocytes and DCs. TLR7 and 8 activation proceeds through the MyD88 dependent signaling pathway.

Experimental evidences show that TLR7 and TLR8 stimulation with specific agonists results in the induction of a different cytokine milieu: TLR7 activation results in a strong production of type I interferon while TLR8 activation provokes the release of TNF $\alpha$ , IL12, IL1 and IL6. [40, 41]

### 3.2.6. TLR9

TLR9 is localized in the intracellular endolysosomal compartment, it's activated by unmethylated CpG motifs within single strand DNA. TLR9 is expressed on pDC, B cells and monocytes/macrophages. CpG DNA is recognized in the endosome following nonspecific uptake into the cells and the activation of TLR9 leads to the induction of MyD88 dependent signaling pathway. Stimulation of TLR9 triggers B-cell proliferation and secretion of antibodies, secretion of Type 1 cytokines and chemokines. [41]

## 3.3. Role of pDC in HIV infection

Recognition of microbial components by TLRs triggers the activation of both innate and adaptive immunity. The signals for adaptive immunity are mainly provided by dendritic cells.

pDC represent 0.2-0.5% of circulating PBMC and following activation they are able to produce large amount of IFN $\alpha$  and pro-inflammatory cytokines like TNF $\alpha$  and IL6. DCs can be subdivided in two subpopulations, mDC and pDC. pDCs are involved in immune response to viral infections. The term "plasmacytoid" refers to

their plasma cell-like morphology, resulting from an abundant cytoplasm with well-developed endoplasmic reticulum.

pDCs lack markers of PBMC lineages (CD3 for T cells, CD19 for B cells, CD14 for monocytes, CD56 and CD16 for NKs) but they are positive for CD123 (IL3-R $\alpha$  chain) and HLA-DRII.

Immature dendritic cells are localized in peripheral tissue, they have high capacity of endocytosis which allow them to easily uptake the antigens. Microbial recognition induces DC maturation, mature DC migrate to the draining LN where they can present the antigen associated to MHC to naïve T lymphocytes. Naïve T lymphocytes require two signal for their activation: the first one is the antigen-specific signal coming from the binding of TCR with peptide presented by MHC while the second signal is provided by costimulatory molecules CD80 and CD86 that are up-regulated on DC surfaces subsequently TLR activation. Once CD4<sup>+</sup> T cells are activated they differentiate into effector Th1 or Th2 cells.

Activated tissue pDC migrate in the LN where they can interact with other cell types thus favoring HIV transmission. Homing to LN is guaranteed by the fact that HIV up-regulates the expression of CCR7.

pDCs represent the linkage between innate and adaptive immunity, they influence T cell polarization, humoral immunity and immune tolerance. pDCs are able to induce different T cell responses according to the signals that they transmit, virus-stimulated pDCs induce naïve T cell to produce IFN $\gamma$  and IL10 while in presence of IL3 they stimulate Th2 differentiation. HIV-specific pDCs can induce the differentiation of Treg cells from naïve CD4<sup>+</sup> T cells thanks to the upregulation of IDO expression [42], a gene whose transcription is dependent on TLR7 signaling pathway. Induction of Treg development, it's useful to control immune activation.

Activated pDCs exert antiviral activity through the production of type I interferon (IFN $\alpha$  and IFN $\beta$ ) but they are also able to secrete pro-inflammatory cytokines. The production of type I interferon is mediated by the transcription factor IRF7, which is constitutively expressed at high level by pDC, after the activation of TLR7/8 pathway, IRF7 is translocated into the nucleus where it regulates the expression of IFN $\alpha$  and pro-inflammatory cytokines genes. Activated pDCs show a typical phenotype, they up-regulate the expression of costimulatory molecules such as CD80 and CD86, MHC I, MHC II and CD40. IFN $\alpha$  works in an autocrine and paracrine way because it acts on target cell with an antiviral mechanism and it is also a survival factor for the same pDCs that produce it [43, 44].

Furthermore IFN $\alpha$  might induce apoptosis in CD4<sup>+</sup> T cells thanks to different mechanisms, it's well known that it up-regulates the expression of p53, a nuclear protein that regulates apoptotic pathway in cycling cells, and of TRAIL/Fas mediated pathways.

Prolonged pDCs activation and type I interferon production have been associated with the upregulation of activation markers on CD8<sup>+</sup> T cells, the suppression of functional T-cell responses by Treg cells and the progressive depletion of CD4<sup>+</sup> T cells through apoptotic mechanisms such as TRAIL or Fas/FasL. Experimental evidences suggest that the induction of apoptotic pathways due to IFN $\alpha$  action might explain the persistent cell depletion observed during HIV infection. Moreover type I interferons exert a double action: they play a positive effect in destroying infected CD4<sup>+</sup> T cells that have become infected by infectious HIV-1 while but when pDCs and CD4<sup>+</sup> T cells bind non infectious HIV-1, uninfected pDCs will produce IFN $\alpha$  and CD4<sup>+</sup> T cells will express TRAIL and DR5, resulting in the immunopathogenic apoptosis of uninfected T helper cells. Because the frequency of circulating noninfectious HIV-1 particles exceeds that of infectious HIV-1 by a factor of 10<sup>2</sup>-10<sup>4</sup>, the binding of noninfectious HIV-1 to CD4 on both pDC and CD4<sup>+</sup> T cells would be expected to occur much more frequently than the binding of infectious virus to these important antiviral cells. Therefore, the apoptosis of HIV exposed but uninfected CD4<sup>+</sup> T cells would outpace the apoptosis of productively infected CD4<sup>+</sup> T cells. This predominant death of uninfected CD4<sup>+</sup> T cells would provide a distinct advantage for the virus, by destroying T helper cells that are specific for opportunistic pathogens, as well as those that recognize HIV-1 antigens. It has been demonstrated that this effect on CD4<sup>+</sup> T cells might be unique to HIV-1 because this virus uses the CD4 molecule as its primary receptor and also that only the binding of viral gp120 to cellular CD4 is required to induce IFN- $\alpha$ . [45, 46, 47]

The persistence of HIV virion provides a chronic stimulus for DCs, pDCs express CD4 receptor, CXCR4 and CCR5 co-receptors so they are ideal target of HIV virus and they can be infected by both R5 and X4 strains of HIV-1. Viral gp120 interacts with CD4 receptor and HIV is accumulated in early endosomal compartments where it starts its replication.

pDCs express TLR7, TLR8 and TLR9 at intracellular level, thus they can be activated by the interaction of viral RNA by TLR7. [38, 48]

In HIV infection, pDCs are responsible for different effects. First of all they produce high quantities of IFN $\alpha$ . Even if the role played by IFN $\alpha$  in HIV infection remains controversial, it's thought that in the acute phase, high level of IFN $\alpha$  provides a protective antiviral effect and sustain the adaptive immune response while during chronic phase, high levels have been correlated with both T cell activation and disease progression.

The production of high level of IFN $\alpha$  in response to high level of HIV virions, might be considered beneficial as it upregulates TRAIL expression in uninfected T cells and thus apoptosis. In addition as TRAIL expression



is upregulated even on the pDC surface they can kill both HIV-infected and uninfected T CD4<sup>+</sup> cells as previously explained. [49]

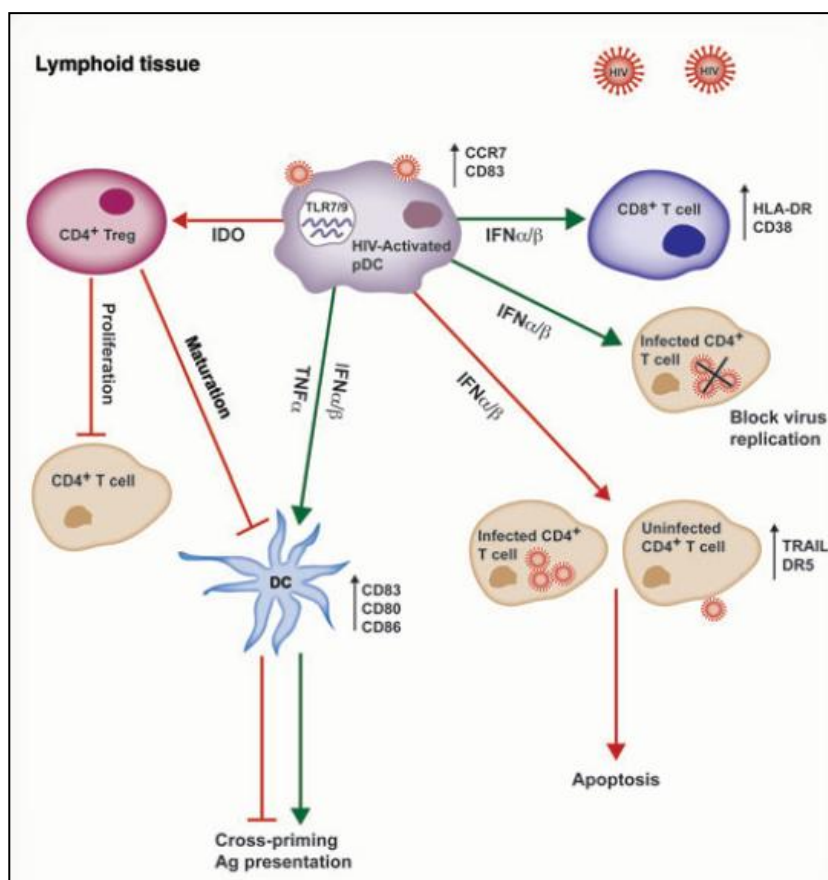


Figure 10. The role of pDCs during HIV infection. Red arrows indicate detrimental effects while green arrows indicate beneficial effects

HIV-infected patients are characterized by low level of circulating pDC and reduced ability to produce IFN $\alpha$ , it has been suggested that this depletion might be due to the homing to lymphoid tissue in order to control and fight the infection. However in LN of HIV infected patients it hasn't been detected a raise of pDCs; maybe because of a contemporary increased pDCs death rates. However despite a decrease in circulating pDCs, there's an over-expression of IFN $\alpha$ ; in fact in a study conducted by Lehmann [50], it has been shown that HIV-infected patients present 100-fold higher levels of IFN $\alpha$  than uninfected control but probably with reduced antiviral properties. As previously said, CD4-gp120 interaction induces the production of IFN $\alpha$ , it should be noted that soluble gp120, in monomeric or trimeric isoform, displays an inhibitory effect on IFN $\alpha$  production by pDCs. Moreover chronic stimulation of pDCs with IFN $\alpha$  may lead to hyporesponsiveness of these cells upon exposure to HIV particles of TLR9 ligand. [51]

Moreover, it has been demonstrated that CD4<sup>+</sup> T cell and pDC count are positively correlated, suggesting that HIV-induced mechanisms may lead depletion of both cellular populations, and between pDC count, viral load and disease progression.

Clinical studies have shown that women display a faster progression to AIDS than men, it's thought that strong pDCs activation and sustained production of type I interferon might be the causes. [43, 46]

#### 4. HIV AND IMMUNE SUPPRESSION: ROLE OF TREG CELLS

Treg cells control immune responses to self- and non self antigens and they play an important role in maintaining the right balance between immunity and tolerance. They are essential for maintaining peripheral tolerance to self antigens, preventing autoimmune diseases and limiting chronic inflammatory diseases.

Treg cells operate by modulating the intensity and the quality of immune reactions and they are identified by specific superficial markers.

Treg cells belong to CD4<sup>+</sup> T lymphocyte subset, so they are CD4 positive and they express CD25 and FoxP3. CD25 is the marker for alpha chain of IL2 receptor while FoxP3 is a nuclear transcription factor fundamental for Treg cells differentiation. CD25 is expressed also by effector T cells but at lower level, that's why Treg cells are defined as CD25<sup>high</sup> cells. FoxP3 and IL2 receptor are fundamental for the development, function and survival of Treg cells and mutations or polymorphisms in the genes encoding this molecules predispose to autoimmune disease: for example mutations in FoxP3 gene provoke the IPEX syndrome. [52] There are two main subsets of Treg cells: naturally occurring Treg cells (nTregs) and induced/adaptive Treg cells (iTregs).

nTregs represent 2-5% of the CD4<sup>+</sup> T lymphocytes, they originate in the thymus and they are present from birth, they play a pivotal role in the maintenance of immunological self-tolerance and in the modulation of immune responses. nTregs recognize self antigens because of their development and selection in the thymus but also self antigens of injured tissue released in acute disease [53]

nTregs show an activated/memory status and also high level of CTLA-4, GITR, CCR4 and CD62L (L-selectin). The main ligands of CCR4 are CCL17 and CCL22 which are secreted by mature APC and the circuit CCR4/CCL17-CCL22 is involved in homing of nTregs to secondary lymphoid organs and inflamed areas. CD34 and MadCam1 are ligands of CD62L expressed by endothelial cells, their mutual binding is fundamental for extravasation of nTregs through high endothelial venules to peripheral inflamed tissue and also to homing them to lymph nodes. By contrast, in a study directed by Benoist [54] it's affirmed that CCR4 isn't expressed by thymic Treg cells but only by extralymphoid Treg cells, thus further investigations are required.

Several studies are trying to define their generation but until now it seems that they require both TCR ligation and strong co-stimulation through CD28 to develop from immature precursor. [55, 56, 57]

iTregs develop in periphery from CD4<sup>+</sup> T cells following antigenic stimulation during inflammatory processes and unlike nTregs they don't require CD28-mediated co-stimulation to develop or function.

iTregs can be subdivided into two different subsets according to the specific cytokine production: Tr1 Th3.

Tr1 and Th3 share the mechanism of development: both of them require a repeated TCR stimulation and the presence of a specific cytokine environment, IL10 and TGFβ for Tr1 and Th3 respectively.

Once differentiated, Tr1 are able to produce IL10 and TGFβ. Th3 produce high levels of TGFβ and less quantities of IL4 and IL10, considering their localization at mucosal sites, they are necessary to control immune response at mucosal level, their absence could determine the onset of autoimmune or inflammatory disease in the gut. [55, 58]

The key difference between nTregs and iTregs is the specificity of TCR in antigen recognition and signals involved in generation and maintenance of these cells. TCR of nTregs recognize self antigens in the thymus with high avidity while iTregs work mainly in periphery and they show a TCR pool similar to the one that characterizes conventional T cells.

To date it was reported that the generation and survival of Treg cells are regulated by TGFβ and IL2 and by costimulatory molecules belonging to the B7:CD28 family. Recently two groups have tried to clarify the differentiation pathway of Treg cells. They suggest a two-step process in which CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>-</sup> T cells, repeatedly stimulated through their TCR, are exposed to IL2 leading to the complete differentiation in FoxP3<sup>+</sup> cells. They suggested a key role for IL2 in the complete differentiation of Tregs and the minor role for TGFβ which should work in the neonatal period for the development of thymic Treg cells.

By contrast in another work it was suggested that IL2 and TGFβ are important for iTregs development but not for nTregs differentiation as mice deficient for these two cytokines were still able to produce nTregs. So important signals to obtain Foxp3<sup>+</sup> iTregs could be both IL2 and TGFβ.

Co-stimulatory molecules such as CD28, CTLA-4 and ICOS play an essential role in the differentiation steps of both nTregs and iTregs. Mice models show that a strong CD28 co-stimulation is fundamental for nTregs differentiation while CTLA-4 favors iTregs generation. ICOS has been recently included in co-stimulatory signals useful for Tregs differentiation. [59, 60]

The development of Treg cells depends on several factors and the fine regulation of their actions is fundamental. Apart from the role played by cytokines, specific transcription factor are also important and show different effects. Transcription factor NF-κB, and the pathway that it activates, is important for the

differentiation of Tregs. Studies conducted in mice put in evidence that the absence of molecules involved in NF- $\kappa$ B activation leads to the lack of Treg cells and they suggest that the strength of the NF- $\kappa$ B pathway sets a threshold for thymocyte differentiation in Treg or conventional CD4<sup>+</sup> T cells. The signal of proper intensity is required for FoxP3 expression induction and nTregs development. [61]

By contrast Akt pathway has been recently defined like an important suppressor of thymic differentiation of Tregs because it down-regulates the expression of TGF $\beta$ . [54]

Ox40 is an other member of TNF-TNFR superfamily that displays effect on Treg cells development, studies conducted in mice show its negative action on Treg cells generation and function. Ox40 signaling pathway blocks iTregs generation and it abolishes the capability of nTregs to block T cell proliferation and cytokine secretion. [60]

The development of Treg cells depends on Foxp3 expression which is finely regulated by different factors. IL12, IFN $\gamma$  and pro-inflammatory cytokines like IL1 and IL6 are able to influence Foxp3 expression, transforming Treg cells in other type of effector cells. The ability of these cytokines to transform Treg cells in other effector cells is related to specific activated STAT and nuclear transcription factors.

Above of all, in mice it has been demonstrated that IL6 combined with TGF $\beta$  reduces the suppressor activity of nTregs and they are transformed in pro-inflammatory Th17 cells. By contrast iTregs are resistant to IL6-induced Th17 transformation. IL6 is one of the principal mediator during inflammation, its ability in transforming Treg cells in pro-inflammatory cells could have an important effect for the outcome of diseases. IL6 different effect on nTregs and on iTregs might play a precise role in adaptive immune response. Following microbial invasions, nTregs conversion in Th17 activates immune responses capable to eliminate pathogens while in a second time the activity of iTregs is useful to terminate antigen-specific immune response and to avoid an exaggerated reaction of the immune system. IL1 exerts a similar activity to IL6, while IL12 and IFN $\gamma$  promote Th1 cell differentiation. IFN $\gamma$ , in particular, induces STAT1 activation and Tbet expression, nuclear transcription factor characteristic of Th1 cells. These are preliminary works that give an idea of the plasticity of immune cells anyway further studies are needed to gain more detailed information. [60, 62]

#### **4.1. Mechanism of action of Treg cells**

Treg cells use different mechanisms to control immune response (fig.11).

##### **4.1.1. Direct cell-to-cell contact**

The first mechanism involves cell elimination by cell-to-cell direct contact at the site of inflammation, iTregs use Fas/Fas-L pathway to induce apoptosis in target cells while activated nTregs express granzyme A and so they kill the effector cells through perforin/granzyme action after the adhesion of CD18. The best known and studied mechanism of cytolysis is the one mediated by granzyme anyway the involvement of TRAIL/DR5 pathway in promoting Treg-mediated apoptosis seems to be as much important. Treg cells express high level of TRAIL while activated T cells express increasing level of DR5 and in experimental models it has been demonstrated that using a blocking antibody for DR5, apoptosis and consequently the suppressor activity of Tregs are significantly reduced both in vitro and in vivo. Moreover, nTregs can suppress effector T cells through the intercellular transport of cAMP via gap junctions. Treg cells are characterized by high level of cAMP because the enzyme (cAMP-cleaving enzyme phosphodiesterase 3B) responsible of its degradation is strongly reduced. In T lymphocytes, endogenous cAMP displays different effect like inhibition of cell activation, cytokine production and it interferes with the activation of molecules (i.e. Ras and Rap1) involved in cell proliferation. When Tregs interact with target cells through gap junctions, cAMP can enter into lymphocytes where cAMP levels increase resulting in immune suppression. Reduction of adenyl cyclase in Tregs, resulting in low level of cAMP, abrogates Tregs suppression. [63]

##### **4.1.2. Inhibition of cytokine production by target cells**

The second mechanism is based on the inhibition of proliferation and cytokine production by target cells. IL2 is a cytokine, mainly produced by activated CD4<sup>+</sup> T cells, that plays a pivotal role in survival and proliferation of lymphocytes. It acts as an autocrine and paracrine growth factor and an alteration in its availability alters cell homeostasis. Upon TCR stimulation, activated antigen-specific-effector T cells start to synthesize and secrete IL2 and to up-regulate CD25 expression, receptor of IL2. Clonal expansion of antigen-specific T and B lymphocytes, induced by IL2, is an important step in immune response because it allows immune system to quickly eliminate non self antigens. In addition IL2 is able to control the immune responses through the action of Treg cells. Treg cells constitutively express IL2R- $\alpha$  on their surface so they are always able to receive IL2 signals and they represent a way to block the action of antigen-specific cells when the infection is completely eradicated.

So Treg cells undergo the action of IL2 and they survival seems to depend on it but they can also block or reduce the quantities of IL2. Tregs could be the main acceptor of IL2 thanks to the high expression of CD25,

they subtract this important growth factor to T cells inducing them into a state of anergy. When the absence of IL2 stimulus lasts for a long time T cells can be induced to apoptosis.

#### 4.1.3. Modulation of cytokine environment

The third mechanism of Treg action involves the modulation of the cytokine environment through direct secretion of IL10 and TGF $\beta$ . TGF $\beta$  performs different actions: it reduces cytokine secretion by activated CD4<sup>+</sup> T cells blocking IL2 production and it induces IL10 production in effector cells, a cytokine that influences negatively effector T cell function. TGF $\beta$  exists in two forms, a membrane bound and a secretory one. Membrane bound TGF $\beta$  suppresses cell activity through cell-to-cell contact while the secretory bind its receptor on T cells and it induces apoptosis through an extrinsic apoptosis pathway.

IL10 down-regulates IL12 production by APC and inhibits the development of Th1 responses, it shows a synergic effect with TGF $\beta$  and it upregulates TGF $\beta$  receptor expression [52, 64, 65]

At last Treg cells can induce metabolic disruption in target cell. Depletion of IL2 can also be included in this type of action considering that its absence causes starvation in active effector T cell and consequently their apoptosis.

A pivotal role is played by adenosine nucleosides which bind to adenosine receptor 2A. Adenosine nucleosides is a component of nucleic acids coming from the action of specific ectonucleotidases CD39 and CD73 expressed on Tregs cell surface. CD39 degrades nucleoside tri- and diphosphates like ATP into adenosine monophosphate (AMPs) and the subsequent action of CD73 catabolizes the conversion of AMP into adenosine. Adenosine-adenosine receptor 2A binding blocks effector T cell functions and it abolishes the expression of pro-inflammatory cytokines like IL1 and IL6, the proliferation of Th1 cells and the production of TNF or IFN $\gamma$ . Considering that the presence of ATP within the extracellular environment indicates tissue damage and represent a pro-inflammatory marker, the generation of adenosine and its interaction with adenosine receptor 2A expressed by effector T cells favors the activity of Tregs in order to suppress immune responses. [63, 64]

#### 4.1.4. Suppression by targeting dendritic cells

Treg cells might also modulate the maturation and function of DCs, which are required for the activation of effector T cells. Treg interacts with DC and suppress their activity with a mechanism involving the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), which is constitutively expressed by Treg cells. Studies have suggested that lymphocyte-activation gene 3 (LAG3) may block DC maturation. LAG3 binds MHC II molecules and it has a negative regulatory T-cell intrinsic function and is required for maximal Treg-cell suppression. Binding of LAG3 to MHC II expressed by immature DCs induces an immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signaling pathway that suppress DC maturation and their immunostimulatory capacity. Treg cells are able to modulate DC function, they could condition DCs to express indoleamine 2,3-dioxygenase (IDO) resulting in the suppression of effector T cells through a mechanism dependent on interactions between CTLA4 and CD80/CD86. [64]

Cell-to-cell contact is the mechanism of action mainly used by nTregs while iTregs use indifferently all the mode of actions.

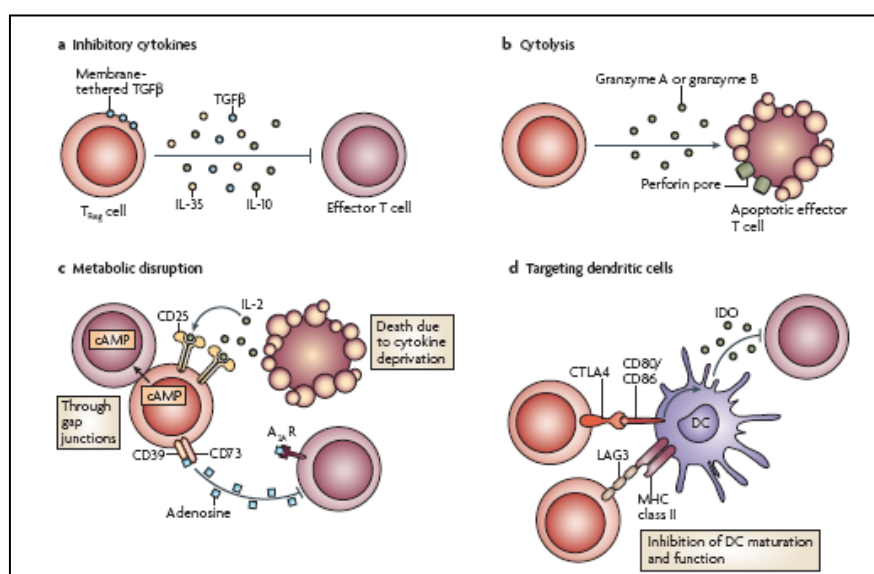


Figure 11. Basic mechanism of action used by Treg cells.

## 4.2. TLR expression on Treg cells

TLRs are expressed by different cellular type and recently they have been discovered also on Treg cells. Treg cells express higher level of TLR4, TLR5, TLR7 and TLR8 in comparison with effector CD4<sup>+</sup> T cells. TLRs ligands can exert a positive or negative effect on Treg cells activity. Activation of TLR5 provokes an increase in Treg immune suppression while activation of TLR2 and TLR4 leads to cells proliferation and augments Treg activity. The influence on Treg number and activity was confirmed in studies conducted with animal models, mice knockdown for TLR2 or TLR4 were characterized by fewer Treg cells than control mice and the administration of TLR2 or TLR4 ligands (respectively bacterial lipoprotein and LPS) in wild type mice increases Treg cell number. Moreover the treatment with LPS increases the immunosuppressive ability of Treg by 10-fold. By contrast TLR8 and TLR9 activity antagonizes Treg immune suppression, TLR8 acts directly on Treg while TLR9 attenuates suppressor activity of Tregs and at the same time it induces expansion of effector T cells rendering them more resistant to the regulation exerted by Tregs. It's supposed that negative effects on Treg-mediated immune suppression could be realized through an alteration in Foxp3 expression but how it happens still remains unclear. The modulation of Treg activity can be carry out with an indirect mechanism involving DCs. TLR-induced activation of DCs leads to the secretion of soluble factor like IL6 or IL1 which, as previously described, display negative effects on Treg activity. [65, 66, 67, 68]

## 4.3. PD1 pathway, Treg cells and their involvement in HIV infection

PD1 (Program Death receptor 1) is an inducible receptor expressed on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NKT, B cells and activated monocytes. It's a monomer with a cytoplasmic domain containing two tyrosine-based motif. PD1 transduces an inhibitory signal when crosslinked with TCR or BCR.

It represents a discriminatory marker for the identification of naïve and activated Treg cells, according to its cellular localization. Naïve Treg cells retain PD1 in intracellular compartments and when they are stimulated via TCR, PD1 is exposed on their surfaces. Cellular ligands of PD1 are PDL1 (B7H1) and PDL2 (B7DC). PD1 is expressed on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NKT cells, B cells and monocytes upon activation. PDLs differ in their expression, PDL1 is expressed much more broadly than PDL2. PDL1 is constitutively expressed and moderately induced upon inflammation on B cells, DCs and macrophages while PDL2 is found on activated DCs and macrophages. PD1-PDL pathway regulates the immune response in both lymphoid and non-lymphoid organs. [69, 70]

Treg cells are also characterized by the presence of both PD1 and PDL1, anyway less is known about their influence on Treg cells function. To date several efforts have been made to clarify the effects of PD1-PDL1 pathway in the regulation of T-cell responses, it's well established that Tregs, through PD1, inhibit T cell proliferation and cytokine production.

### 4.3.1. PD1-PDL1 pathway in HIV infection

PD1 pathway is involved in several cellular processes, it plays a pivotal role in both central and peripheral tolerance where it avoids the onset of autoimmune diseases and it ensures the maintenance to self-tolerance.

Furthermore it's implicated in infectious diseases, in fact several groups studied the existence of differences in PD1 pathway involvement in various infectious disease. It has been demonstrated that the establishment of an infection caused by non persisting virus displays different effects on PD1 expression than that seen in association with chronic infection.

PD1 expression on T cells infected by non persisting virus is lower compared to the one on T cells infected by virus like HIV, HBV and HCV that might cause chronic infection. The higher expression of PD1 on virus-specific T cells can influence antiviral immune response limiting their function and proliferation. The persistent immune suppression leads the cells in a state of exhaustion, characterized by inability to proliferate, to produce cytokines and consequently to direct immune responses. Persistent immune exhaustion may lead T cell apoptosis through the mechanism of AICD. It has been demonstrated that blocking the PD1-PDL1 pathway with specific antibodies reverts the exhausted status of T cells and restores effector function. PD1 also might serve as a useful marker to indicate the degree of T-cell exhaustion and disease severity (fig.12). The level and percentage of PD1 expression on HIV-specific lymphocytes correlates with the viral load and declining of CD4<sup>+</sup> cell count. Moreover it's evident that HAART therapy reduces PD1 expression on HIV-specific lymphocytes so it can be assumed that high viral load stimulates PD1 expression and functional exhaustion. Similarly PD1 might be a marker for disease progression in HCV infection, as it's upregulated on HCV-specific CD8<sup>+</sup> T cells and it declines in patients with resolving infection but it remains high in patients who develop chronic infection. [71, 72, 73]

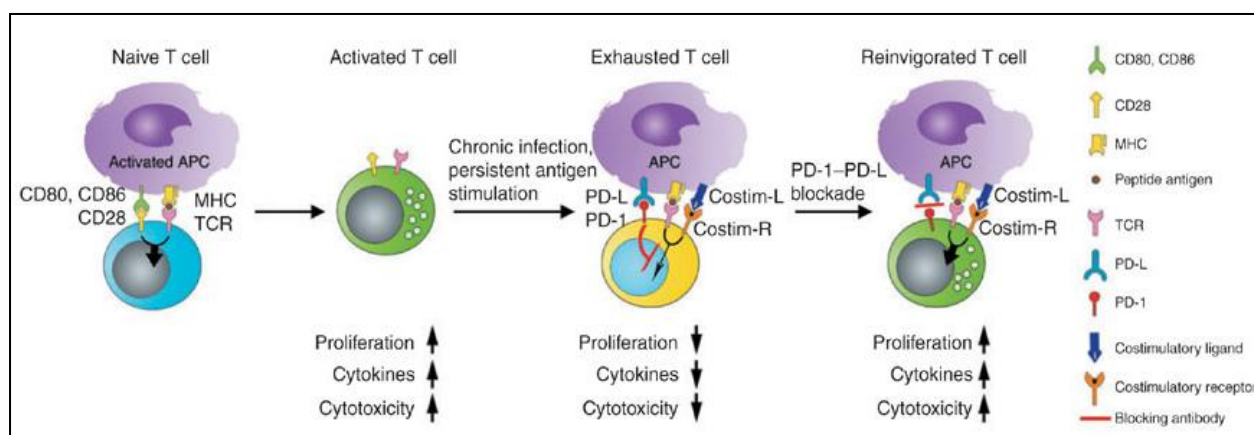


Figure 12. PD1/PDL1 pathway and its role in immune exhaustion.

Clerici's group analyzed PDL1 expression during HIV infection and they proposed PDL1 as a marker of disease progression because of its increasing expression during the course of infection. The data obtained demonstrated that PDL1 was up-regulated on PBMC of HIV-infected patients and that this increase was associated with a reduced CD4 cell count and an augment of HIV plasma viremia. Furthermore HIV infection leads to an impairment of immune responses with an increased secretion of type 2 cytokines like IL10. IL10 displays immune suppressive actions towards Th1 activation and it enhances HIV entry into target cells through the up-regulation of CD4 and CCR5. Concomitantly HAART therapy was highly effective in enhancing CD4<sup>+</sup> T cell count and in reducing IL10 production, this was positively correlated with the reduction in PDL1 expression observed. That findings supports the idea of the direct involvement of PD1-PDL1 pathway in the degree of HIV infection. [74]

#### 4.3.2. Treg cells in HIV infection

Several factors have been implicated in the impaired immune response that characterizes HIV infection. Treg cells-mediated immune suppression plays an essential role in the incapability of immune system to overcome the infection. Long efforts have been made to define how and when this process can influence the disease.

The initial phase of infection is characterized by a strong immune activation in primary sites of infection, the mucosal tissues, that causes CD4<sup>+</sup> T cell depletion and disease progression. Because of this chronic immune activation, the expansion of Treg cells can be interpreted as a protective mechanism trying to control and attenuate the HIV-induced chronic immune activation.

Treg cells express both CXCR4 and CCR5 co-receptors and so they are suitable target for HIV-1 infection. In particular, *in vitro*, it has been discovered [75] a greater susceptibility to HIV infection by X4 viral strain than by R5 strain, this allows to suppose that in the early phase of infection, typically characterized by R5 viral strain infecting mucosal macrophage and CCR5-expressing DCs, Treg cells could be protected from HIV-1. HIV-1 might display several effects on Treg functionality and localization. HIV-1 replicates successfully in activated Treg cells while in resting Treg cells it results in abortive infection, moreover it has been demonstrated that HIV-1 infected Treg cells have an increased expression of homing receptors, CD62L and  $\alpha\beta 7$ , to peripheral lymph nodes and mucosal tissue. These two receptors are expressed also by conventional CD4<sup>+</sup> T cells and cross-linking of  $\alpha\beta 7$  induces apoptosis in these cells, by contrast *in vitro* Tregs resulted to be resistant to apoptosis. Treg cells resistance to apoptosis induced by  $\alpha\beta 7$  cross-linking worsen the immune dysfunctions characterizing HIV infection. [76]

Survival of Treg cells is also guaranteed by the induction of bcl-2 expression and because of the up-regulation of Foxp3 expression.

Treg can directly interact with HIV through Foxp3 action and CD4-gp120 interaction.

Foxp3 is able to influence the expression of several HIV gene. FoxP3 is a transcription factor containing prolin-rich amino terminal domain reported to function as a nuclear factor of activated T cells (NF-AT) and NF- $\kappa$ B binding domain, a central region important for protein-protein interactions and a carboxyl-terminal forkhead domain required for nuclear localization and DNA-binding activity. FoxP3 with its aminoterminal domain might establish protein-protein interactions with NF-AT and NF- $\kappa$ B factors and acting like a transcriptional repressor. Regarding HIV gene expression, it is dependent on NF- $\kappa$ B activity and it's regulated by two NF- $\kappa$ B sites located within LTR sequences. Considering that LTR sequences play the role of enhancer of HIV genes transcription, when FoxP3 interacts with them binding to the NF- $\kappa$ B sites through its amino terminal domain it suppresses the viral transcriptional activity.

As Foxp3 interferes with the activation of NF- $\kappa$ B pathway, Treg cells might be resistant to HIV replication, moreover the negative effect played by Foxp3 on NF- $\kappa$ B could protect Treg cells from HIV-mediated cell death. The same effect played by FoxP3 has been observed with HTLV-1 infection, in this case its inhibitory action is directed on the CREB pathway. [77] By contrast a study by Holmes et al. assumes that FoxP3 enhances HIV-1 gene expression by modulating chromatin structure and NF- $\kappa$ B activity. [78]

It can be supposed that Foxp3 interacts with distinct HIV LTR sequences and, according to type of interaction and cellular type, it's possible to obtain repressor rather than inducing effect. Anyway further investigations are required. [79]

It has been verified that, thanks to the interaction between CD4 and gp120, HIV is directly responsible of increased FoxP3 expression, survival and immune suppressor activity of Treg cells. Gp120 and Tat play a pivotal role because, as previously described, gp120 increases the expression of FoxP3 and inhibits Treg apoptosis while in DCs Tat increases CTLA-4 expression and the suppressive strength of Treg cells.

This explains why during HIV infection Treg cells depletion or impairment are not observed despite the persistent impairment of immune responses able to eradicate the infection. [76, 79, 80]

Experimental evidences, coming from a study directed by Tsunemi, suggested that HIV infection leads to the induction of Treg that inhibits antiviral immune responses in early stage disease, while in progressive disease, Treg function is decreased which results in immune activation and CD4<sup>+</sup> T cell lymphocytopenia.

In particular, it has been underlined the interplay between HIV infection and Treg activity. Patients characterized by a progressive HIV infection, identified by high level of plasma HIV RNA (>50 copies/ml), showed an increase in Treg cells frequency and this was inversely correlated with CD4<sup>+</sup> T cell count but may be these Treg cells has lower immune suppressive capacity seen the progression of the disease and according to data obtained. [81]



## 5. HIGHLY ACTIVE ANTIRETROVIRAL THERAPY – HAART

Several efforts have been made to eradicate HIV virus and up to now many goals have been reached. Each step of HIV replication represents a potential target for antiretroviral drugs and so they are classified according to the viral life-cycle phase they inhibit. Drugs are divided into two main categories: drugs that inhibit the virus replicative cycle and drugs that interfere with the adhesion process (fig. 13).

Drugs able to influence the replicative cycle of HIV include

- Inhibitors of the Reverse Transcriptase, nucleotide (NRTI) and non nucleotide (NNRTI)
- Integrase strand transfer inhibitors (I) of integration of proviral DNA into cellular DNA
- Inhibitors of assembly, maturation or fusion of viral particles: protease inhibitors (PI), fusion inhibitors (FI), entry inhibitors (EI), maturation inhibitors (MI) and chemokine R5 inhibitors (CCR5 inhibitors).

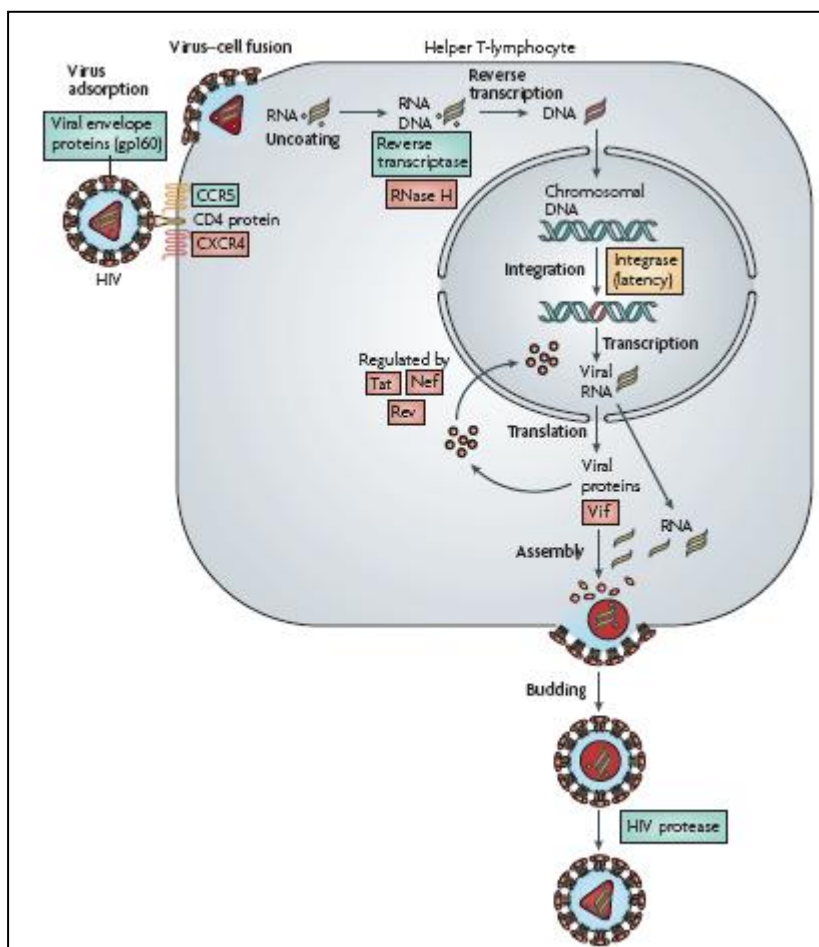


Figure 13. Replication cycle of HIV with indicated targets of antiretroviral drugs.

**NRTIs** are the first class of antiretroviral drugs developed and they act on HIV reverse transcriptase as competitive inhibitors by blocking the addition of nucleosides to the DNA chain during reverse transcription of the HIV genome. Actually commercial drugs include: Abacavir (ABC), Didanosine (DDI), Emtricitabina (FTC), Lamivudina (3TC), Stavudina (d4T), Tenofovir (TDF) and Zidovudine (ZDV).

**NNRTIs** interfere with the activity of RT through a non competitive mechanism, they block hydrophobic pocket of p66 subunit of RT inducing a conformational change that alters the catalytic site of the enzyme. The main drugs belonging to this category are Delavirdina (DLV), Efavirenz (EFV), Etravirine (TMC125) and Nevirapine (NVP).

**INSTIs**, integrase strand transfer inhibitors act on viral integrase, a protein involved in the integration of viral genome in the DNA of the host cell, Raltegravir (RAL) is one of these.

**PIs** bind to the catalytic site of HIV protease blocking its ability in originate viral proteins from the cleavage of precursor protein. HIV protease regulates the generation of mature core and capsid proteins and consequently the assembly of infectious virions, the absence of protease activity leads to the production and release of immature, non infectious virions. Atazanavir (AZT), Darunavir (DRV), Fosamprenavir (FPV),



Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV) and Tipranavir (TPV) are drugs belonging to this category.

**IFs** work in competition with gp41 blocking fusion and entry process of the virus into host cells, Enfuvirtide (ENF) belongs to this group.

**CCR5 inhibitors** block the entry of HIV into CD4 T cells by binding to CCR5 protein. Maraviroc (MVC) is one of this.

NRTIs NNRTIs and PIs are the three main antiretroviral classes currently used to treat HIV-1 infection in both treatment-naïve and treatment-experienced patients. Administration of a NNRTI or NRTI alone is able to reduce plasmatic level of viral RNA but not to block disease progression; while a therapy based on a single PI isn't recommended given the high risk of early onset of mutant strains of the virus.

Nowadays the therapeutic approach is based on a combined therapy. The combined therapy is based on the administration of drugs with synergistic effects and it's called Highly Active AntiRetroviral Therapy (HAART). Current international guidelines suggest to combined 2 NRTI and 1 NNRTI or 2 NRTI and 1 PI because are combinations well tolerated and with less drug interaction, furthermore the PI might be boosted with Ritonavir to increase the power of treatment. Ritonavir is poorly tolerated because of its high toxicity and so it has to be administer in sub-therapeutic dose.

HAART is an effective way of treatment to control viral replication and to increase CD4<sup>+</sup> T cell count.

Several parameters are monitored to decide when to start HAART therapy administration but the main ones are CD4<sup>+</sup> T cell count (<350 cells/ml) and viral load. Several clinical trials have been conducted to elucidate which are the positive and negative parameters implicated in a successful therapy: a decrease in CD4<sup>+</sup> T cell is an important factor that should always be considered in initiating an antiretroviral therapy, anyway it must be underlined that there isn't a threshold at which starting it.

Therapy is recommended regardless of CD4<sup>+</sup> T cell count in the following settings: increased risk of disease progression associated with a rapid decline in CD4<sup>+</sup> T cell count or a plasma HIV-1 RNA level greater than 100.000 copies/ml, older than 60 years, pregnancy, chronic HBV and HCV coinfections, opportunistic infections and heightened risk of HIV transmission (ie, HIV-serodiscordant couples). [82, 83, 84]

Nevertheless initiating the therapy with a higher number of 350 cells/ml CD4<sup>+</sup> T cells might have beneficial effects like maintaining a high T cell count, reducing the risk of HIV-associated complications, coinfections and transmission.

Moreover studies conducted in SIV-infected macaques showed that initiation of early therapy might mitigate the early and profound loss of T cells within gastrointestinal lymphoid tissue and enhance tissue repair critical to restoring the mucosal immune system. This aspect is of key importance in controlling and reducing the immune activation that characterizes HIV infection.

To date in literature have been published only 4 randomized controlled trials. All of them underlined the fact that early therapy reduces viral load, immune activation and increases CD4<sup>+</sup> T cell count. Benefits in early starting HAART have been proved but data regarding virological control after treatment interruption are still conflicting.

Among benefits there are potential disadvantages of early treatment such as HAART-related toxicity and drug resistance important factors in determining the failure of therapy.

To avoid drug resistance, current guidelines suggest to test genotyping drug resistance at the time of HIV diagnosis and before initiation of HAART, this might be useful in choosing the right drug combinations to have positive effects in controlling HIV replication and in inducing T cell recovery. Drug-drug interactions are a specific problem for PIs and NNRTIs, because both classes are metabolized through the cytochrome P450 hepatic metabolic enzyme system and they exhibit inductive or inhibitory activities on specific subsets of this system in complex. Ritonavir is a strong inhibitor of the cytochrome P450 system. [85]

Responsiveness to antiretroviral treatment should be evaluated after 4 weeks and 3 months after initiation of therapy through the control of HIV-RNA levels. Generally HAART display its efficacy in 4-6 months with suppression of viral replication less than 50 copies/ml and an increase of 100-150 cells/mm<sup>3</sup>/year of cell count.

## 5.1. Immune reconstitution with HAART

The kinetic of immune reconstitution with HAART is a two phase process (fig.14).

In the first 2-3- months a reduction in viral load (1-2 log<sub>10</sub> of viral copies) can be observed and usually this is coupled with an increase in CD4<sup>+</sup> T lymphocytes count, with an average rate of 20 to 30 cells/μl monthly. This phase is accompanied by a reduction of circulating pro-inflammatory cytokines such as TNFα, IL6 and MCP-1. This trend is observed each time HAART therapy is initiated independently from the phase of the infection. It has been demonstrated that the increase of CD4<sup>+</sup> T cells is negatively correlated with the viral load before the treatment as well as the extent of CD4<sup>+</sup> T depletion pre-HAART.

The second phase of immune reconstitution occurs over several years and the extent of recovery depends upon the magnitude and sustainability of viral control but not on the extent of CD4 depletion before treatment. CD4<sup>+</sup> T cell count over 200 cell/mm<sup>3</sup>, which guarantees a protection against opportunistic

infections, is reached in 2-4 years of therapy and it depends on the rate of the immune system impairment at the beginning of antiretroviral therapy, the control of viral replication and age.

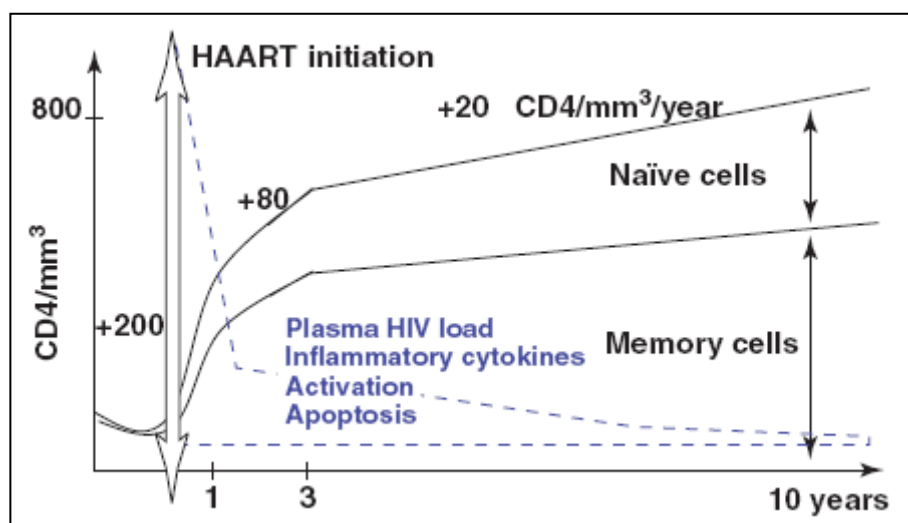


Figure 14. Schematic representation of the mechanism of  $CD4^+$  T cell reconstitution after HAART initiation.

Several studies have demonstrated that most of the initial reconstitution seems to be the consequence of a redistribution of memory  $CD4^+$  T cells from the lymphoid tissue toward the blood compartment, this was confirmed by immunophenotypic analysis showing an increase in the expression of CD45RO and in a down-regulation of adhesion molecules like VCAM-1, ICAM-1 and of CCR7, CD62L lymphoid-homing receptor by  $CD4^+$  T lymphocytes. These adhesion molecules play a role in maintaining  $CD4^+$  T cells into secondary lymphoid organs, as their down-regulation leads to the release of  $CD4^+$  T lymphocytes into the periphery.

Memory  $CD4^+$  T cells are retained in the lymphoid tissue to set immune responses against viral replication, so their redistribution might be correlated with a viral load reduction secondary to HAART therapy.

The repopulation of  $CD4^+$  T pools is a double step process. In the first step the increase is related to memory cells while the second step is up to the  $CD45RA^+$  naïve pool, leading to the achievement of a normal ratio between naïve and memory T lymphocytes.

Experimental evidences explained that the increase of naïve  $CD4^+$  T cells is due to a direct thymic activity and not to conversion of  $CD45RO^+$   $CD4^+$  T cells. The thymic output of naïve  $CD4^+$  T cells can be monitored through TREC level analyses. TREC stands for TCR Rearrangement Excision Circle, they are episomal DNA sequences resulting from TCR gene rearrangement in the thymus, so naïve T cells with high content of TREC refer to recent thymic output. Moreover TREC level is inversely correlated to viral load and it is higher in younger than older HAART-treated patients because in the last one thymic production is impaired.

IL7/IL7R pathway is involved in thymic output too. HAART decreases IL7 level and it has been observed that higher level of IL7 pre-HAART is positively correlated with higher ex novo production of thymic  $CD4^+$  T cells and of TREC level. [86, 87]

Important evidences come out from a study conducted by Tan, he enrolled HIV-infected patients starting antiretroviral therapy at the time of the recruitment and analyzed immunologic and virologic responses after 3 to 9 months from HAART initiation. Tan observed that the type of immunologic and virologic responses in this short period might be predictive of long-term clinical outcomes relative to disease progression. Patients without suppression of viral load and  $CD4^+$  T cell recovery were predisposed to a faster negative progression of the disease. He supposed that the first months of antiretroviral treatment are the more important to determine the clinical outcome and that this is independent from the therapeutic regimen adopted. [88]

## 5.2. Failure of HAART: Immunological Non Responder patients

The 15-30% of HIV-infected HAART-treated patients have discordant responses to long-term treatment consisting of a lack in  $CD4^+$  T cells count increase despite a full suppression of HIV replication, they are called immunological non responder (INR).

Several factors have been associated with immunological failure during HAART: age,  $CD4^+$  T cell nadir, adherence to antiretroviral therapy, concurrent viral hepatitis, failure in de novo  $CD4^+$  T cell production and excessive  $CD4^+$  T cell destruction.

Older age is negatively correlated with de novo  $CD4^+$  T cell production because older patients have a restrict pool of naïve T cells which is insufficient to compensate for  $CD4^+$  T cell depletion induced by HIV.

$CD4^+$  T cell nadir is the number of  $CD4^+$  T lymphocytes which are present in a HIV-infected patient before initiating antiretroviral therapy. It has been proved that low  $CD4^+$  T cell nadir is inversely correlated with

lymphocyte recovery. Recent evidences demonstrate that CD4<sup>+</sup> T cell count greater than 500 cells/ $\mu$ l and long-term combination of antiretroviral therapy might be positive factors for a better outcome of the disease: presence of 500 cells/ $\mu$ l after 6 years of HAART has been associated with the same rates of mortality as among the general uninfected population. Thus, CD4<sup>+</sup> T cell nadir influences not only the possibility to normalize cell count in response to therapy but also affects the time needed to reach this goal and the time between HAART initiation and achievement of CD4<sup>+</sup> T cell count more than 500 cells/ $\mu$ l was found to be significantly longer in individuals with lower baseline lymphocyte count.

Moreover the presence of concurrent viral infections and mainly viral hepatitis, might contribute to the immunological failure during HAART as the presence of coinfection contributes to a persistent immune activation. At last loss of adherence to antiretroviral therapy provokes viral rebounds that worsen the situation [89, 90].

### 5.2.1. Failure in de novo CD4<sup>+</sup> T cell production

Failure in de novo CD4<sup>+</sup> T cell production may be due to persistent bone marrow impairment, smaller thymuses and lower thymopoietin levels, normal plasmatic IL7 levels and defective IL-7R expression. [91]

Marziali conducted a pivotal study which clarified some of the mechanisms involved in the failure of de novo cell production. He suggested that IL7/IL7R pathway plays a fundamental role in this process. IL7 is a key cytokine in the regulation of T-cell homeostasis because of its effects on thymopoiesis and a survival factor for naïve and memory lymphocytes. Moreover IL7 acts as a co-stimulatory molecules after T-cell activation. IL7R $\alpha$  is IL7 receptor and an impairment in IL7/IL7R $\alpha$  pathway has been directly correlated with CD4<sup>+</sup> T cell depletion. In this study he observed that INR patients showed a reduction in IL7R $\alpha$  expression both on naïve and memory CD4<sup>+</sup> T cells and a positive correlation between the percentage of CD4<sup>+</sup> T cells and the expression of IL7R $\alpha$  receptor as well as an inverse correlation between IL7 level and CD4<sup>+</sup> cell count. Thus defects in the expression of IL7 receptor is directly involved in the failure of CD4<sup>+</sup> T cell recovery observed in INR patients. [92]

In another work Marchetti et al. demonstrated, that INR are characterized by high level of plasma IL7 because of a reduced consumption of this cytokine due to a decrease in the expression of its receptor rather than an increased production. [93]

Recently Rajasuriar et al. studied the correlation between the 4 haplotypes of IL7R $\alpha$  gene and immune recovery. Haplotype 2 was significantly associated with more rapid immune recovery. IL7R $\alpha$  exists also in soluble form and in that case it may limit the availability for IL7 to mediated its effects on T cells. HIV-infected patients homozygous for haplotype 2 have significant lower sIL7R $\alpha$  levels than those presented by non-haplotype 2 carriers so there's a great availability of IL7 to bind to the membrane-bound receptor. Moreover it has been proved that high IL7 level downregulates IL7R $\alpha$  expression on lymphocytes so low pre-HAART IL7 level is directly correlated with improved thymic function. [94]

In 2008 Isgrò et al. clarified that the impairment of naïve T cell depends on an alteration in bone marrow cellular components mainly due to apoptotic mechanisms and changes in cytokine production. HIV-infected bone marrow cells secrete large amounts of proinflammatory cytokines, such as TNF $\alpha$ , which inhibit hematopoiesis by the induction of apoptosis through the FasL-Fas pathway on the bone marrow progenitor cells. This cytokine unbalance is mainly dependent on TNF $\alpha$  and IL2. TNF $\alpha$  has a suppressive effect on bone marrow progenitors, it influences cell viability, modulates the expression of cytokine receptors on the cell surface and induces the expression of Fas receptor on progenitors cells. By contrast, IL2 is a strong growth and survival factor. HAART treatment induces an amelioration of stem cell activity, a restoration of stromal cell pattern and functions. Regarding cytokine environment, antiretroviral treatment reduces TNF $\alpha$  and increases IL2 levels but these changes do not occur in immunological non responder patients. Conversely, INR patients are characterized by TNF $\alpha$  augmented level, IL2 reduced level and alteration of IL7/IL7R $\alpha$  pathway. Furthermore, CD4<sup>+</sup> T cell depletion observed in INR patients cannot be reverted by augmented thymic output because the reduced clonogenic potential of bone marrow lymphoid progenitors cannot replenish the loss of thymocyte progenitors. [93, 94, 95, 96]

### 5.2.2. Excessive CD4<sup>+</sup> T cell destruction

Excessive CD4<sup>+</sup> T cell destruction may be provoked by ongoing viral replication, CD4<sup>+</sup> T cell hyperactivation, persistent antigenic stimulation (LPS) and immune regulatory mechanisms.

Several factors are implicated in the immunological impairment characterizing HIV infection.

INR have been shown to have higher levels of proviral DNA in total, memory and naïve CD4<sup>+</sup> T cells, reinforcing the role of increased HIV antigen-driven CD4<sup>+</sup> T cells in INRs. The persistence of residual undetectable low-level viral replication contributes to the maintenance of HIV-1 reservoir in these patients and triggers immune activation. It has been observed that proviral DNA is associated with CD4<sup>+</sup> T cell activation, with the increase of CD4<sup>+</sup> T effector cells and consequently it influences the immune reconstitution. Furthermore, the frequency of CD4<sup>+</sup> T cells containing HIV-1 proviral DNA is inversely correlated with CD4:CD8 ratio suggesting that persistent HIV-1 replication is a crucial factor in determining

the CD4<sup>+</sup> T lymphocyte depletion. Moreover in absence of disease progression and CD4<sup>+</sup> T cell recovery, CD8<sup>+</sup> T cells predominate in order to maintain total T cell homeostasis even if it interferes with regeneration of CD4<sup>+</sup> T cells. Ostrowski confirmed this findings investigating the associations between low level viremia, proviral DNA, CD4<sup>+</sup> T cell gain and T cell subsets in INR patients. He found that reduction in CD4<sup>+</sup> T cell gain might be due to a constant ongoing viral replication that persists even in the presence of antiretroviral drug treatment. Ongoing viral replication influences also the homeostatic balance of immune cells in fact INR patients were characterized by an increase in percentage of memory and activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells despite a reduction in the percentage of naïve T cells, indicating that proviral DNA level interferes with immune reconstitution. Furthermore another study [97] confirmed that higher CD4<sup>+</sup> T cell activation and proviral DNA lead to a naïve T lymphocytes decrease and an increase in cell death due to hyperexpression of Fas death, thus these might be critical parameters for the lack of immune recovery characterizing non responder patients. [91, 98, 99]

HIV induced immune activation might be one of the mechanism responsible for damaging the immune system. CD4<sup>+</sup> T cell hyperactivation has been demonstrated to persist even after HAART-induced virological suppression and to play a significant role on CD4<sup>+</sup> T cell count recovery during antiretroviral treatment. CD4<sup>+</sup> T cell depletion may occur both for persistent activation and for virus-directed cytopathic effect, as a consequence the immune system tries to replenish cells depletion stimulating the activation and proliferation of naïve and central memory lymphocytes. Experimental evidences, coming from a study conducted by Hunt, confirmed that INR patients were characterized by a higher level of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells than FR patients but that this increase was not accompanied by CD4<sup>+</sup> T cell recovery, which is normally induced by a successful antiretroviral therapy. This lack in CD4<sup>+</sup> T cell gain might be due to major susceptibility of HIV-infected cells to apoptosis, besides the increase in activated CD8<sup>+</sup> T cells was associated with lower CD4<sup>+</sup> T cell gains confirming once more how CD8<sup>+</sup> T cell activation represents the ideal marker to predict disease progression. Moreover the presence of coinfections increases CD4<sup>+</sup> T cell hyperactivation, and worsen the situation. [100, 101]

Excessive destruction might also be due to an exaggerated apoptosis, Pitrak and colleagues demonstrated that INR patients are characterized by high levels of apoptosis and they infer that this mechanism plays an important role in lymphocyte destruction because of the existence of a negative correlation between apoptosis and CD4<sup>+</sup> cell count. [102]

HAART reinvigorates cellular pool by blocking HIV replication and virus-induced hyperactivation, however in INR patients, immune activation is reduced compared to HAART-naïve patients but it never return to physiologic level. This persistent basal state of immune activation is identified as “residual immune activation” and several mechanisms might provoke it. Persistent low levels of virus replication and microbial translocation are two of them. Persistent virus replication may depend on the size of reservoir compartment established prior of HAART initiation suggesting that a more potent antiretroviral regimen and an early initiation of therapy might be more effective in reducing both virus replication and immune activation. [34]

Moreover, as previously said, the acute phase of HIV infection is characterized by a strong activation of lymphocytes localized at the mucosal surfaces and T cell activation has been associated with disease progression in untreated HIV-infected individuals. Untreated HIV infected individuals are characterized by high level of lymphocytes proliferation and apoptosis due to ongoing T cell activation and enhanced HIV replication in infected cells caused by their increase susceptibility.

In HAART-treated patients with persistent CD4<sup>+</sup> T cell depletion, the level of immune activation of memory cells returned to physiologic level but remained elevated in the gastrointestinal tract. HIV replication in mucosal tissue might be the major reason behind the persistently low levels of mucosal CD4<sup>+</sup> T cells in HIV-infected individuals on HAART with undetectable virus in blood. Experimental evidences coming from studies conducted on SIV-infected macaques and further on HIV-infected individuals, suggested that mucosal CD4<sup>+</sup> T-cell reconstitution may be achieved if therapy is started enough early. Mucosal CD4<sup>+</sup> T cells are able to repopulate the MALT as long as their depletion is stopped before the restoration capacity of the immune system is irreversibly compromised. Mehandru and colleagues conducted a study on HIV-infected patients which initiated early antiretroviral treatment and they identified reconstitutor and non reconstitutor patients according to the ability in reconstituting lymphocytes at mucosal level. They observed that in non reconstitutors there wasn't a complete restoration of gut lymphocyte's pool. The conclusion was that maybe several factors were implicated in this process: high level of CD8<sup>+</sup> T cell activation, persistence of inflammatory responses, altered cellular recruitment and also increased cell death due to ongoing viral replication or immune activation in the effector sites. Finding the right combination of antiretroviral drugs might be useful to control viral replication and immune activation in order to reinvigorate mucosal lymphocytes. [103]

It has been supposed that non-responsiveness to therapy observed in INR patients might be due to the abnormal T cell activation and that this can be induced by a persistent antigenic stimulation. Recently a

pathogenetic model of increased translocation of microbial products from the gastrointestinal lumen has been proposed as a mechanism continuously triggering immune system in HIV-infected patients. LPS is the major microbial product involved in the impairment in the gastrointestinal mucosal barrier. Considering that mucosal lymphocytes are the first to be destroyed by HIV action, mucosal barrier becomes leaky, allowing microbes to invade the organism. Microbial translocation may be one of the driver of immune activation and consequently of CD4<sup>+</sup> T cell loss. [28, 30]

Brenchely demonstrated that in HIV-infected individuals microbial translocation correlated with the rate of immune activation. Furthermore, continuous hyperactivation due to antigenic stimulation contributes to the persistent differentiation of naïve T cells into effector cells thereby accelerating the depletion of naïve T cell pool. Moreover after initiation of HAART, it was observed that LPS level decreased but they remained elevated compared uninfected individuals, this increase in LPS concentration was directly correlated with the poor CD4<sup>+</sup> T cell reconstitution. [27, 36, 86, 91]

HAART therapy is able to correct immune activation and cellular apoptosis characterizing HIV infection. Rajasuriar and colleagues showed that pre-HAART LPS level reflect the extent of gastrointestinal lymphoid tissue damage sustained in the absence of HAART. They demonstrated that lower level of LPS are positively correlated with a faster immune recovery and so the degree of residual microbial translocation after initiation of antiretroviral therapy is associated with the degree on immune restoration. [94]

This allows to conclude that HAART might be able to induce a partial repair in mucosal barrier and to control LPS level, anyway lack of CD4<sup>+</sup> T cell recovery observed in INR patients is due to a combination of several factors.

Treg cells are specialized T cell subpopulation with the ability to down-modulate immune activation and function. Several studies concentrated on their role in controlling immune activation occurring in HIV infection and conflicting data have been reported. Some studies reported a significant reduction in Tregs count in INR compared to FR patients, allowing to suppose that low level of T-regulatory activity might be linked to failure in CD4<sup>+</sup> T cell reconstitution, others studies support the opposite hypothesis.

HAART displays its effect on Treg cells, experimental evidences shows that Treg frequency is reduced after 6 month of effective HAART therapy and that is correlated with the increase in CD4<sup>+</sup> T cell count and a decrease in immune activation, typical of full responder patients but not of immunological non responders to HAART. Considering that Treg activity plays an important role in controlling immune responses, reduction of immune activation, due to effective HAART, diminished the necessity of a sustained immune response and consequently of a immune suppressive action. [104]

By contrast, in a study conducted by Kolte [105] on HIV-1 infected patients after 5 years of HAART, emerges an increase in Treg levels although in presence of viral load suppression, normalized CD4 counts, immune activation and cytokines patterns. It has been suppose that this augment in Tregs level might be correlated with the increase thymic output of naïve lymphocytes occurring in full responder individuals.

Different studies have been conducted to elucidate the interplay between immune activation and immune suppression. The gastrointestinal tract of HIV infected patients naïve to HAART therapy is shown to be distinguished by a high concentration of mucosal Treg cells while in HAART-treated patients the frequency of this cellular populations is similar to that characterizing healthy subjects. In the periphery there's a normal or a small increase of Treg level. This allows to suppose that mucosal recruitment and the subsequent accumulation of Treg is driven by HIV replication in order to control the infection even this implies a reduction in the immune response at this level and so a high susceptibility to gastrointestinal opportunistic infections in untreated HIV-infected patients. The same findings were observed by an other group of researchers working on lymphoid tonsillar biopsies and in addition they also pointed out the higher expression level of typical markers of Treg activity. [106, 107]

Moreover in a study conducted by Weiss, it has been suggested that in presence of HAART, the activity of Treg cells in controlling residual immune activation is promoted while in absence or after interruption of antiretroviral therapy viral replication re-induces immune activation, this might be due to a decrease in absolute number of Tregs. [108]

## 6. HYDROXYCHLOROQUINE TREATMENT

The first study in which the possible application of Chloroquine (CQ) and Hydroxychloroquine (HCQ) in the treatment of HIV infection was tested dates back to 1990. CQ and HCQ are antimalarial drugs most widely used in the world. To date, CQ has been dismissed because of continuous emerging of CQ-resistant *Plasmodium Falciparum* strains while HCQ is commonly used also in autoimmune diseases like rheumatoid arthritis and Lupus Erythematosus Systemicus (LES). HCQ has practical advantages, it's economic, well distributed and not stigmatizing so a possible application in third world could have a positive effect in controlling HIV spreading.

### 6.1. Structure and biological effects

HCQ is a weak base that affects acid vesicles leading to dysfunction. At extracellular level it's in a protonated form unable to cross the plasma membrane, just the non-protonated form can enter in the cell where it's protonated in a manner inversely proportional to the present pH. HCQ is so accumulated in acid organelles like endosomes, lysosomes, Golgi vesicles where the pH is low, it affects several enzymes and post-translational modification of proteins by increasing the pH of these acid vesicles. HCQ can be extruded through these organelles thanks to the action of specific transporter or exocytosis mechanism.

On the immune system, HCQ displays an anti-inflammatory action. Its accumulation in lymphocytes and macrophages reduces the secretion of TNF $\alpha$  and the expression of TNF $\alpha$  receptor on monocytes. HCQ can cause muscle weakness and retinopathy if administered in high doses or in prolonged therapy. [109, 110]

### 6.2. Anti-HIV effects of HCQ

CQ and HCQ exert similar anti-HIV effects. They act both on X4, R5 and X4/R5 HIV-1 isolates from subtypes A-E, against HIV-2 and at different stages of infection. CQ and HCQ can affect the early stage of HIV infection or the replicative cycle of the virus, they: 1) augment pH in acid vesicles, 2) alter gp120 production, 3) act on Tat and integrase, 4) reduce intracellular iron, and 5) reduce pro-inflammatory status.

HCQ augments pH in acid vesicles of non infected and infected cells in a dose-dependent manner. It has been demonstrated that HIV-1 passed through HCQ-treated cells is non-infectious.

The main role of HCQ is directed on gp120 glycosylation, considering the effect on pH of acid organelles, it alters the functionality of several enzymes necessary for post-translational modification of proteins like gp120. The production of gp120 is reduced in cells treated with HCQ and so newly production of virion is compromised or anyway new virus are not able to infect other cells because of the altered conformation of gp120.

Inhibitory effect of HCQ is directed to 2G12 epitope of gp120, this epitope is a conserved portion important for HIV infectivity. [111, 112]

HCQ/CQ displays an inhibitory effect also on LTR activation, it reduces Tat-mediated transactivation of HIV-1 LTR. LTR function as regulator of HIV genome transcription and define the interaction with host enzymes, their activation is directed by Tat activity. Moreover Tat is able to induce pro-inflammatory cytokine secretion when it translocates in the cytosol from endosome: HCQ/CQ blocks the translocation of Tat and consequently cytokine secretion and the immune activated status typical of HIV infection. [113, 114]

HIV uses host cellular enzymes for its replicative cycle but cofactors such as iron are also necessary. This tissue accumulation increases HIV transcription and decreases host immunological defense. HCQ/CQ reduces the accumulation of iron because it interferes with cytoplasmic release from ferritin and with transferrin-transferrin receptor uptake. Ribonucleotide reductase is an enzyme involved in HIV replicative cycle it contains an iron ion in its catalytic site, in the absence of iron this enzyme is inhibited and as a consequence there's a decrease in the integration of pro-viral DNA in host genome. [115]

### 6.3. HIV immune activation and HCQ

HIV infection is characterized by a strong and persistent immune activation that provokes apoptosis in continuously activated cells and the establishment of a pro-inflammatory state. HCQ/CQ is known to suppress immune activation by several mechanisms like inhibition of TLR signaling and inflammatory cytokine secretion.

As previously said immune activation can be detected thanks to specific markers expressed on cell surface (CD38) or at intracellular level (Ki67), it can be induced in presence of persistent antigenic stimulation and microbial translocation.

Recently in literature it has been published a study based on a double-blind, randomized placebo-controlled trial on chronically HIV-infected subjects receiving CQ or placebo. In this clinical trial it was verified that the administration of CQ decrease in the expression of CD38 on CD8<sup>+</sup> T memory cells and of Ki67 on both lymphocyte population. HCQ/CQ reduces TLR signaling pathway, and this in turn might be responsible for the reduction in immune activation. [116]

HCQ/CQ blocks the translocation of TLR from endoplasmic reticulum to early/late lysosomes because it alters the acid pH present in the endosome useful for the binding between TLR and viral nucleus acid products. In vitro studies confirmed that CQ inhibits pDC activation and maturation, the MyD88 pathway signaling molecules IRF-7 and IRAK4, IFN $\alpha$  production and IDO synthesis. Inhibition of pDC activation and maturation is due to the ability of CQ to block endosomal fusion and acidification leading to a suppression in HIV-1 TLR signaling with consequently inactivation of pDC due to the failure in upregulating IRAK 4 and IRF 7. Moreover IFN $\alpha$  production is induced by the activation of genes regulated by TLR signaling pathway and in previously studies it has been demonstrated that immune activation of CD8<sup>+</sup> lymphocytes is correlated with IFN $\alpha$  action. Thus, it can be infer that CQ is able to reduce immune activation thanks to the reduction in IFN $\alpha$  concentration.

A consequence of HIV infection is the induction of the IDO enzyme in virus activated pDC which directly inhibits T-cell proliferation thanks to the induction and stimulation of Tregs, and the induction of PDL1/PD1 pathway. HIV-1 up-regulates the expression of PDL-1 on pDC surface which in turn can exert immune suppressive activity in T target cells expressing PD1 receptor. CQ, inhibiting IDO and PDL1 expression on pDC, reverts immune suppression improving T cell survival. In conclusion CQ is able to control HIV-induced immune activation and immune suppression, consequently the use of CQ as an adjuvant in combination with HAART could be an effective and inexpensive approach to control immune activation and reduce the risk of comorbidities for HAART-HIV-infected individuals. [117, 118]

#### **6.4. Combined therapies: HCQ and antiretroviral drugs**

HCQ shows anti-HIV effects and absence of cross-resistance with other HIV-1 drugs, so several studies have been conducted to evaluate a possible and positive combined therapy.

It has been demonstrated that in vitro HCQ could have different effects depending on the association with specific antiretroviral drugs.

In vitro HCQ shows synergistic effect with protease inhibitors (PIs) because it inhibits efflux mediated by two important cell surface drug transporters P-glycoprotein and MRP-1. These transporters are responsible for the efflux of intracellular antiretroviral pools to extracellular compartments, HCQ blocks their activity leading to an increase of intracellular concentration of antiretrovirals. This discover is important for the treatment of drug-experienced HIV infected patients who have developed multiple resistances to antiretroviral drugs and thus have limited therapeutic options. [119]

Several pilot studies concerning the treatment of HIV infected patients with hydroxychloroquine are documented in literature. The first study was directed by Sperber in 1995. It was a randomized, double-blind, placebo-controlled study in which HCQ or placebo was given to HIV infected patients asymptomatic, with CD4<sup>+</sup> T cell counts between 200 and 500 cells/mm<sup>3</sup>, and not under antiretroviral therapy for at least 2 month because HCQ was given at higher dose (800mg/d) than the one normally used in the treatment of rheumatic disease (400mg/d). Anti-viral effect of HCQ was demonstrated by the significant decrease in plasmatic levels of viral RNA and by the marked reduction in plasmatic concentration of IL6 and viral RNA in HCQ group compared to placebo one despite a stable CD4<sup>+</sup> T cell count. [120]

In an other study, 144 weeks long, it was evaluated a combined therapy with HCQ, Hydroxyurea (HU) (an inhibitor of ribonucleotide reductase) and Didanosine (DDN). At the end of the study positive effects on immune system responses, were observed for example a reduction of CD8 percentage accompanied by a significant increase in CD4<sup>+</sup> cell count, moreover this drug combination was well tolerated and safe when administered in asymptomatic HIV infected patients. [121]

Paton and colleagues concentrated on a possible association between HCQ, HCM and DDN. HCM (also called HU) is an inhibitor of ribonucleotide reductase while DDI is a NRTI. HIV infected patients were treated with this drug combination for 48 weeks. After 12 weeks it was observed an increase in the percentage of CD4<sup>+</sup> T cell count and a reduction in viral load, these positive effects were sustained up to week 48. Even if the recovery of CD4<sup>+</sup> cell count is less than that obtained during HAART therapy alone, HCQ shows slow development of resistance so it increases the durability of this regimen. Above all, this drug combination is well-tolerated with mild side-effects none of which resulted in discontinuation of treatment so it can be easily used in developing countries even because of its low cost. [122]

In conclusion combined therapy based on the association of HCQ and antiretroviral drugs might be useful in the treatment of HIV infected patients which show multi-drug resistance or in developing countries where there are economic and drug-manufacturing problems

***Aim***



HIV infection is characterized by a profound and continuous depletion of CD4<sup>+</sup> T lymphocytes and by an impairment of immune response. To date antiretroviral therapy is a combined therapy able to persistently reduce viral replication and induce CD4<sup>+</sup> T cell recovery. Despite the efficacy of this therapy, demonstrated by several clinical studies, 15%-30% of patients have discordant responses to long-term HAART consisting in the lack of CD4<sup>+</sup> T cell increase despite a full suppression of HIV replication; these patients are known as immunological non responders (INR).

Several factors are thought to be involved in the failure of the immune recovery, the most important are: failure in de novo CD4<sup>+</sup> T cell production, excessive in CD4<sup>+</sup> T cell destruction, CD4<sup>+</sup> T cell nadir pre-therapy and adherence to antiretroviral therapy. Failure in de novo production of CD4<sup>+</sup> T cells might be provoked by damage of primary lymphoid organs and damage in cytokine circuit that should ensure cell survival (mainly IL7 and IL7R pathway). To date it's supposed that persistent depletion of CD4<sup>+</sup> T cells is correlated with an excessive hyperactivation of immune system, so that CD4<sup>+</sup> T cells are induced to apoptosis by the presence of HIV infection and continuous hyperactivation depletes naïve T cell pool leading to the failure of immune system to properly respond to the infection.

The aim of this study was to better understand the mechanisms responsible for the excessive CD4<sup>+</sup> T cell destruction observed in INR patients. According to what described in the literature, CD4<sup>+</sup> T hyperactivation, ongoing viral replication, persistent antigenic stimulation and immune-regulatory mechanism, are all factors that might explain this process.

Moreover, recent evidences show that HCQ, an anti-malarian drug, has been widely used for the treatment of various autoimmune diseases because of its ability in modifying pH in endosomal vesicles. HCQ has immune-modulating properties and it has been also demonstrated that HCQ has anti-HIV activity.

Considering the role played by HCQ in the treatment of HIV infection, the second part of this study aims to determine whether HCQ might be useful in augmenting CD4<sup>+</sup> T cell count in immunological non responder patients and in modifying the most common parameters usually altered in HIV infected patients characterized by a cell count less than 200 cells/ $\mu$ l.

Thus, in the first part of the study we are going to compare HAART-treated patients presenting CD4<sup>+</sup> cell counts more than 500 cells/ $\mu$ l and HAART-treated patients presenting CD4<sup>+</sup> cell counts less than 500 cells/ $\mu$ l, analyzing:

- Treg cell percentage
- apoptosis
- immune activation
- microbial translocation

In the second part of the study, our attention will be focused on the population of HIV-infected patients immunological non responder to HAART with an absolute CD4 count less than 200cells/ $\mu$ l and treated with HCQ to evaluate:

- markers of immune activation
- presence of a pro-inflammatory environment
- changes in microbial translocation in INR patients
- modulation of TLR7 and TLR8 pathway of expression

***Material and methods***

## 1. HAART AND IMMUNE RECOVERY

### 1.1. Study population

All individuals of this study were enrolled by the Departments of Infectious diseases of the Luigi Sacco Hospital in Milan and of the San Gerardo Hospital in Monza and written informed consent was obtained before enrolment.

For the first part of this project 67 HIV-infected patients with suppressed viremia (<50 copies/ $\mu$ l) were enrolled and all of them were treated with combined HAART according to currently guidelines (NRTI plus PI or NRTI plus NNRTI). In all patients, HIV plasma viremia has been under the threshold for at least 7 years and they were stratified into two groups on the basis of the CD4 cell counts (<500 cells/ $\mu$ l or >500 cells/ $\mu$ l).

### 1.2. Sample collection and processing

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) (Becton Dickinson, Rutherford, NJ, USA). Samples were centrifuged 1400rpm for 10 minutes, plasma obtained was collected and stored at -20°C for subsequent analyses. The remaining sample, composed by erythrocytes, PBMC, granulocytes and platelets, was diluted in PBS (phosphate buffered saline) (PBI International, Milano, Italy) and separated thanks to a density-gradient centrifugation on lymphocyte separation medium (Ficoll-Hypaque) (Cedarlane Laboratories Limited, Hornby, Ontario Canada) for 25 minutes at 2300rpm. PBMC are localized between the phase of ficoll-hypaque and the phase including granulocytes and erythrocytes, they are collected and washed twice in PBS for 10 minutes at 1900 rpm. Cellular pellet was resuspended in PBS and washed twice in phosphate-buffered saline (PBS) (PBI International, Milano, Italy). Cellular count was conducted using an automatic cell counter.

### 1.3. Cell count

The number of viable leukocytes was determined by the use of an automatic cell counter ADAM-MC (Digital-Bio, NanoEnTek Inc., Corea). ADAM-MC automatic cell counter measures total cell numbers and cell viabilities by cutting edge detection technologies. The instrument is able to count the cells thanks to the use of propidium iodide, a fluorescent dye that intercalates in the DNA of cell characterized by damage cell membranes. It's based on two different solution, AccuStain Solution T is composed by propidium iodide and lysis solution while AccuStain Solution N is composed by propidium iodide and PBS. AccuStain Solution T allows to count all cells present in the sample while AccuStain Solution N just non-viable cells.

To evaluate cell concentration, a small quantity of sample (30 $\mu$ l) is combined with an equal volume of each solution. Samples prepared in this way are directly transferred in a disposable slide and this is read by the instrument. A green laser (532nm) is focalized on the slide and the cells colored are detected by a CCD camera (B/W CCD). On the screen it's shown total cell count, the count of non-viable cells and the vitality of each sample is calculated by ADAM-MC software. The number of cell/ml is obtain with the subtraction of total number of non-viable cells from the number of total cells.

### 1.4. Analyses on PBMC

#### 1.4.1. Stimulation of PBMC

PBMC were incubated for 18 hours in the presence/absence of a pool of gag/env peptides (HIV), CMV protein (Microbics Biosystems inc., Toronto, Ontario, Canada). For cytokine analyses, 10 $\mu$ g/ml Brefeldin A (Sigma-Aldrich) was added to cell cultures during the last 6 hours to block vesicular transport of cytokines between the Golgi apparatus and endoplasmic reticulum. In some experiments PD1 or PDL1 neutralizing antibodies (eBiosciences Inc., San Diego, CA, USA) were added to the cultures to evaluate the PD1-PDL1 pathway activity.

#### 1.4.2. Identification of Treg cells

Unstimulated, basal and stimulated PBMC were washed in PBS and subsequently incubated with 5 $\mu$ l anti-CD4 PC7, 5 $\mu$ l of anti-CD25 ECD, and 5 $\mu$ l of anti-PD1 PE mAbs for 15 min at RT. After the incubation, cells were washed with PBS and they were resuspended in fix/perm solution (eBioscience Inc.) for 30 minutes. Cells were then washed twice with PBS (PBI International) and with 10-fold diluted Perm buffer solution (eBioscience Inc.), 5 $\mu$ l of intracellular mAbs for PD1 and FoxP3 (respectively labeled with FITC and PC5 fluorocromes) were added. After the 30-minutes incubation, cells were washed, resuspended in PBS and read in an hour. All the incubations were conducted at 4°C in the dark.

Intracellular or surface co-staining of PD1 and intracellular FoxP3 was performed by flow cytometry on CD4<sup>+</sup>CD25<sup>bright</sup> gated T cell.

### 1.4.3. Evaluation of TLR2 and TLR4 expression on Treg cells

Both basal and stimulated PBMC were resuspended in PBS (PBI International) and stained for anti-CD4 PC7, anti-CD25 ECD, anti-FoxP3 PC5, anti-TLR2 FITC, anti-TLR4 PE. See paragraph 1.5.2. for details about the methods.

### 1.4.4. Intracellular cytokine expression

Antigen-stimulated PBMC were stained for CD4 expression. After a 15-minute incubation at room temperature in the dark, cells were fixed in 100µl of 1% PFA (Sigma-Aldrich) and incubated for 15-min at 4°C in the dark. Cells were then washed, permeabilized with 100µl of 0.5% saponin (Sigma-Aldrich) and incubated with 5µl of anti-TGFβ PE and of anti-IL10 FITC mAbs for 45 minutes at 4°C. After this incubation cells were washed, fixed 1% PFA (Sigma-Aldrich) and analyzed by flow cytometry.

### 1.4.5. Ki67 evaluation

The protocol used to evaluate Ki67 expression is the same described in the paragraph 1.5.4. Basal and stimulated PBMC were stained with 5µl of the following mAbs: anti-CD4 PC7, anti-Ki67 FITC or mouse FITC-coupled IgG1 (as isotype control).

### 1.4.6. Identification of viable, early apoptotic and late apoptotic cells

Stimulated PBMCs were washed and resuspended in D-PBS (Euroclone, Siziano, Pavia, Italy). They were then stained with 5µl of CD4 FITC, 5µl of annexinV PE and 5µl of 7AAD mAbs. After 20-minutes incubation at room temperature in agitation, cells were washed in cold D-PBS and resuspended in D-PBS. The cells were read immediately by flow cytometry. Lymphocytes population were identified based on the FS and SS characteristic and an immunological gate was create considering SS characteristic and CD4 expression. In this gate, 7AAD and Annexin V positivity were considered at the same time.

### 1.4.7. Detection of activated caspases 8 and 9

The FLICA Apoptosis detection kit (Immunochemistry Technologies, Bloomington, Minnesota, USA) was used to analyze activated caspases. FLICA reagent were prepared according to manufacture's instructions and added to the resuspended cells followed by 1 hour incubation at 37°C under 5% CO<sub>2</sub>. After this incubation cells were washed twice with wash buffer, resuspended and stained with CD4 mAb for 30-min in ice. Cells were immediately analyzed by flow cytometry.

## 1.5. Cytometric analyses

Flow cytometry analyses were performed using FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with a double 15-mW argon ion laser operating at 456 and 488nm interfaced with Intercorp Computer (Intercorp, Verona, Italy). For each analyses 20000 events were acquired and gated on CD4, CD8 and CD14 expression and SSC properties. Green fluorescence from FITC (FL1) was collected through a 525-nm band pass filter, red fluorescence from PE (FL2) collected by 575-nm band pass filter, orange fluorescence from ECD (FL3) was collected through a 620-nm band pass filter, deep-red fluorescence from PC5 (FL4) was collected through a 670-nm band pass filter, red-fluorescence from APC (FL4) was collected through a 650-nm band pass filter and far-red fluorescence from PC7 (FL5) collected by a 770-nm band pass filter. For forward and side scatter were used linear amplifiers, logarithmic amplifiers for FL1, FL2, FL3, FL4 and FL5. Flow data were analyzed by first gating on the lymphocyte or monocyte population as defined by forward and side light scatters. From this population a single color CD4 or CD14 histogram was made, and the T cells or monocytes were selected and put into a two-dimensional dot plot to achieve the results.

## 1.6. Plasma LPS concentration

LPS concentration was measured on plasma samples with LAL Chromogenic Endpoint Assay (Hycult Biotechnology, Uden, The Netherlands). All the samples, standards and reagents were prepared according to manufacture's instructions. Considering that plasma may contain endotoxin inhibiting compounds, it is required to heating the sample at 75°C for 5 minutes in order to neutralize these compounds. Moreover, plasma samples required a 3-fold dilution. 50µl of each diluted sample and standard was plated in duplicate in a 96-well plate and immediately 50µl of LAL reagent was added in each well. After 45-minutes incubation at room temperature, reaction was stopped with the addiction of 50µl of stop solution. Absorbance was measured at 405nm with a spectrophotometer and for the calculation of results, from the average of the duplicate readings for each standard and sample was subtracted the blank optical density. The standard curve was constructed by plotting the optical density on the y-axis against the concentration of the standards on the x-axis (logarithmic scale). LPS concentration was calculated relatively to the standard curve, multiplied by the dilution factor, expressed in EU/mL and subsequently transformed in pg/ml.

### **1.7. Statistical analyses**

Data were analyzed according to standard statistical tests; t test were performed to compare groups. Procedures were based on parametric analyses. The rank-transformed variables were analyzed if distributions were not normal. To account for different patient's characteristics between groups, an analysis of variance was run through a general linear model, including sex and age as independent variables. Possible relationship were evaluated using Pearson's correlation test.

## 2. HCQ THERAPY

### 2.1. Study population

The second part of this study regarded the evaluation of efficacy of hydroxychloroquine treatment in increasing percentage of CD4<sup>+</sup> T cells in HIV patients non responder to HAART therapy.

20 HAART-treated HIV-infected patients with an absolute CD4 count less than 200 cells/ $\mu$ l during the last year of therapy and with suppressed viremia (less than 37 HIV RNA copies/ml) were enrolled. All patients were treated with combined antiretroviral therapy according to the current guidelines: a NNRTI plus PI or NRTI plus NNRTI. Exclusion criteria included glucose-6-phosphate dehydrogenase deficit, pregnancy, breast feeding, acute infectious disease, or 5% CD4<sup>+</sup> T cell count increase in the last year.

All patients received 400 mg/die of HCQ daily for 6 months. Virologic and immunologic parameters were evaluated at baseline, at the end of the treatment and at 2 month after HCQ suspension.

All patients were enrolled by Infectious Diseases Units at Luigi Sacco Hospital, Milan, Italy which approved this study; written informed consent was obtained before enrollment in accordance with the Declaration of Helsinki.

### 2.2. Sample collection, processing and cell count

See paragraphs 1.2 and 1.3 for details about the methods.

### 2.3. Immunophenotypic analyses

Lymphocytes subsets and monocytes were evaluated using 50 $\mu$ l of EDTA-treated peripheral blood incubated for 10 minutes at room temperature with 5 $\mu$ l of fluorochrome-labeled mABs: anti-CD4, -CD8 and -CD14 PC7; anti-CD69 APC; anti-HLA-DRII, -CD38, -TLR4 and -TLR5 PE; and anti-CD8, -CD16, -CD45RO and -TLR2 FITC, according to the different analyses. Erythrocyte lysis and fixation of stained cells were performed at the end of the incubation period using Immunoprep Epics Kit and Q-Prep Workstation (Coulter Electronics).

### 2.4. Stimulation of PBMC

PBMC were incubated for 18 hours in the presence/absence of a pool of gag/env peptides (HIV), single strand RNA (ssRNA) (InvivoGen, San Diego, CA, USA), LPS antigen (Sigma-Aldrich, St. Louis, MO, USA). For cytokine analyses, 10 $\mu$ g/ml Brefeldin A (Sigma-Aldrich) was added to cell cultures during the last 6 hours before cell analyses to block cytokines secretion.

#### 2.4.1. Evaluation of TLR4, TLR7 and TLR8-expression on stimulated monocytes

PBMC were stimulated for 18 hours with LPS (Sigma-Aldrich), agonist of TLR4, and ssRNA (InvivoGen), agonist of TLR7 and TLR8. Evaluation of TLR4 were conducted on PBMC stimulated with LPS while TLR7 and TLR8 on ssRNA stimulated PBMC. For TLR4 evaluation mABs used were: 5 $\mu$ l of anti-CD14 PC5 and of anti-TLR4 PE. For the identification of TLR7 and TLR8 expression mABs used were: 5 $\mu$ l of anti-CD14 PC5 of anti-TLR8 PE and of anti-TLR7 FITC. The protocol used for the evaluation of TLR expression is the same explained in the paragraph 1.5.4.

#### 2.4.2. Intracellular cytokines expression

Antigen-stimulated PBMC were stained for CD4 or CD14 expression. After a 15-minute incubation at room temperature in the dark, cells were fixed with 100 $\mu$ l of 1% PFA (Sigma-Aldrich) and incubated for 15-min at 4°C in the dark. Cells were then washed, permeabilized with 100 $\mu$ l of 0.5% saponin (Sigma-Aldrich) and incubated with 5 $\mu$ l of anti-TNF $\alpha$  PE, anti-TNF $\alpha$  FITC and anti-IL6 APC mAbs for 45 minutes at 4°C. After this incubation cells were washed and fixed with 500 $\mu$ l 1% PFA (Sigma-Aldrich). For details in the methods see paragraph 1.5.4.

#### 2.4.3. Identification of plasmacytoid dendritic cells and IFN $\alpha$ expression

Analyses were conducted with both basal and stimulated condition with gag/env peptides. For the identification of plasmacytoid dendritic cells, PBMC were washed in PBS (PBI International) and incubated with 5 $\mu$ l of anti-CD123 PECy7, 20 $\mu$ l of lineage- PC5 (mix of 5 $\mu$ l of each following mABs: anti -CD3, -CD14, -CD16, -CD19, -CD56) and 5 $\mu$ l anti-HLA DRII PE for 15 min at RT. After this incubation cells were fixed with 1% PFA (Sigma-Aldrich) and incubated 15 minutes at 4°C in the dark, washed with PBS (PBI International) and permeabilized with 0.5% saponin (Sigma-Aldrich). The addition of anti-IFN $\alpha$  mAb was followed by 45 minutes incubation at 4°C in the dark. At last, cells were washed with PBS (PBI International) and fixed with 500 $\mu$ l of 1% PFA (Sigma-Aldrich). Plasmacytoid dendritic cells were identify by flow cytometry as cells

CD123<sup>+</sup>HLADR<sup>+</sup>LIN<sup>-</sup>, expression of IFN $\alpha$  was evaluated on plasmacytoid dendritic cells. For details about the methods see paragraph 1.5.4.

#### **2.4.4. KI67 evaluation**

Analyses were conducted with both basal and stimulated condition with gag/env peptides. mAbs used were anti-CD4 PC7 to identify lymphocytes population, anti-KI67 and mouse FITC-coupled IgG1 (isotype control) for the intracellular staining. For details about the methods see paragraph 1.5.4.

Flow data were analyzed by first gating on the lymphocyte population as defined by forward and side scatter properties and on CD4<sup>+</sup> T cells. Results were expressed as the percentage of CD4<sup>+</sup>KI67<sup>+</sup> cells on CD4<sup>+</sup> population.

#### **2.4.5. Identification of Treg cells and of TLR-expressing Treg cells**

Analyses were conducted with both basal and stimulated condition with gag/env peptides and LPS antigen. PBMCs were incubated for 15 minutes at room temperature with 5 $\mu$ l of the following mAbs: anti-CD4 PC7, anti-CD25 ECD and anti-PD1 PE. The intracellular staining of PD1 and FoxP3 was performed using 5 $\mu$ l of anti-PD1 FITC and anti-FoxP3 PC5 mAbs. To detect TLR-expressing Treg, PBMCs were stained with anti-CD4 PC7, -CD25 ECD, -TLR4 PE and -TLR2 FITC (5 $\mu$ l of each mAbs). Intracellular staining was performed according to manufacturer's instruction, for details see paragraph 1.5.2.

### **2.5. Cytometric analysis**

See paragraphs 1.5 for details about the methods.

### **2.6. Serological assay**

Proinflammatory cytokines (TNF $\alpha$  and IL6) and IFN $\alpha$  were evaluated in plasma samples using commercial ELISA kits (TNF $\alpha$  and IL6: R&D Systems, Minneapolis, MN, USA; IFN $\alpha$ : Hycult Biotechnology) in 96-well plate.

#### **2.6.1. Plasma TNF $\alpha$ concentration**

All the samples, standards and reagents were prepared according to manufacture's instruction. 50 $\mu$ l of assay diluent was added to each well and subsequently 200 $\mu$ l of each sample and standard were plated in duplicate. After the incubation of 2 hours at room temperature, the plate was washed 4 time with 25-fold diluted wash buffer and 200 $\mu$ l of conjugate was added to each well and the plate was incubated 2 hours at room temperature. Plate was then washed four times and 200 $\mu$ l of substrate solution were added. After the incubation of 20 minutes in the dark, the reaction was stopped with the addition of stop solution (50 $\mu$ l in each well). The optical density of each well was determined within 30 minutes using a microplate reader set to 450nm. To avoid optical imperfections in the plate, wavelength correction was added and set to 540nm. For the calculation of results, from the average of the duplicate readings for each standard and sample was subtracted the average zero standard optical density. Plasma concentration of TNF $\alpha$  was calculated relatively to a standard curve construct by plotting the log of mean absorbance for each standard on the y-axis against the log of the concentration on the x-axis (logarithmic scale). Values was expressed in pg/ml.

#### **2.6.2. Plasma IL6 concentration**

High sensitivity ELISA assay was used for the quantitative determination of IL6 concentration in plasma and all the samples, standards and reagents were prepared according to manufacture's instruction. 100 $\mu$ l of assay diluent and of each sample and standard was added to each singular well in duplicate. The plate was incubated for 2 hours in agitation at room temperature. After the incubation, the plate was washed six times with 10-fold diluted wash buffer and 200 $\mu$ l of conjugate was added to each well. Subsequently 2 hours of incubation at room temperature on the shaker, the plate was washed 6 times with wash buffer and 50 $\mu$ l of substrate solution was added to each well. This incubation lasted 1hour at room temperature and it was followed by the addition of 50 $\mu$ l of amplifier solution. The reaction was stopped after 30 minutes with the addition of 50 $\mu$ l of stop solution to each well. The optical density was evaluated within 30 minutes using a microplate reader set to 490nm and wavelength correction (640nm) was used to avoid optical imperfections in the plate. For the calculation of results, from the average of the duplicate readings for each standard and sample was subtracted the average zero standard optical density. IL6 plasma concentration was calculated relatively to standard curve obtained by plotting the optical density on the y-axis of the standards versus the concentration of the standards on the x-axis (logarithmic scale) and expressed in pg/ml.

#### **2.6.3. Plasma LPS concentration**

For details about the methods see paragraph 1.7.

## 2.7. Gene analysis

### 2.7.1. RNA extraction from PBMC

PBMC were analyzed in both basal and after 3 hours with antigen specific stimulations. Basal and stimulated PBMC were processed to extract total RNA. mRNA was extracted from  $1 \times 10^6$  PBMC by the using of acid guanidium thiocyanate-phenol-chloroform method modified for RNAzol B (guanidine thiocyanate, 2-mercaptoethanol). Samples were resuspended in 200 $\mu$ l of RNAzol B to which 10% of chloroform was added. Samples were incubated at 4°C for 15 minutes and subsequently centrifugated at 13000g for 15 minutes. After centrifugation, three phases can be distinguished: in the upper phase, clear and transparent, there's the RNA, in the intermediate, formed by a white ring, proteins can be found while the lower is composed by DNA and RNAzolB. To the aqueous phase, collected and transferred to a sterile tube, was added an equal volume of isopropanol, samples were so stored at -20°C overnight to allow complete precipitation of RNA. The day after, samples were heated at 4°C for 15 minutes and centrifugated at 13000g for 15 minutes. Supernatant was removed and RNA pellet was washed with 100 $\mu$ l of ethanol (70%). Subsequently the sample was centrifugated for 8 minutes at 12000g at 4°C, once the supernatant was removed, pellet was dried under laminal flow cabin. 10,3 $\mu$ l of hydration buffer (10mM TRIS, 1mM EDTA) (Promega, Madison, Wisconsin, USA) were added to resuspend the pellet and allow a complete hydration.

### 2.7.2. DNase treatment

The RNA was subjected to treatment with DNase to eliminate contamination of genomic DNA. For each sample a reaction mix was prepared containing 1 $\mu$ g of RNA, 0.5 $\mu$ l of TURBO DNase 2U/ $\mu$ l, 1.2 $\mu$ l of 10x TURBO DNase buffer (10mM TRIS-HCl ph 7.5, 25mM MgCl<sub>2</sub>, 5mM CaCl<sub>2</sub>) to obtain a final volume of 12 $\mu$ l. The final volume was established considering that DNase treatment must not exceed 40% of final volume of RT-PCR. Reaction mix was incubated at 37°C for 15 minutes, at the end of which 2 $\mu$ l of DNase inactivation reagent (Ambion Inc., Austin, Texas, USA). Samples were agitated for 2 minutes at room temperature and subsequently centrifugated at 10000g for 2 minutes to pellet DNase inactivation reagent. The supernatants, containing RNA, was transferred in new tubes.

### 2.7.3. Retrotranscription of RNA into cDNA

After DNase treatment, RNA samples were retrotranscribed in cDNA. The protocol of retrotranscription provides a reaction containing 1 $\mu$ g of RNA, 0.5 $\mu$ l of 1mM random hexanucleotide primers and 0.5 $\mu$ l of oligo (dT) primer 1mM. The reaction mix was heated at 70°C for 5 minutes and immediately placed on ice. Subsequently 1 $\mu$ l of 0.5mM dNTP mix, 4 $\mu$ l of 5X M-MLV reaction buffer, 0.5 $\mu$ l of recombinant RNase inhibitor 40U/ $\mu$ l, 1 $\mu$ l of 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV) (Promega) were added. The reaction mix was incubated at 42°C for 1 hour and at the end RT enzyme was inactivated by heating at 95°C for 5 minutes. M-MLV enzyme has a 5'-3' RNA-dependent DNA polymerase activity and a low RNase H activity. Finally each sample of cDNA has been 4-fold diluted.

### 2.7.4. Evaluation of TLR signalling pathway

Evaluation of TLR pathway was performed with 96-well plate array specific for analyses of 84 genes involved in TLR pathway signaling (SABiosciences Corporation, Frederick, MD, USA). A mix was prepared by adding 9 $\mu$ l of SYBR Green PCR mix (Finnzymes, Vantaa, Finland), 2 $\mu$ l of 4-fold diluted cDNA pool and 8 $\mu$ l of double-distilled water. Each well contain a couple of primer specific for a gene of interest (A1-G12 well), 5 housekeeping gene (H1-H5), genomic DNA control (H6), reverse transcription controls (H7-H9) and positive PCR controls (H10-H12).

Quantification of gene expression levels was performed with DNA Engine Opticon 2 (MJ Research, Ro, Nevada, USA) and the thermic profile was as follows: one initial step of denaturation at 95°C for 2 minutes followed by 40 cycles including one step of denaturation at 92°C for 15 seconds, one step of annealing at 55°C for 20 seconds and one step of extension at 68°C for 20 seconds. Fluorescence was measured after extension step at the end of each cycle. Melting curve was constructed from 55°C to 62°C and fluorescence was measured at each 0.5°C increment and it was analyzed for each sample to confirm the specificity of the amplified and the absence of primer-dimer.

Analysis of the results required the elimination of background noise, all samples were normalized by subtracting the background fluorescence, calculated as the mean fluorescence detected from the third to the tenth cycle. The relative efficiency of the gene of interest is calculated taking into account the efficiency of amplification of the target and the difference between Ct of the target normalized to the expression of the housekeeping gene GAPDH.

## 2.8. Statistical analyses

Data were analyzed according to standard statistical tests, t test were performed to compare patients during treatment. Procedures were based on parametric analyses. The rank-transformed variables were analyzed if



distributions were not normal. Statistical analysis was performed using the SPSS statistical package (SPSS Inc.).

## ***Results and discussion***

## 1. HAART AND T CELL RECOVERY

### 1.1. Study population

In this study 67 HIV-infected, HAART-treated individuals were enrolled. In all of them HAART was effective because it maintained the viral load at undetectable level (<50 copies/ $\mu$ l).

According to several evidences present in literature [89, 90], patients were divided in two groups on the basis of the CD4 cell count reached after 7 years of HAART. 32 patients presented CD4 cell count >500 cells/ $\mu$ l while 35 patients had <500 cells/ $\mu$ l.

HIV-infected individuals in whom HAART was not associated with CD4<sup>+</sup> T cells recovery were characterized by a lower CD4 nadir ( $p=0.01$ ). As per inclusion criteria, lower absolute numbers ( $p<0.001$ ) and percentages ( $p=0.002$ ) of CD4<sup>+</sup> T cells/ $\mu$ l. The epidemiologic characterization of the two groups of individuals is presented in table 1.

Parameter	CD4 cell count >500 cells/ml	CD4 cell count <500 cells/ml	P
N <sup>o</sup>	32	35	
Sex	25M 7F	25M 10F	
Age	48.13 $\pm$ 1.12	52.11 $\pm$ 1.39	0.03
Years of HAART	10.34 $\pm$ 0.46	11.51 $\pm$ 0.52	NS
CD4 nadir (cells/ml)	195.84 $\pm$ 24.35	119.37 $\pm$ 15.70	0.01
CD4 cell count (cells/ml)	705.52 $\pm$ 25.87	245.83 $\pm$ 24.31	<0.001
% CD4	20.96 $\pm$ 1.49	12.93 $\pm$ 1.34	0.002

Table 1. Demographic and clinical characteristics of the HIV-infected antiretroviral therapy-treated patients enrolled in the study. Mean values, SE and P are shown.

### 1.2. Evaluation of immune suppression

#### 1.2.1. Identification of naïve and activated Treg cells

Treg cells control immune responses against self and non self antigens, they play a pivotal role in maintaining the right balance between tolerance and immunity.

In physiologic conditions, Treg cells represent 2-5% of CD4<sup>+</sup> T lymphocyte population, while in HIV infection it has been demonstrated an increase in the percentage of Treg cells and an induction of their suppressor activity. It has been formulated the hypothesis that their action might provoke the impairment of CD4<sup>+</sup> T cell recovery observed in HIV infected patients non responsive to antiretroviral therapy. Their immune suppressive activity might be due to the inhibition of T cell proliferation and survival because of reduction in survival cytokines availability, induction of apoptosis through PD1/PDL1 pathway and increase secretion of immune suppressive cytokines by lymphocytes.

Recent data indicating that Treg cells can be further subdivided into two populations based on the intra (naïve)- or extra (activated)-cellular expression of the PD1 molecule, this stimulated us to examine such populations in our patients. We started to evaluate changing in Treg subpopulation percentages between patients with CD4<sup>+</sup> cell counts less than 500cells/ $\mu$ l and patients with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l. Analyses were conducted in both basal and stimulated conditions on PBMC of patients enrolled (see paragraph 1.5.2 in material and methods, for further details).

Treg cells were identified in flow cytometry according to the methods present in literature. Total (CD4<sup>+</sup>CD25<sup>bright</sup>Foxp3<sup>+</sup>), naïve (CD4<sup>+</sup>CD25<sup>bright</sup>Foxp3<sup>+</sup> intracellular PD1<sup>+</sup>), and activated (CD4<sup>+</sup>CD25<sup>bright</sup>Foxp3<sup>+</sup> extracellular PD1<sup>+</sup>) regulatory T cells were analyzed. All the population examined were increased in patients with CD4<sup>+</sup> cell counts less than 500cells/ $\mu$ l compared with individuals with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l. These differences reached statistical significance in the case of total ( $p=0.004$ ) and naïve ( $p=0.040$ ) Treg cells (fig.15).

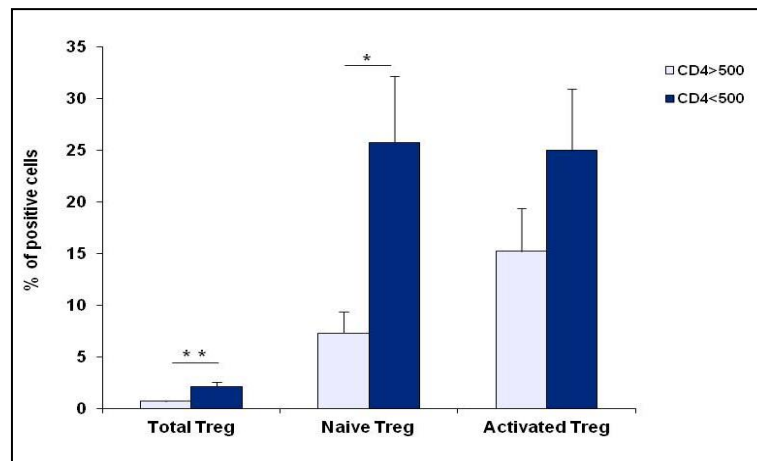


Figure 15. Percentage of Treg subpopulations in patients with CD4 cell counts >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

Total Treg cells was subsequently analyzed after stimulation of PBMC with HIV or CMV. HIV-stimulated, but not CMV-stimulated total Treg cells were significantly increased in patients with CD4<sup>+</sup> cell counts less than 500 cells/ $\mu$ l compared to patients with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l (basal:  $p = 0.045$ , and HIV-specific:  $p = 0.039$ ) as shown in figure 16.

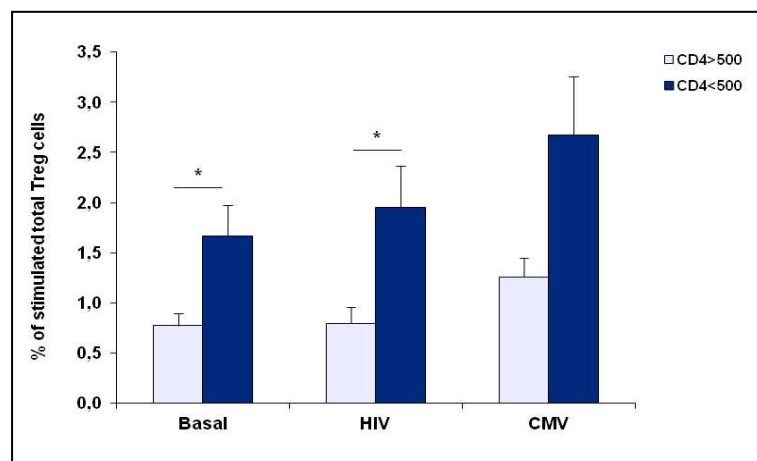


Figure 16. Percentage of total Treg in basal, HIV-, CMV-specific conditions in patients with CD4 cell counts >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p < 0.05$ ).

### 1.2.2. Immune suppressive cytokines

One of the most validated hypothesis to explain HIV-associated immune activation stems from the observation that acute HIV infection is associated with a rapid and probably irreversible destruction of the extensive CD4<sup>+</sup> T cell population that resides in gut-associated lymphoid tissues. The lamina propria macrophages can induce Treg cells differentiation and limit the generation of pro-inflammatory immune responses. Spontaneous IL-10 production by lamina propria macrophages controls their reactivity to various TLR ligands and promotes the generation of tolerogenic IL10-producing T cells and, when combined to TGF $\beta$ , Foxp3 Treg cells. Given the deleterious effects of sustained inflammatory responses, the immune system goes to great length to prevent such responses. The main mechanism involves Treg cells, a subset of CD4<sup>+</sup> T lymphocytes that suppress local T cell activation via direct and indirect mechanisms. Treg cells mediate their effect via two complementary mechanisms: IL10-mediated and TGF $\beta$ -mediated functional impairment of immune cells, and induction of apoptosis of such cells.

IL10 and TGF $\beta$  are the main cytokines produced and used by Treg cells to exert their immune suppressive activities. We decided to analyze the expression of TGF $\beta$  and IL10 in CD4<sup>+</sup> T cells following stimulation for 18 hours with HIV peptides and CMV protein (see paragraph 1.5.4 in material and methods, for further details). The results obtained showed that HIV-specific IL10<sup>+</sup>CD4<sup>+</sup> and TGF $\beta$ <sup>+</sup>CD4<sup>+</sup> T cells were augmented in patients with CD4<sup>+</sup> T cell counts less than 500 cells/ $\mu$ l compared to patients with CD4<sup>+</sup> cell counts more than 500 CD4<sup>+</sup> T cells but only IL10<sup>+</sup>CD4<sup>+</sup> parameter was statistically significant ( $p = 0.047$ ) (fig. 17 panel A); in contrast no differences were observed with CMV stimulation (fig. 17 panel B).

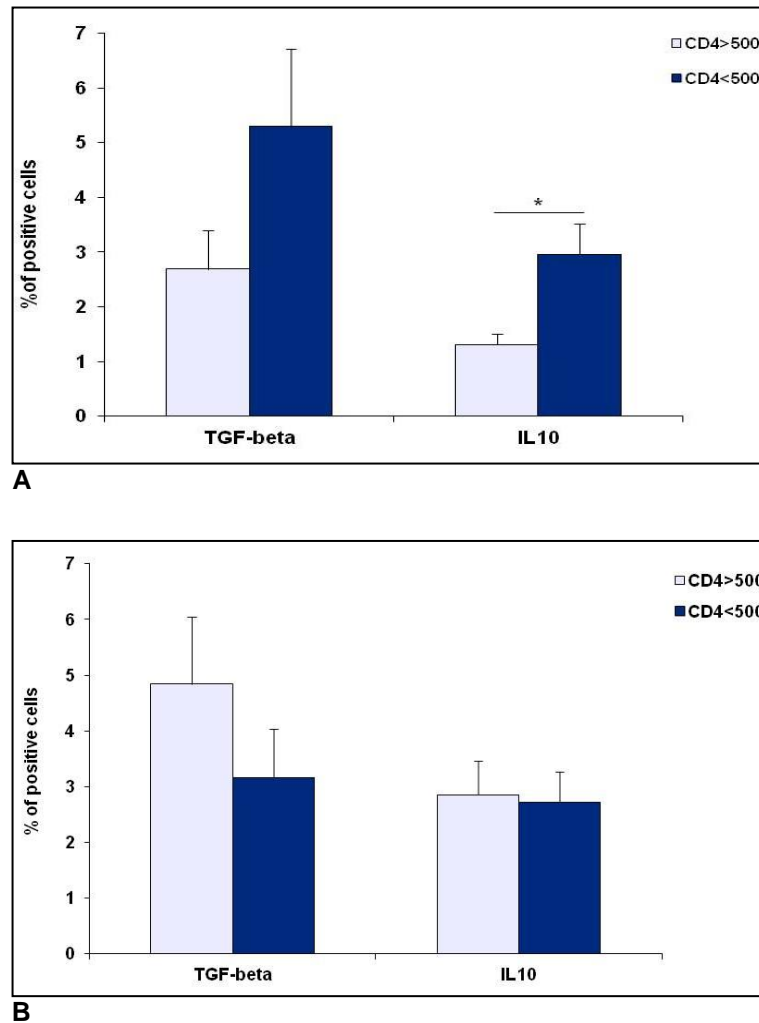


Figure 17, panel A and panel B. Percentage of HIV-specific (A) and CMV-specific (B) immune suppressive cytokines in patients with CD4 cell counts >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p < 0.05$ ).

Our results showed that HIV-stimulated production of both IL10 and TGF $\beta$  was increased in patients with CD4<sup>+</sup> cells counts less than 500 cells/ $\mu$ l in comparison to patients with CD4<sup>+</sup> cell counts more than 500 cells/ $\mu$ l.

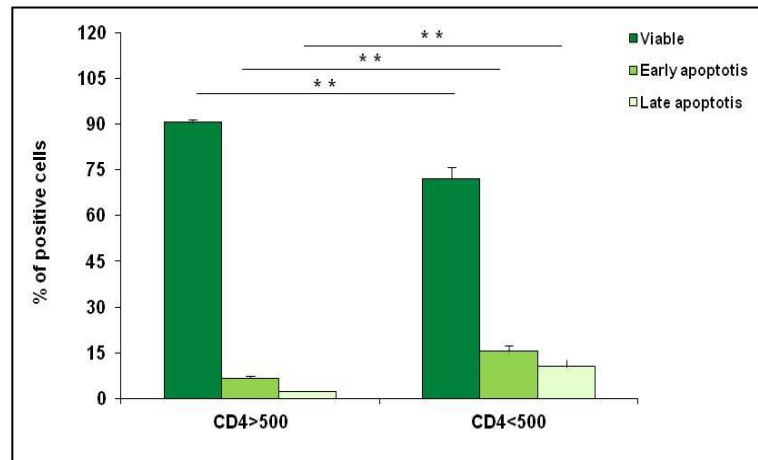
### 1.3. Evaluation of apoptosis

#### 1.3.1. Viable, early apoptotic and late apoptotic T cells

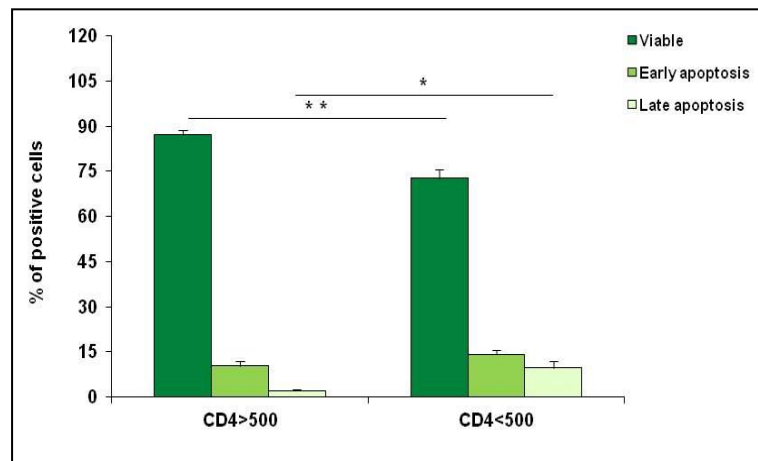
HIV infection is characterized by a profound T cell depletion that can be caused by both direct and indirect action of the virus. HIV proteins might play a role in inducing apoptosis in CD4<sup>+</sup> T cells, this cellular process may be initiated in two different ways: the extracellular way is activated consequently to intracellular signal translation by death receptors expressed on the surface of target cell, while the intracellular one is due to impairment in mitochondria permeability. Apoptosis process plays an important role in CD4<sup>+</sup> T cell depletion observed during HIV infection. We decided to investigate the role of apoptosis in the lack of CD4<sup>+</sup> T cell recovery characterizing immunological non responder patients. The distinguish between viable and necrotic cells was effectuated using 7AAD/Annexin V method (for detail see paragraph 1.5.6 in material and methods). Annexin V binds to phosphatidylserine, a phospholipid usually expressed in the intracellular side of the plasma membrane but it is the first relocated at the extracellular side during the initial phase of apoptosis. Annexin V lets to identify cells entering in the early phase of apoptosis. 7AAD is a vitality dye that enters in the cell which presents a strong impairment of the cellular membrane. Annexin V and 7AAD allow to identify cells in apoptosis and in necrosis. PBMC were evaluated in basal condition and after stimulation with HIV peptide and CMV protein. Viable cells were identified as CD4<sup>+</sup>/AnnV<sup>-</sup>/7AAD<sup>-</sup>, cells in early apoptosis as CD4<sup>+</sup>/AnnV<sup>+</sup>/7AAD<sup>-</sup> and late apoptotic cells as CD4<sup>+</sup>/AnnV<sup>+</sup>/7AAD<sup>+</sup>.

Comparing individuals with CD4<sup>+</sup> T cell count less than 500 cells/ $\mu$ l to patients characterized by T cell counts more than 500 cells/ $\mu$ l, both in basal conditions, upon HIV and CMV stimulation (fig.18 panel A, panel B, and

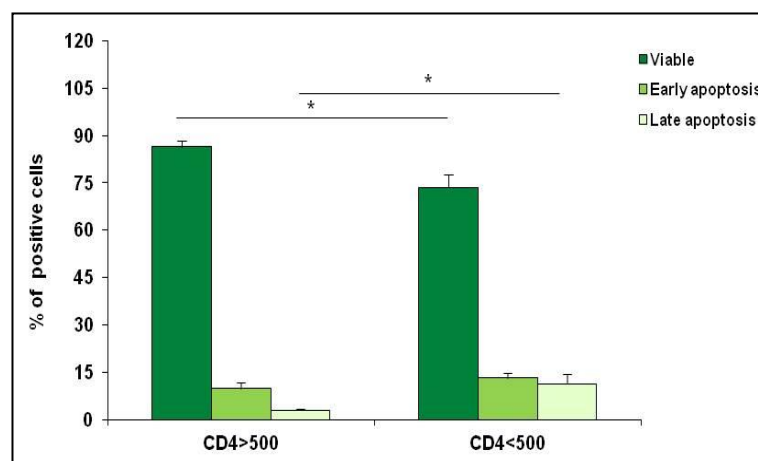
panel C), viable  $CD4^+$  T cells were diminished (viable cells: basal:  $p=0.002$ ; HIV-specific:  $p=0.003$ ; CMV-specific:  $p=0.05$ ), whereas early apoptotic and late apoptotic  $CD4^+$  T cells were augmented in individuals with  $CD4^+$  T cell counts less than  $500\text{cells}/\mu\text{l}$  (early apoptotic: basal:  $p=0.005$ ; late apoptotic cells: basal:  $p=0.005$ ; HIV-specific:  $p=0.05$ ; CMV-specific:  $p=0.05$ ).



A



B



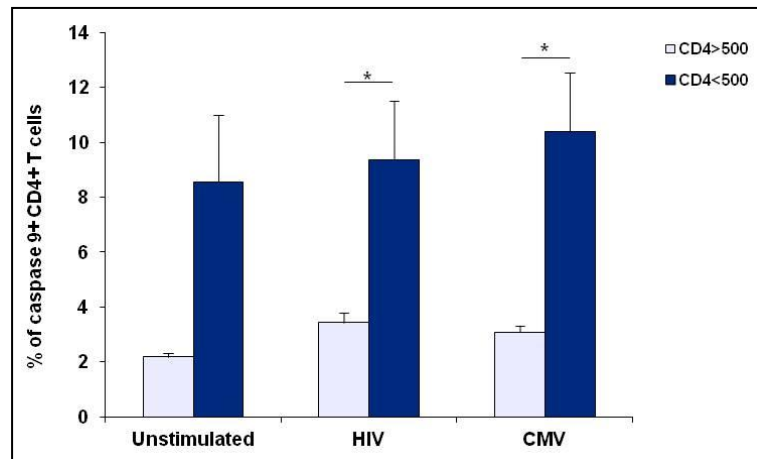
C

Figure 18, panel A, panel B and panel C. Percentage of viable ( $AnnexinV^+7AAD^-$ ), early apoptotic ( $AnnexinV^+7AAD^+$ ) and late apoptotic ( $AnnexinV^+7AAD^+$ )  $CD4^+$  T cells in patients with  $>500\text{ cells}/\mu\text{l}$  and with  $<500\text{ cells}/\mu\text{l}$ , in basal condition (A) and HIV- (B) and CMV-specific stimulation (C). Mean values, SE and P are shown (\*  $p<0.05$ , \*\*  $p<0.01$ ).

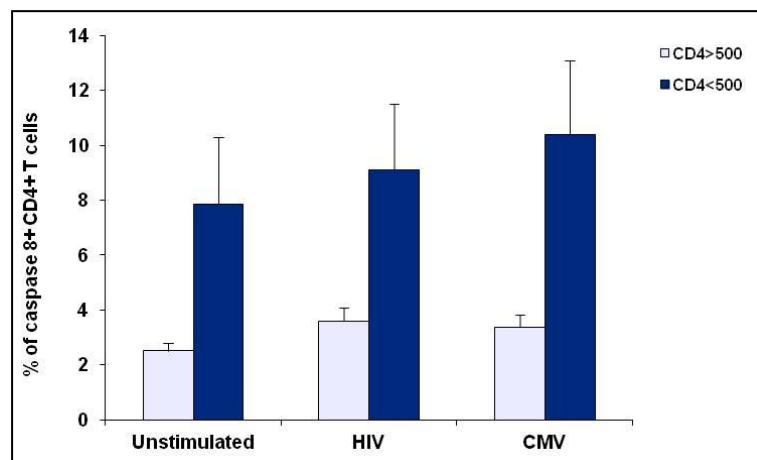
Apoptotic process is driven by specific cellular enzymes, called caspases. Caspases are enzymes involved in the apoptotic mechanism, in particular caspase 8 being specific for the extracellular way while caspase 9 being linked to the intracellular pathway.

We decided to identify the activation of both of these caspases (for details see paragraph 1.5.7 in material and methods section).

In our experiment we evaluated the presence of caspase 8 and 9 activated in CD4<sup>+</sup> T cells, the analyses were conducted in basal condition and after HIV and CMV stimulation. Results showed that expression of caspase 8 and 9 was augmented in cells of patients with CD4<sup>+</sup> T cell counts less than 500 cells/ $\mu$ l, these differences reached statistical significance for HIV-specific and CMV-specific caspase 9<sup>+</sup> CD4<sup>+</sup> cells (fig. 19 panel A, HIV-specific:  $p=0.044$ ; CMV-specific:  $p=0.026$ ). No statistical significance was reached when the expression of caspase 8 was analyzed (fig. 19 panel B).



A



B

Figure 19, panel A and panel B. Percentage of caspase 9<sup>+</sup> CD4<sup>+</sup> (A) and of caspase 8<sup>+</sup> CD4<sup>+</sup> (B) in unstimulated-, HIV- and CMV-condition in patients with CD4 cell count >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p<0.05$ ).

### 1.3.2. Blocking of PD1/PDL1 pathway and its effect on cell vitality

Because activated Treg cells express PD1, and the PD1/PDL1 pathway is fundamental importance in inducing apoptosis of CD4<sup>+</sup> T cells, we analyzed this pathway adding to the cultures antibodies able to block this interaction. PD1 blocking resulted in an increase of viable and a decrease of early and late apoptotic cells in individuals with CD4<sup>+</sup> T cell counts less than 500 cells/ $\mu$ l (fig. 20 panel B), whereas no effects were observed in patients with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l (fig. 20 panel A).

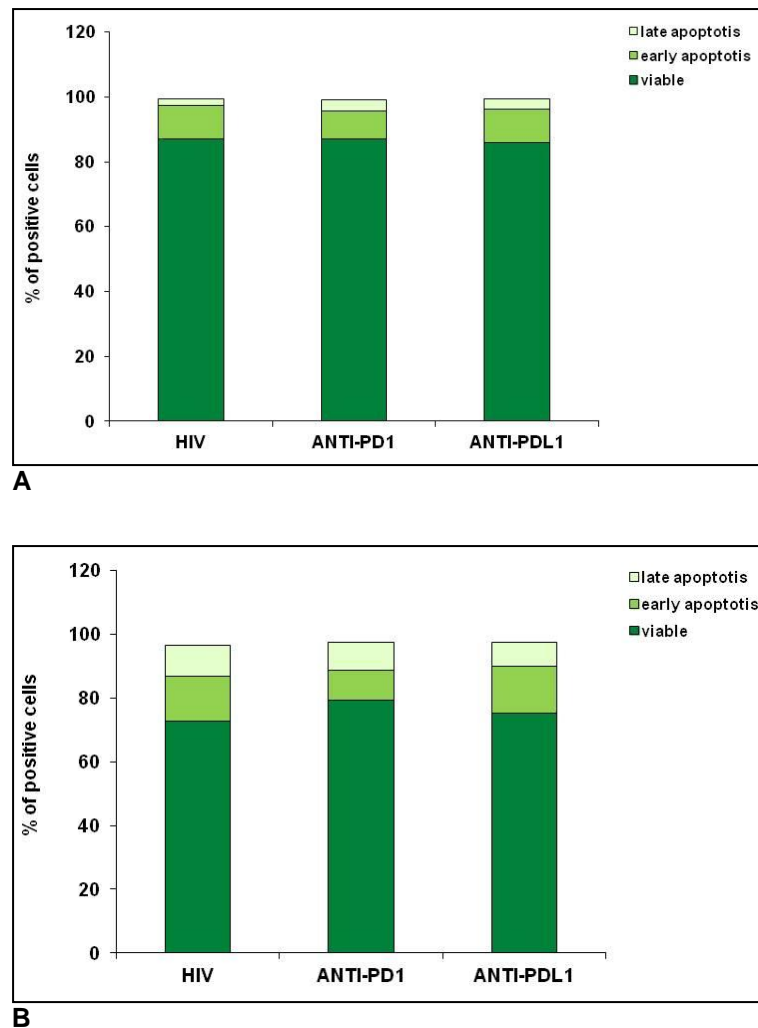


Figure 20, panel A and panel B. Percentage of viable ( $AnnexinV^+7AAD^-$ ), early apoptotic ( $Annexin^+7AAD^-$ ) and late apoptotic ( $AnnexinV^+7AAD^+$ ) in HIV stimulated condition in absence/presence of anti-PD1 or anti-PDL1 antibodies in patients with  $CD4$  cell count  $>500$  cells/ $\mu$ l (A) and with  $<500$  cells/ $\mu$ l (B). Mean values are shown.

Considering all these data just presented herein it can be inferred that all populations of Treg cells were indeed augmented in patients in whom HAART does not increase  $CD4^+$  cell counts, suggesting a full-blown effort of the immune system in the attempt to reduce immune activation. Treg cells mediate their effect via two complementary mechanisms: IL-10-mediated and  $TGF\beta$ -mediated functional impairment of immune cells, and induction of apoptosis of such cells. Our results show that both mechanisms are active in patients in whom HAART does not increase  $CD4^+$  cell counts. Thus, in these patients HIV-stimulated production of IL10 and  $TGF\beta$  was increased, and expression of caspases 8 and 9 (apoptotic cells), as well as early and late apoptotic  $CD4^+$  T cells, were augmented.

Preliminary results showing that in individuals with  $CD4^+$  T cells less than 500 cells/ $\mu$ l the blockage of the PD1-PDL1 pathway, a mechanism of pivotal importance in inducing apoptosis, induces an increase in viable and a decrease of early and late apoptotic cells indicate that this pathway likely plays a role in lack of  $CD4$  recovery in HAART-treated individuals.

#### 1.4. Immune activation in HIV infection

HIV infection is strongly characterized by a persistent immune depletion of  $CD4^+$  T cells that mainly occurs in the gastrointestinal tract during the early phase of the disease. It has been supposed that  $CD4^+$  T cell depletion and progression to AIDS might be dependent on chronic generalized immune activation.

Generalized immune activation might be due to direct viral action and, considering that loss of  $CD4^+$  T cells occurs mainly in mucosal sites, also for impairment of the mucosal barrier. The mucosal barrier represents a defense mechanism by which the organism can be preserved from microbial invasion and consequently a damage of the barrier allows microbial translocation. Microbial products invade submucosal tissues and induce the activation of the immune system.



### 1.4.1. Percentage of activated CD4<sup>+</sup> T lymphocytes

One of the first event, characterizing early phase of HIV infection, is the hyperactivation of CD4<sup>+</sup> T lymphocytes in response to persistent antigenic stimulation. T lymphocytes activation was evaluated by KI67 expression in both basal and stimulated conditions (see paragraph 1.5.5 in material and methods, for further details). The percentage of CD4<sup>+</sup>/KI67<sup>+</sup> T cells was significantly augmented in individuals with CD4<sup>+</sup> cell counts less than 500cells/ $\mu$ l compared with individuals with more than 500cells/ $\mu$ l. As shown in fig. 21, this difference was detected both in unstimulated cells and upon stimulation with HIV (unstimulated:  $p=0.032$ ; HIV-specific:  $p=0.027$ )

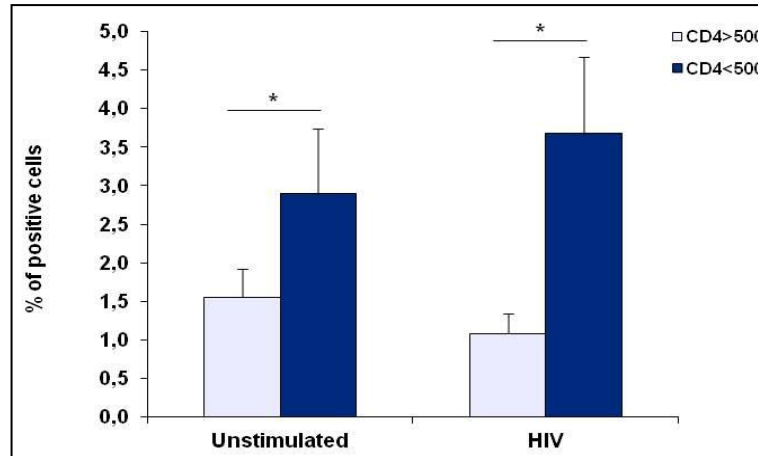
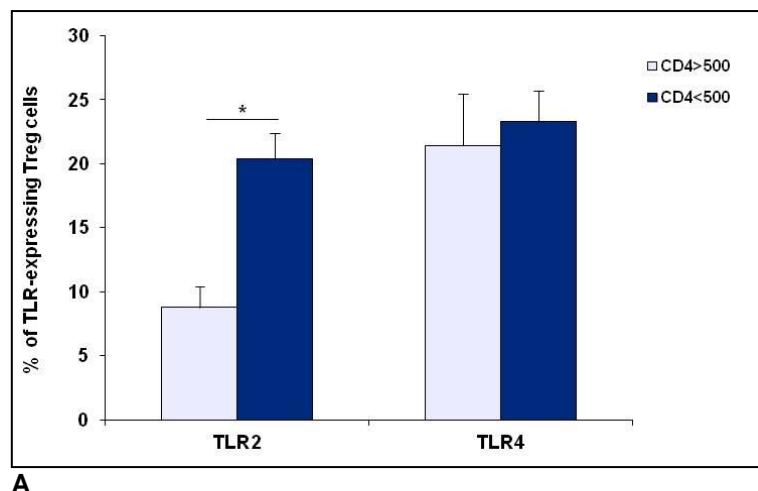


Figure 21. Percentage of KI67<sup>+</sup>CD4<sup>+</sup> T cells in patients with CD4 cell count >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l in basal and HIV-specific stimulated condition. Mean values, SE and P are shown (\*  $p<0.05$ ).

### 1.4.2. Identification of TLR-expressing Treg cells

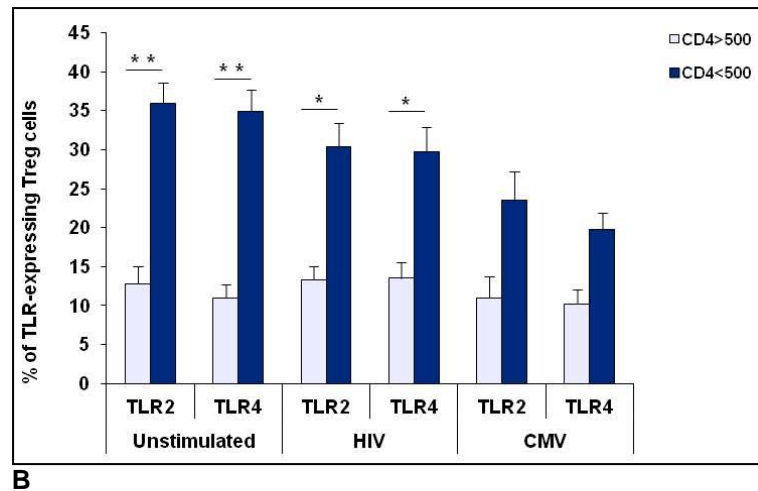
Loss mucosal integrity results in impaired local cellular immunity and translocation of microbial products, including LPS, which in turn contributes to persistent inflammation through TLR activation. Because LPS ligates TLR4, a molecule expressed on a variety of immune cells, the LPS-TLR4 axis has repeatedly been postulated as being responsible for HIV-associated immune activation. Recent evidences in literature report the expression of TLR2 and TLR4 on Treg cells and underline how TLR expression might display different effect on Treg activity, Treg cells activation mediated by TLR induce an increase their survival and their immune suppressive actions.

We analyzed the expression of these two TLRs on Treg cells in both basal and after 18 hours of antigen-specific stimulations (see paragraph 1.5.3 in material and methods, for further details). As shown in figure 22 panel A, basal TLR2- and TLR4-expressing Treg cells were augmented in patients with CD4<sup>+</sup> cell counts less than 500 cells/ $\mu$ l compared with patients with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l (TLR2:  $p=0.05$ ). After 18 hours of stimulation, as shown in figure 22 panel B, results showed that unstimulated TLR2-expressing and TLR4-expressing and HIV-stimulated Treg cells were significantly increased in patients with CD4<sup>+</sup> cell counts less than 500 cells/ $\mu$ l compared with patients with more than 500 CD4<sup>+</sup> T cells/ $\mu$ l (unstimulated: TLR2:  $p=0.005$  and TLR4:  $p=0.001$ ; HIV-specific: TLR2:  $p=0.037$  and TLR4:  $p=0.048$ ).



A

Figure 22, panel A. Percentage of TLR2- and TLR4- expressing Treg cells in basal condition in patients with CD4 count >500 cells/ $\mu$ l and with CD4 count <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p<0.05$ ).



B

Figure 22, panel B. Percentage of TLR2- and TLR4- expressing Treg cells in unstimulated and upon HIV-, CMV-specific stimulation in patients with CD4 count >500 cells/ $\mu$ l and with CD4 count <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

We observed significantly increased percentages of HIV-specific TLR2 and TLR4-expressing Treg cells in patients in whom HAART does not increase CD4<sup>+</sup> T lymphocytes.

#### 1.4.3. Plasma LPS concentration

Brenchley was the first that proposed the hypothesis that HIV immune activation might mainly be due to a disruption in the gastrointestinal barrier. Early stage of HIV infection is characterized by an impairment of mucosal barrier that allows translocation of microbial products, like LPS. Microbial products contribute to maintain an inflammatory and hyperactivation status leading to a profound depletion of gut CD4<sup>+</sup> T lymphocytes.

Plasma LPS concentration is an ideal index to detect microbial translocation and its augmented concentration has been directly correlated with an alteration of gut permeability.

According to this hypothesis we decided to evaluate plasma LPS concentration in our population enrolled (see paragraph 1.5.7 in material and methods, for further details).

Our data showed that plasma LPS concentrations were significantly higher in individuals with CD4<sup>+</sup> cell counts less than 500 cells/ $\mu$ l compared with individuals with more than 500 cells/ $\mu$ l (fig. 23,  $p < 0.001$ ).

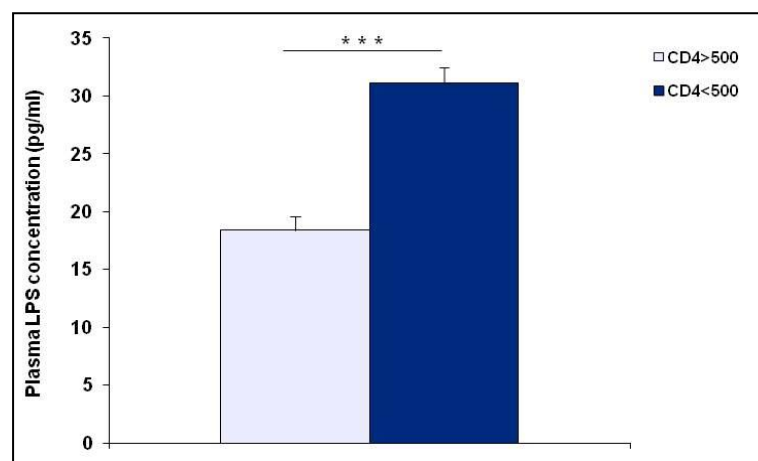
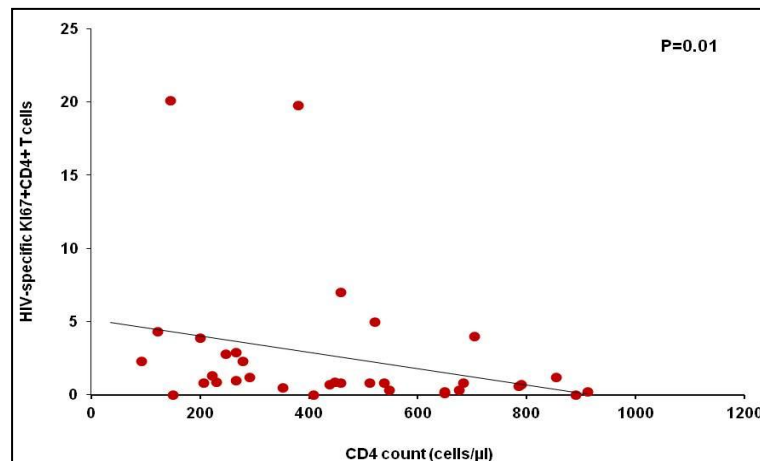
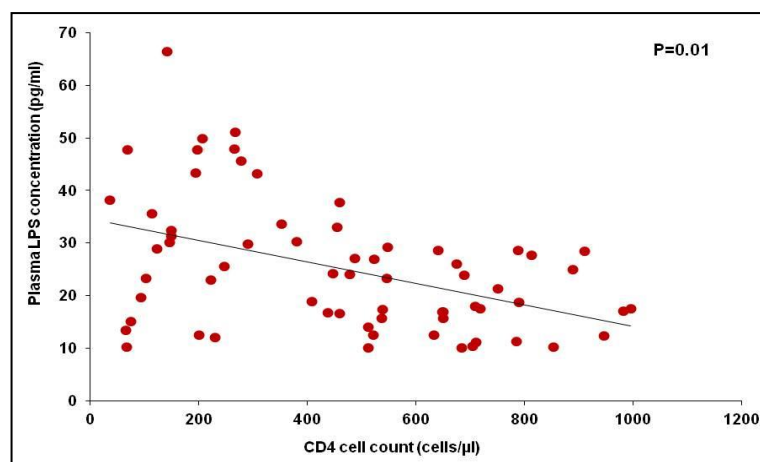
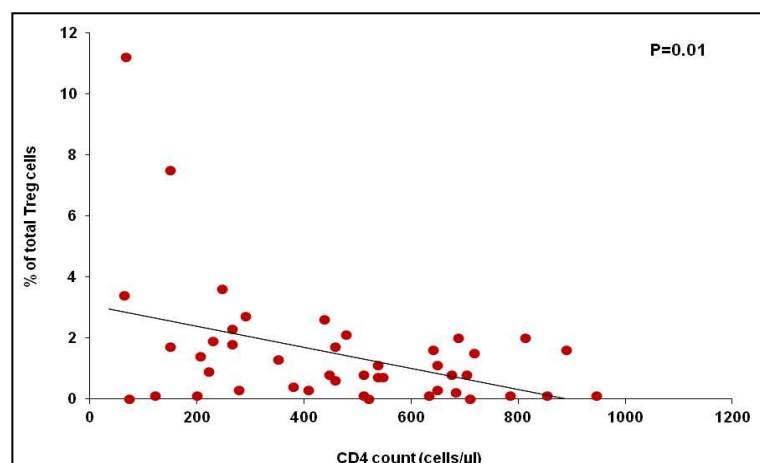


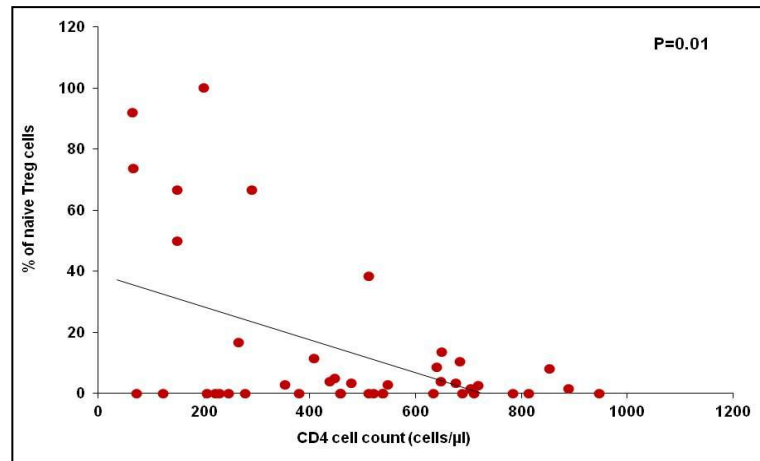
Figure 23. Plasma LPS concentration in patients with CD4 cell count >500 cells/ $\mu$ l and with <500 cells/ $\mu$ l. Mean values, SE and P are shown (\*\*\*)  $p < 0.001$ .

Notably, serum concentrations of LPS were significantly increased as well in the same patients in whom HAART does not increase CD4<sup>+</sup> T lymphocytes. Altered gut permeability resulting in increased LPS serum concentrations would trigger TLR4-mediated immune activation of multiple cell types, including Treg cells. The presence of significant negative correlations between CD4<sup>+</sup> T lymphocytes, immune activation, microbial translocation, and CD4 cell counts indicate that these parameters likely play a pathogenetic role in these patients.

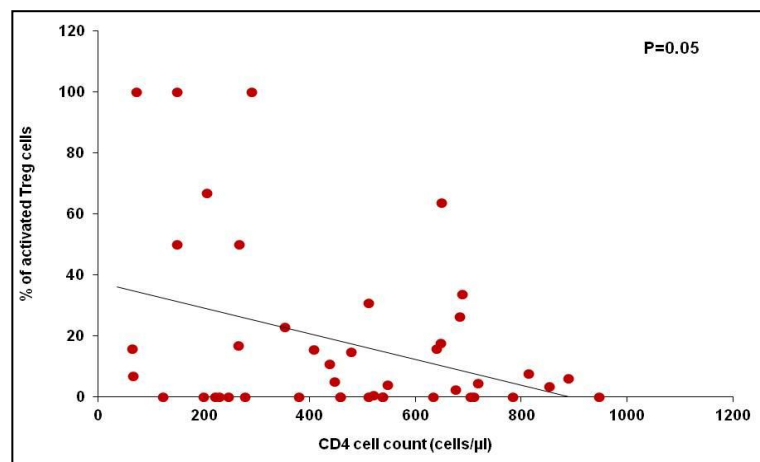
### 1.5. Correlations

Impairment of immune responses observed during HIV infection is supposed to be due to several mechanism. In this first part of the study we have evaluated different parameters linked to immune activation and immune suppression. It's thought that they are both strictly correlated. Correlation analyses were conducted between CD4<sup>+</sup> T cells, lymphocyte activation, LPS concentration, Treg cells and apoptosis. Significant negative correlations were observed between CD4<sup>+</sup> T cell counts and HIV-specific CD4<sup>+</sup>/KI67<sup>+</sup> cells (fig. 24 panel A, Pearson correlation:  $P=0.01$ ), plasma LPS concentration (fig. 24 panel B), percentage of total Treg cells (fig. 24 panel C, Pearson correlation:  $P=0.01$ ), percentage of naive Treg cells (fig. 24 panel D, Pearson correlation:  $P=0.01$ ), percentage of activated Treg cells (fig. 24 panel E, Pearson correlation:  $P=0.05$ ) and percentage of HIV-specific late apoptotic CD4<sup>+</sup> T cells (fig. 24 panel F, Pearson correlation:  $P=0.01$ ).

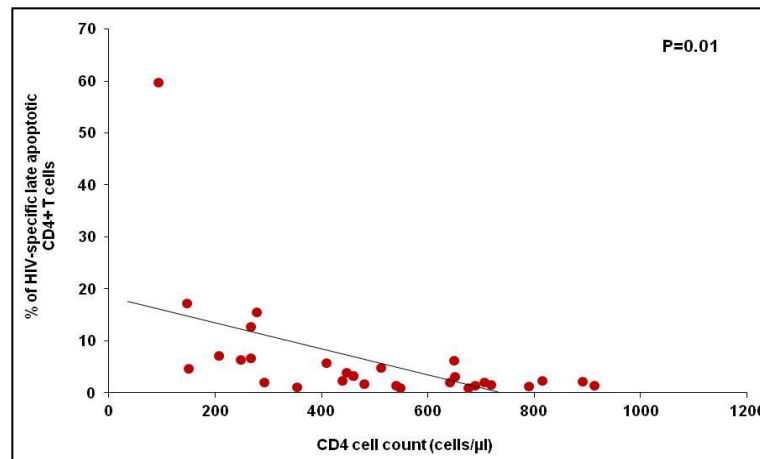
**A****B****C**



D



E



F

Figure 24, panel A, panel B, panel C, panel D, panel E and panel F. Correlation between percentage of HIV-specific  $KI67^+CD4^+$  cells (A), plasma LPS concentration (B), percentage of total Treg cells (C), percentage of naïve Treg cells (D), percentage of activated Treg cells (E) and percentage of HIV-specific late apoptotic  $CD4^+$  cells (F) and CD4 cell count in HIV-infected HAART-treated patients. Pearson correlation values are shown.

Analyses of correlation clearly showed the strict negative relationship between immune activation, microbial translocation, immune suppression, apoptosis and nadir of  $CD4^+$  T cells previously supposed: a low  $CD4^+$  T cell counts pre-therapy is a negative prognostic factor of a better outcome of the disease. Results herein suggest a multifactorial model explaining failed  $CD4$  recovery in successfully HAART-treated patients.

Thus, altered gut permeability resulting in increased LPS serum concentrations would trigger TLR4-mediated immune activation of multiple cell types, including Treg cells. Activated Treg lymphocytes could prevent full CD4<sup>+</sup> T cell reconstitution via both direct (apoptosis) and indirect (immunosuppressive cytokines) mechanisms. The observation that, with the exception of caspase 9-expression, all the immune parameters examined are altered upon HIV but not CMV stimulation, deserves further analyses and underlines the extreme expansion of HIV-specific lymphocytes in this infection. From a clinical standpoint, the observation that lower nadir likely result in a more difficult increase of CD4<sup>+</sup> T cells heightens the suggestion that the CD4 threshold actually used to start HAART could be reassessed, and reinforces the need for effective immune modulators in the therapy of HIV infection.

## 2. IMMUNE MODULATORY EFFECTS OF HCQ

### 2.1. Study population

HCQ is an antimalarial drug which displays also immune modulating and antiviral effects. We decided to test its activity in patient non responder to antiretroviral therapy.

The clinical and demographic characterization of the patients enrolled in the study is presented in table 2. HCQ was well tolerated in all patients with the exception of one individual who reported a maculopapular exanthema after 10 days of treatment. The individual was excluded from the final analyses. HIV viremia was undetectable throughout the study period.

Parameter	HCQ patients (n=20)
Age (year)	49.78±2.92
Sex (male/female)	14/6
HIV infection	5.33±1.38
Duration of HAART (year)	4.43±1.22
<b>Viral load, copies HIV RNA/ml</b>	
Baseline	<37
6 months of HCQ treatment	<37
2 months after HCQ interruption	<37
<b>Coinfections</b>	
HBV	1/20
HCV	4/20
<b>HAART treatment, n</b>	
PI	10
NNRTI	7
Other	3

Table 2. Clinical and epidemiologic characteristics of HCQ-treated patients

The percentage of CD4<sup>+</sup> T cells was significantly augmented after six months of HCQ treatment ( $p<0.001$  fig. 25 panel A) without changes in total lymphocytes counts as shown in figure 25 panel B and this increase in percentage was retained 2 months after suspension ( $p=0.001$ ). A similar trend was observed in CD4<sup>+</sup> T cell count even if changes did not reach statistical significance (fig. 25 panel C).

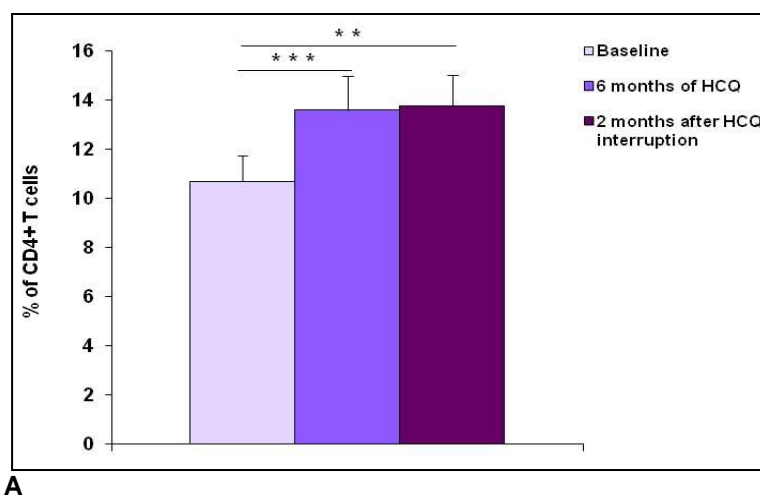


Figure 25, panel A. Percentage of CD4<sup>+</sup> T cells in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown ( \*\*  $p<0.01$ , \*\*\*  $p<0.001$ ).

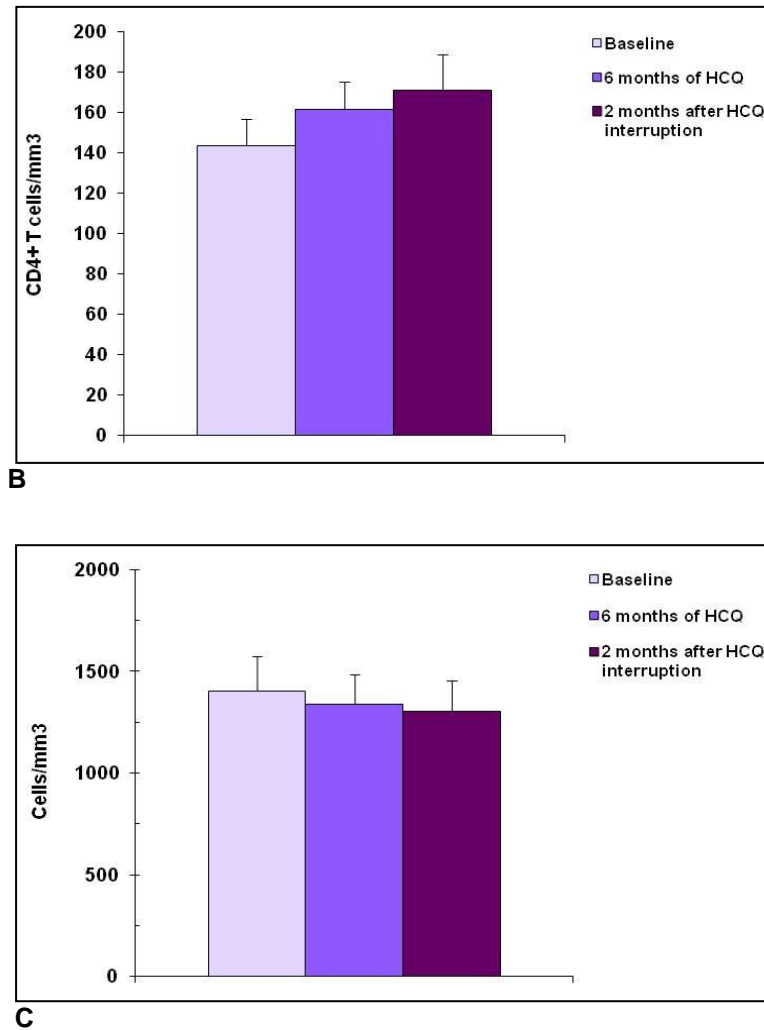


Figure 25, panel B and panel C. Percentage of CD4<sup>+</sup> T cell counts (B) and total lymphocytes count (C) in HIV-infected INR patients enrolled in the study. Mean values and SE are shown.

## 2.2. Evaluation of immune activation

### 2.2.1. Identification of activated lymphocytes and monocytes

Immune activation results in the detection in peripheral blood of increased quantities of subsets of immune cells expressing particular proteins. To evaluate a possible immunomodulatory effect of HCQ on this immune activation, we analyzed such subsets in all the individuals enrolled in the study. The best markers of immune activation are HLA-DRII, CD38 and CD69. HLA-DRII is constitutively expressed on APC, it's not expressed by most T cells but a subset of activated T cells become HLA-DRII<sup>+</sup> during immune response and it's a hallmark of HIV disease progression. CD38 is constitutively expressed by naïve T cells, down-regulated in memory cells and upregulated in activated cells. CD69 is a glycoprotein expressed following activation in all bone marrow-derived cells except erythrocytes. CD69 crosslinking induces TGF- $\beta$  production in both subpopulation of T cells and in macrophages. Finally activation of CD4<sup>+</sup> T lymphocytes is detected with the intracellular marker KI67 [123, 124].

CD4<sup>+</sup> T cell activation was evaluated in unstimulated cells and HIV-specific stimulated cells. As shown in figure 26 panel A and panel B, the percentage of KI67-expressing CD4<sup>+</sup> T cells (activated T cells) was significantly reduced after 6 months of HCQ in both unstimulated (panel A,  $p=0.005$ ) and in stimulated condition (panel B, HIV-specific:  $p=0.003$ ). This effect was retained 2 months after HCQ interruption (unstimulated,  $p=0.024$ ; HIV-specific:  $p=0.044$ ).

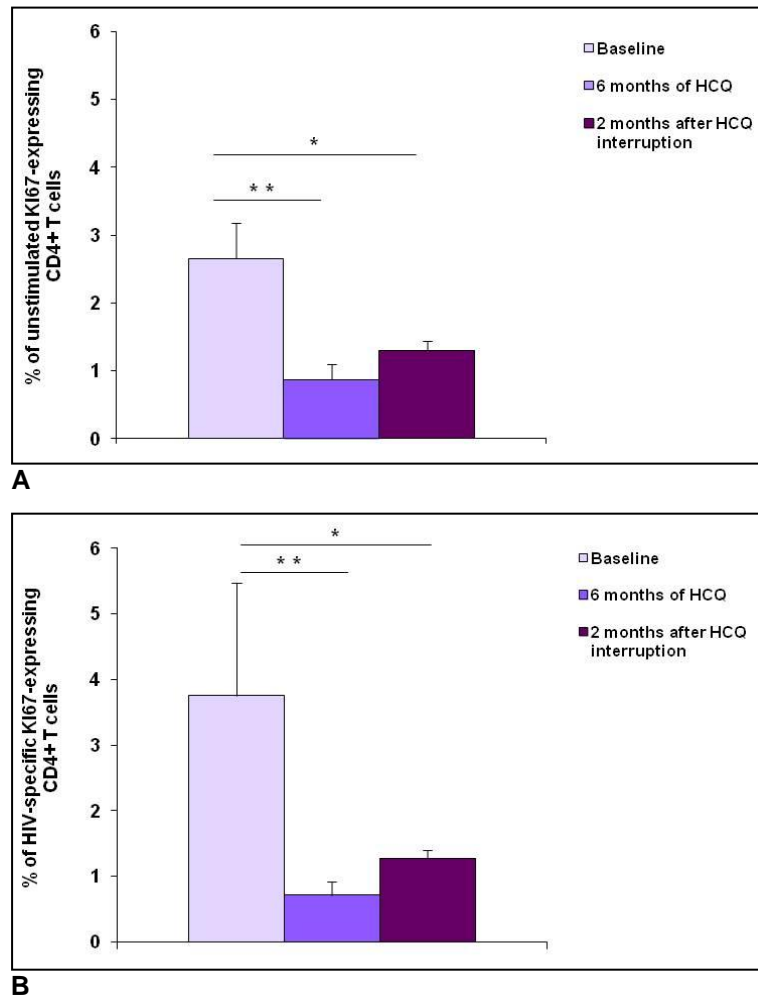


Figure 26, panel A and panel B. Percentage of KI67<sup>+</sup>CD4<sup>+</sup> T cells in unstimulated (A) and HIV-specific stimulation (B) in HIV-infected INR patients enrolled in the study. Mean values, SE and P values are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

The percentage of HLA-DRII-, CD69-, and CD38/CD45RO-expressing CD8<sup>+</sup> T cells decreased as well, albeit not significantly, after 6 months of HCQ treatment. A different pattern was seen when these populations were analyzed 2 months after HCQ interruption. Thus, HLA-DRII<sup>+</sup>CD8<sup>+</sup> cells were significantly reduced both in comparison with baseline and with month 6 ( $p = 0.013$  and  $p = 0.05$ , respectively, figure 27 panel A), whereas CD69<sup>+</sup>CD8<sup>+</sup> (figure 27 panel B) and CD38<sup>+</sup>CD45RO<sup>+</sup>CD8<sup>+</sup> (figure 27 panel C) T cells were significantly augmented compared with month 6 ( $p = 0.043$  and  $p = 0.039$  respectively).

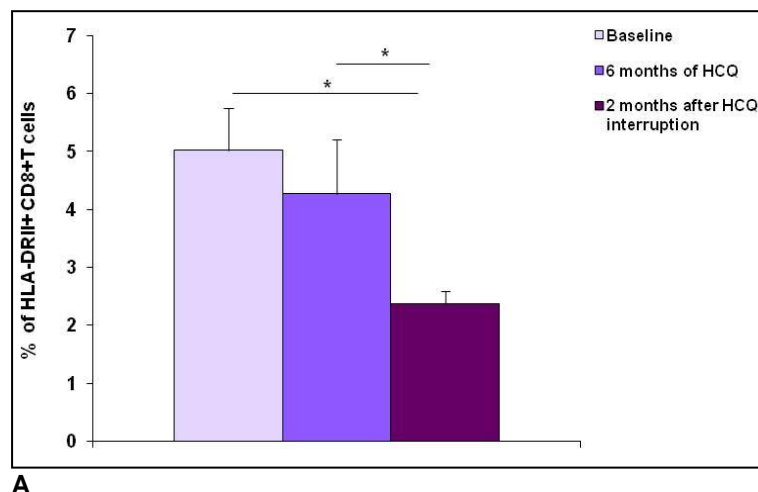
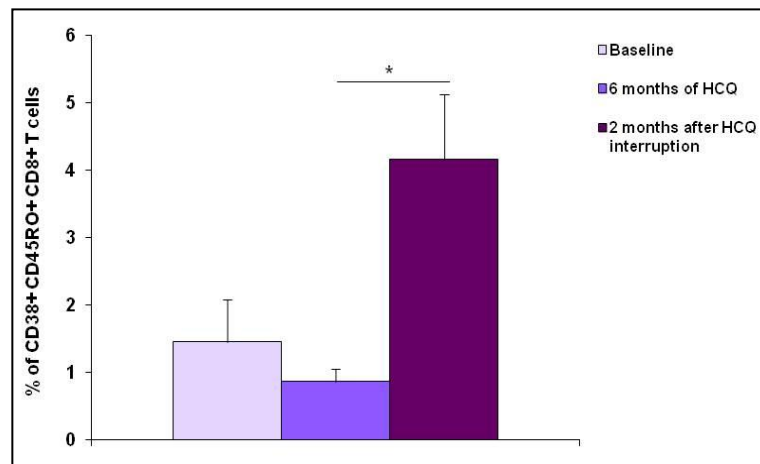
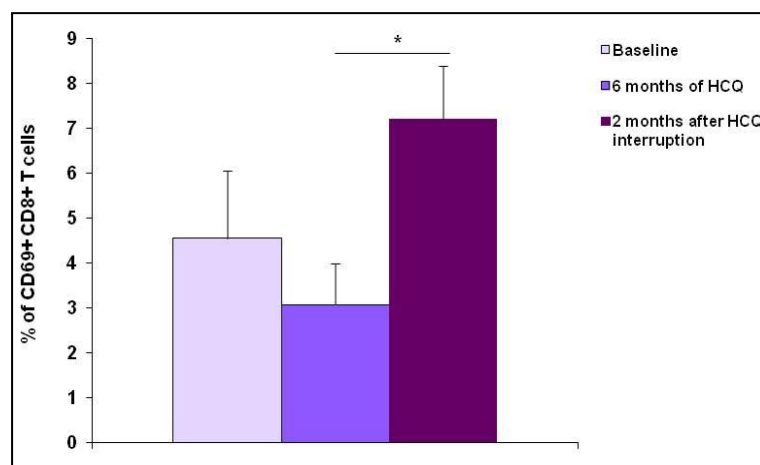


Figure 27, panel A. Percentage of HLA-DRII<sup>+</sup>CD8<sup>+</sup> T cells in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ ).





B

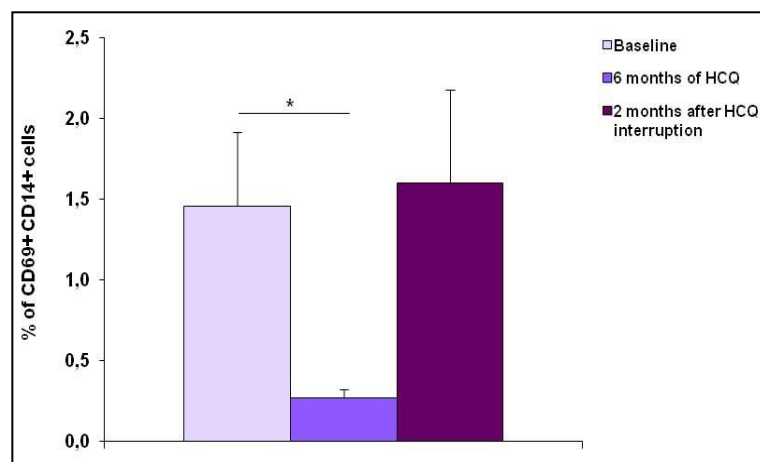


C

Figure 27, panel B and panel C. Percentage of  $CD69^+CD8^+$  T cells (B) and percentage of  $CD38^+CD45RO^+CD8^+$  T cells (C) in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ ).

The existence of a positive correlation between the activated status of monocytes and progression of HIV infection [125], is well documented.

$CD69$ -expressing  $CD14^+$  cells (figure 28) were also significantly reduced after 6 months of HCQ treatment ( $p=0.041$ ). Percentages of these cells returned to baseline values after 2 months of HCQ interruption.



D

Figure 28. Percentage of  $CD69^+CD14^+$  cells in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ ).

### 2.2.2. TLR expression on monocytes

It has been documented that HCQ down-modulates the expression of intracellular TLRs in vitro as well as in murine models. The effect of this compound in HIV infection was analyzed by evaluating TLR2, TLR4, TLR5, TLR7 and TLR8 expression on CD14<sup>+</sup> cells both in whole blood and on stimulation with specific agonist. Results obtained in whole blood showed that HCQ induced a significant reduction in TLR2-, TLR4-, and TLR5-expressing CD14<sup>+</sup> cells as shown in fig. 29 panel A and panel B (TLR2,  $p=0.032$ ; TLR4,  $p=0.004$ ; TLR5,  $p=0.008$ )

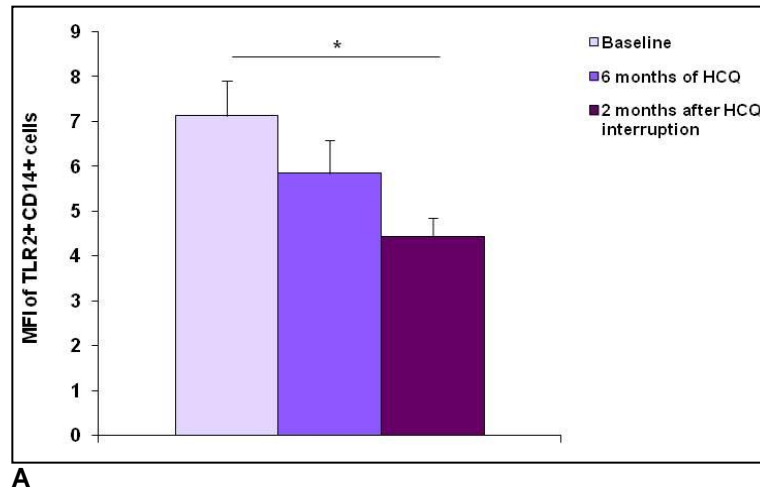


Figure 29, panel A. MFI of TLR2 on CD14<sup>+</sup> cells in whole blood in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p<0.05$ ).

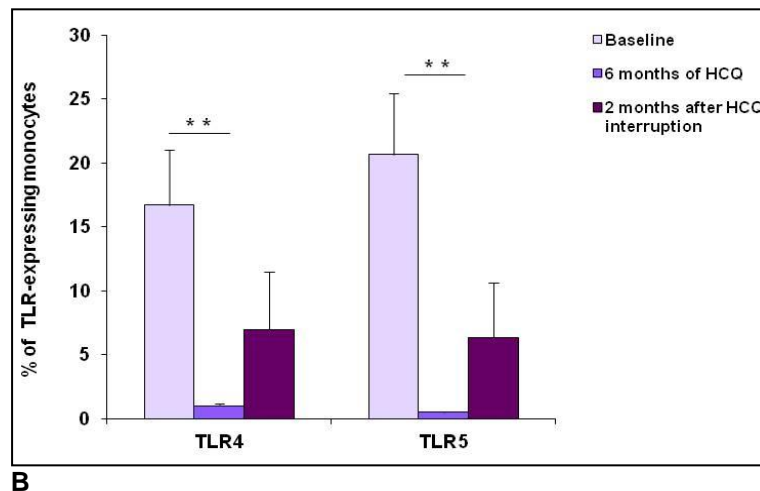
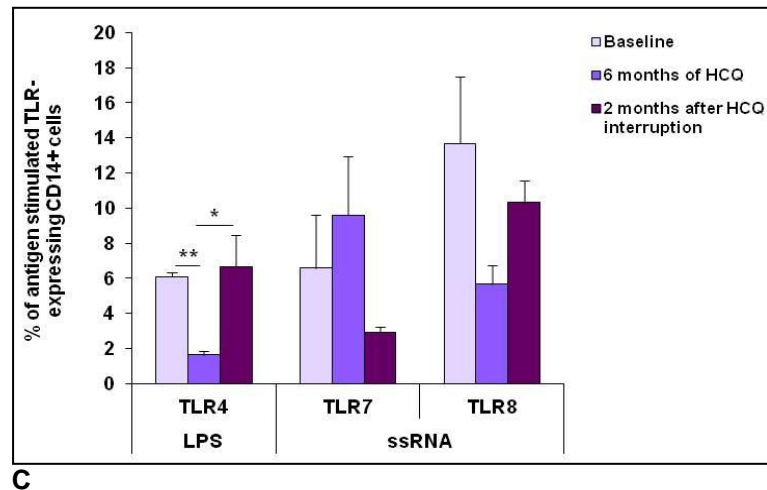


Figure 29, panel B. Percentage of TLR4- and TLR5-expressing monocytes in whole blood in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*\*  $p<0.01$ ).

Results obtained when cell cultures were stimulated with TLR agonists showed a significant reduction in LPS-stimulated cells alone (figure 29 panel C, TLR4,  $p=0.006$ ) that vanished 2 months after interruption of HCQ treatment ( $p=0.050$ ). In contrast, no changes in ssRNA-stimulated TLR7 and TLR8-expressing CD14<sup>+</sup> cells were detected (fig. 29 panel C).



C

Figure 29, panel C. Percentage of antigen stimulated TLR4-, TLR7-, TLR8- expressing monocytes (C) in HIV-infected INR patients in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

The data so far obtained allow to confirm the ability of HCQ in modulating the expression of TLRs and most of all of TLR4, directly involved in responses to microbial translocation and further analyses are required to investigate if HCQ might influence TLR intracellular signaling pathway.

Notably, HCQ-associated reduction of immune activation was mostly retained after therapy interruption and was accompanied by a real increase in CD4<sup>+</sup> T cell percentage, as total lymphocyte counts did not change during the study period. This reduction of immune activation was also associated with a similar trend, albeit not reaching statistical significance, in CD4<sup>+</sup> T cell counts. These data suggest that HCQ is effective in reducing CD4<sup>+</sup> T cells activation, resulting in an increase of CD4<sup>+</sup> T cell percentage.

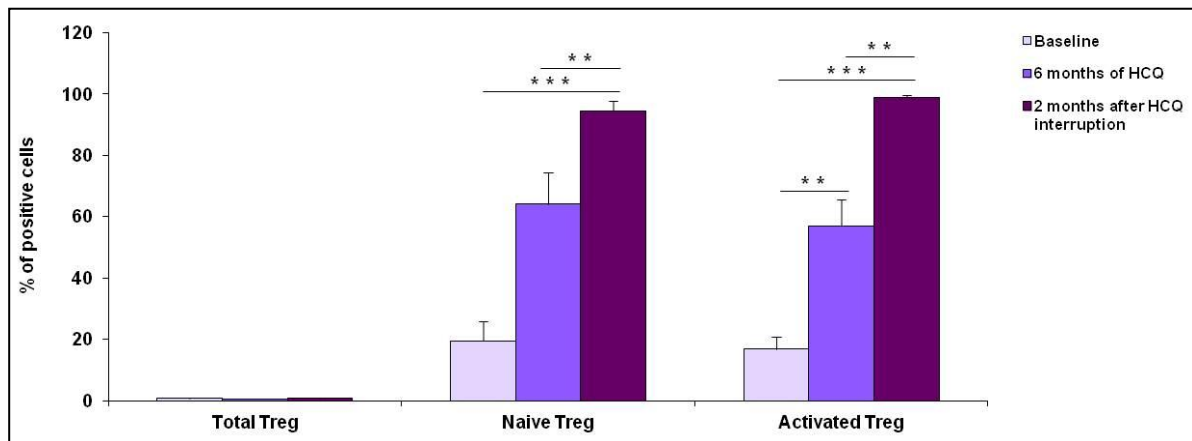
We have shown the presence of LPS/TLR-dependent immune activation in HIV-infected individuals lacking CD4 normalization during HAART, and recently a reduction in immune activation and a decrease in CD38<sup>+</sup> CD8<sup>+</sup> T cells and of KI67 memory CD4<sup>+</sup> T cells was reported in HAART-treated and naïve HIV-infected patients treated with HCQ [116]. Data herein confirm and expand those results and indicate a possible therapeutic benefit of HCQ in HAART-treated individuals in whom a satisfactory recovery of CD4<sup>+</sup> T cells is not achieved. HCQ was effective in reducing the percentage of proliferating CD4<sup>+</sup> T lymphocytes and of activated (CD69<sup>+</sup>) monocytes, whereas its effect on CD8<sup>+</sup> T cells was marginal, indicating a possible lack of effect of HCQ on this lymphocytes population.

## 2.3. Evaluation of immune suppression

### 2.3.1. Identification of Treg cells

As previously said, Treg cells are a subpopulation of CD4<sup>+</sup> T cells that exert immune regulatory activity, controlling different cellular processes.

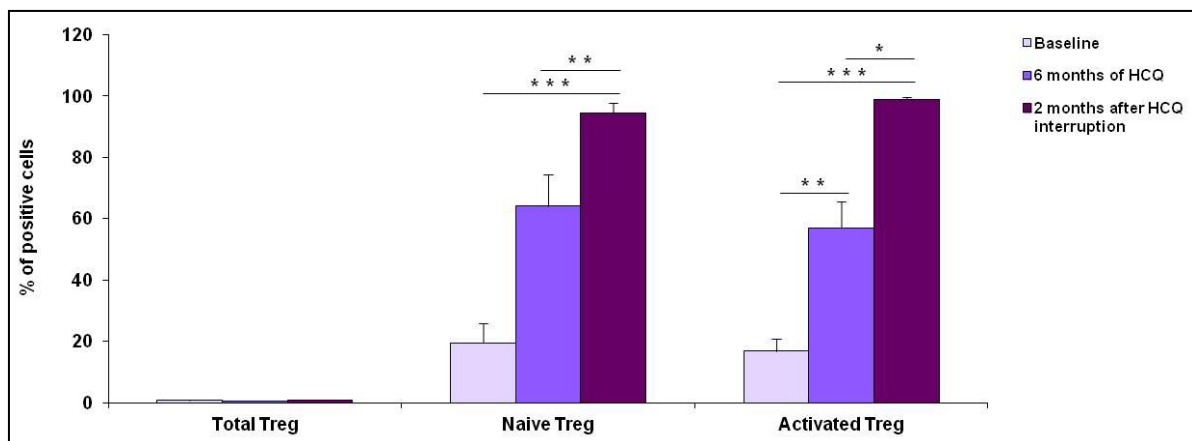
According to the immune modulating properties of HCQ we studied if Treg cells percentage was influenced by HCQ therapy. Treg cells were evaluated both in basal and with HIV-specific stimulation. Both naïve and activated Treg cells were significantly increased after HCQ; this effect persisted 2 months after HCQ interruption. In unstimulated condition (figure 30 panel A), HCQ induced a strong increase in activated Treg cells percentage after 6 month therapy (activated:  $p = 0.002$ ) and this effect was retained also after suspension of the treatment (naïve:  $p < 0.001$ ; activated:  $p < 0.001$ ) when compared to baseline. Moreover comparing the percentage of Treg cells 2 months after treatment interruption with 6 months of HCQ, a significant increase in the percentage of both naïve and activated subpopulations emerged (naïve:  $p < 0.001$ ; activated:  $p = 0.004$ ).



A

Figure 30, panel A. Percentage of subpopulations of Treg cells in unstimulated condition in HIV infected INR patients enrolled in the study. Mean values, SE and P are shown (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

Upon HIV-specific stimulation (fig. 30, panel B), we observed the same trend showed in unstimulated conditions. Comparing 6 months of treatment with baseline we observed a significant increase in the percentage of activated Treg cells ( $p = 0.001$ ); a significant augment of both naïve and activated Treg cells was found when comparing 2 months of treatment suspension with baseline (naïve:  $p < 0.001$ ; activated:  $p < 0.001$ ); and furthermore comparing 2 months after HCQ suspension with 6 months of treatment we found a significant increase in both naïve ( $p = 0.004$ ) and activated ( $p = 0.011$ ) Treg subpopulations. Percentage of total Treg cells remain unchanged throughout the study period in both unstimulated and HIV-specific stimulated condition.



B

Figure 30, panel B. Percentage of subpopulations of Treg cells in HIV-specific stimulated condition in HIV infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

Data regarding analysis of Treg subpopulations showed that HCQ induces an increase in the percentage of both naïve and activated Treg cells, supporting its role as an immune modulating agent.

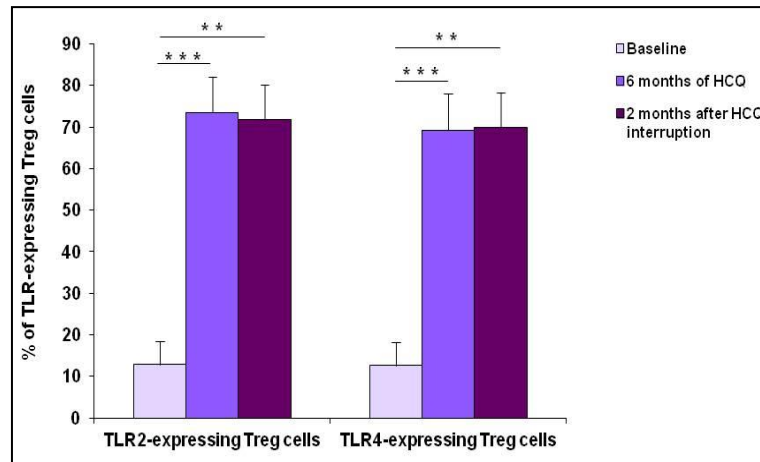
### 2.3.2. Identification of TLR-expressing Treg cells

Several studies underlined that TLR activation on Treg cells increases their survival and leads to the induction of their suppressor activity.

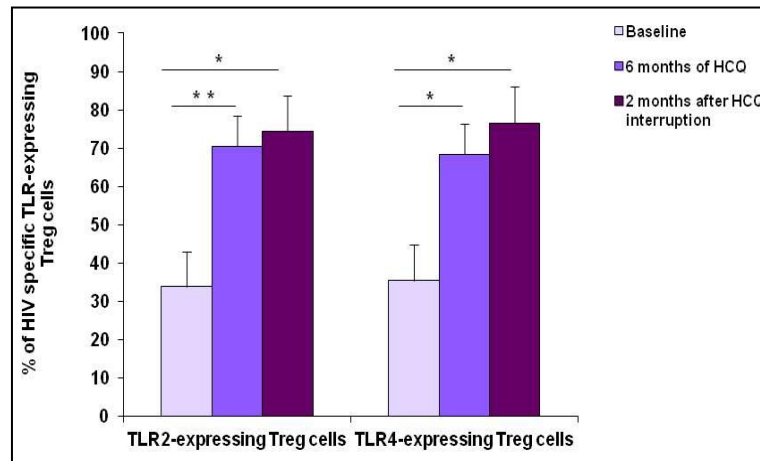
Analyses were conducted in both unstimulated (fig. 31, panel A) and stimulated conditions with HIV peptides (fig. 31, panel B) and LPS (fig. 31, panel C) (see paragraph 2.4.5. in material and methods, for details).

Our data showed that percentages of TLR2-, TLR4-expressing Treg cells in both unstimulated and stimulated conditions were significantly augmented after HCQ, and this increased was maintained 2 months after HCQ suspension in all culture conditions. In detail, in both unstimulated and HIV specific stimulation (figure 31 panel A and B), a statistically significant increase was observed when comparing 2 months after HCQ suspension with baseline (unstimulated: TLR2,  $p = 0.009$  and TLR4:  $p = 0.009$ ; HIV-specific: TLR2,  $p = 0.038$  and TLR4:  $p = 0.033$ ) and 6 months of HCQ therapy with baseline (unstimulated: TLR2,  $p < 0.001$  and TLR4,  $p < 0.001$ ; HIV-specific: TLR2,  $p = 0.005$  and TLR4,  $p = 0.019$ ). Moreover, as shown in figure 31 panel C,

the percentage of LPS stimulated TLR4-expressing Treg cells increased throughout the entire period of the study and reached a statistically significant difference when comparing 2 months after HCQ treatment interruption with baseline ( $p=0.002$ ).

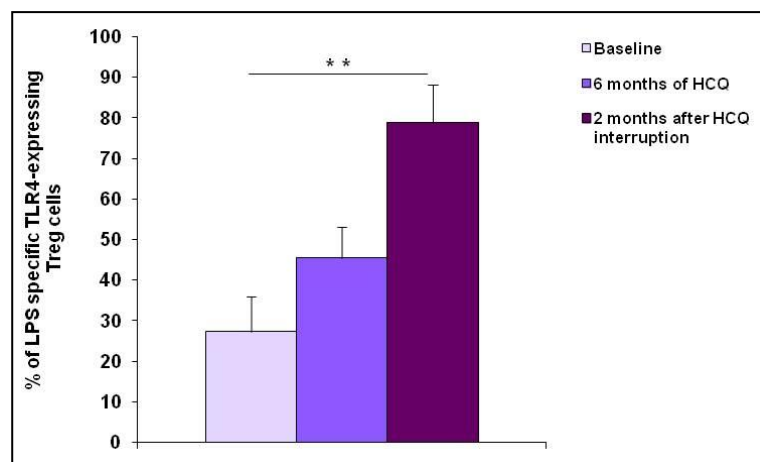


A



B

Figure 31, panel A, panel B. Percentage of TLR2 and TLR4 expressing Treg cells in both basal (A) and HIV-stimulated condition (B) in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



C

Figure 31, panel C. Percentage of LPS stimulated TLR4-expressing Treg cells in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*\*  $p < 0.01$ ).

Thus, the observed increase in Treg cells induced by HCQ was at least partially supported by TLR2- and TLR4-expressing cells.

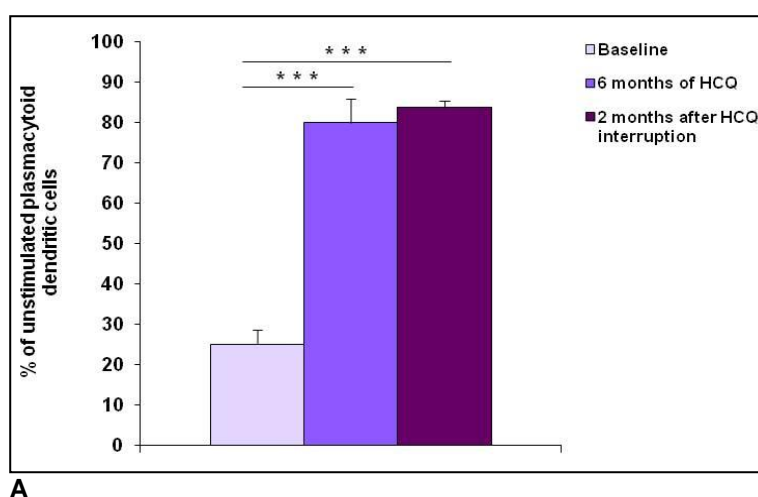
Of note, the use of this compound resulted in increased percentages of circulating Treg cells. The role of these cells in HIV infection is still not totally clarified but it is likely that the destruction or inactivation of Treg cells by HIV would result in a lack of control over immune activation. Treg-mediated suppression of antigen-specific responses *in vitro* was shown to be more effective with cells isolated from relatively healthy HIV infected individuals compared with later stage AIDS patients, suggesting that Treg cells (total or HIV-specific) are depleted and/or dysfunctional later in HIV disease [126]. HCQ treatment induced an increase in naïve and activated Treg cells both in unstimulated and in HIV-specific populations. These subpopulations of Treg cells are endowed with strong immunosuppressive activity and likely play a role in the HCQ ability to reduce immune activation. Of note, the increases in Treg cells seen as a results of HCQ were at least partially supported by TLR2- and TLR4-expressing cells; these subpopulations of Treg cells were recently shown to mediate a strong immunosuppressive activity.

## 2.4. Evaluation of antiviral response

### 2.4.1. Percentage of pDCs and expression of IFN $\alpha$

pDCs are low-frequency cell found in peripheral blood and lymphoid tissues that are best known for their ability to produce large quantities of IFN $\alpha$  in response to viruses. We decided to analyze the percentages of pDC and pDC-expressing IFN $\alpha$  during all the study and unstimulated conditions and presence of HIV-specific stimulation (see paragraph 2.4.3. in material and methods, for further details).

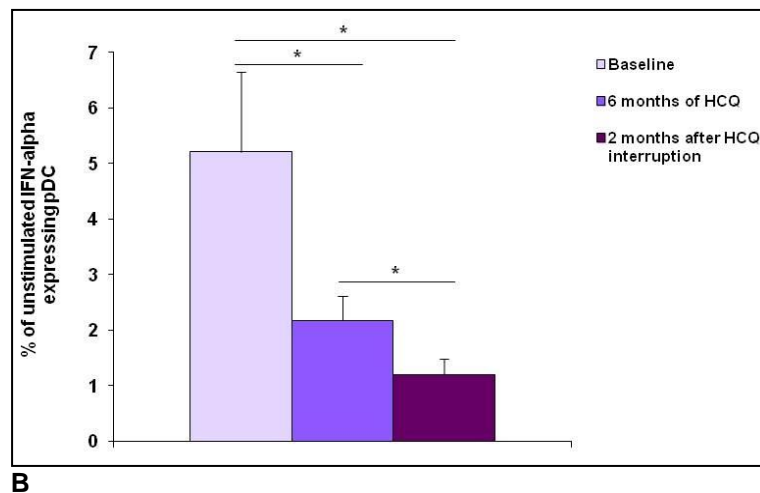
The percentage of pDCs was significantly augmented after 6 months of HCQ treatment; this effect persisted 2 months after HCQ interruption ( $p < 0.001$  in both cases, figure 32 panel A).



A

Figure 32 panel A. Percentage of plasmacytoid dendritic cells in unstimulated condition in HIV-infected INR patients enrolled in the study. mean values, SE and P are shown (\*\*\*)  $p < 0.001$ .

Furthermore, considering that pDCs are the main producer of IFN $\alpha$ , we wanted to analyzed if there were changes in the expression of this interferon by pDCs. Of note, as shown in figure 32 panel B, IFN $\alpha$ -secreting pDCs were diminished during HCQ treatment and 2 months after HCQ suspension (6 months of HCQ vs 2 months after HCQ treatment interruption,  $p = 0.036$ , baseline vs 6 months of HCQ  $p = 0.05$ , and baseline vs 2 months after HCQ suspension  $p = 0.021$ ).



**B**  
Figure 32 panel B. Percentage of IFN $\alpha$  expressing pDCs in unstimulated condition in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ ).

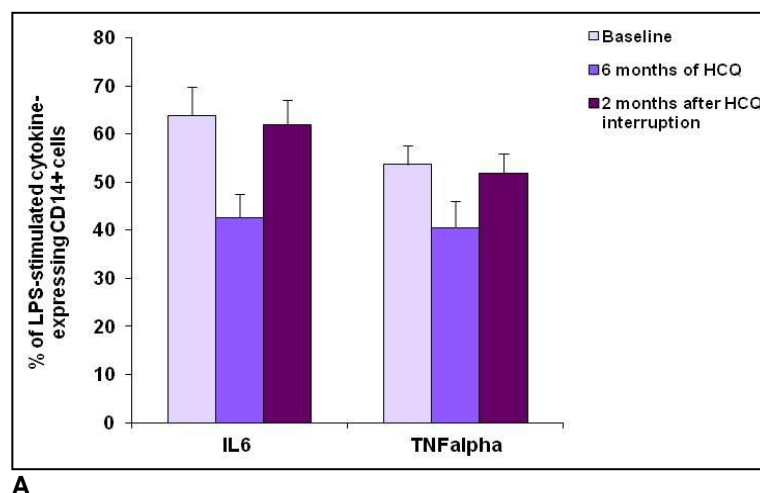
The increase in pDCs percentage is consistent with their antiviral activity and moreover supports HCQ ability in restoring immune responses. The reduction in IFN $\alpha$  expression by pDC is a positively aspect because it has been demonstrated that elevated level of this cytokine in advanced HIV disease is associated with microbial translocation and immune activation, factors worsening the progression of the disease.

An impairment of pDC function was described in HIV infection and the pre-HAART era pDCs were shown to inversely correlate with CD4<sup>+</sup> T counts and viral loads. These observations, together with the facts that HAART increases pDC counts and the higher levels of pDC are seen in long-term non progressor, led to the suggestion that pDCs play a protective role against the progression of HIV infection [127, 128, 129, 130, 131]. Although IFN $\alpha$  reduces viral burden in the acute phase of infection, elevated level of this cytokine in advance HIV disease is associated with increased microbial translocation and could contribute to immune activation. Of note, despite increasing the number of pDCs, HCQ reduced IFN $\alpha$  production by these cells. Finally, whereas pDCs activated through TLR7 and TLR9 work as antiviral cells via IFN $\alpha$ , it was recently suggested that non-IFN $\alpha$  producing pDCs can act as tolerogenic cells promoting Treg cells activation via the indoleamine 2,3-dioxygenase and PD1 pathways [118]. Data herein suggest that this phenomenon could take place in vivo in HCQ-treated individuals.

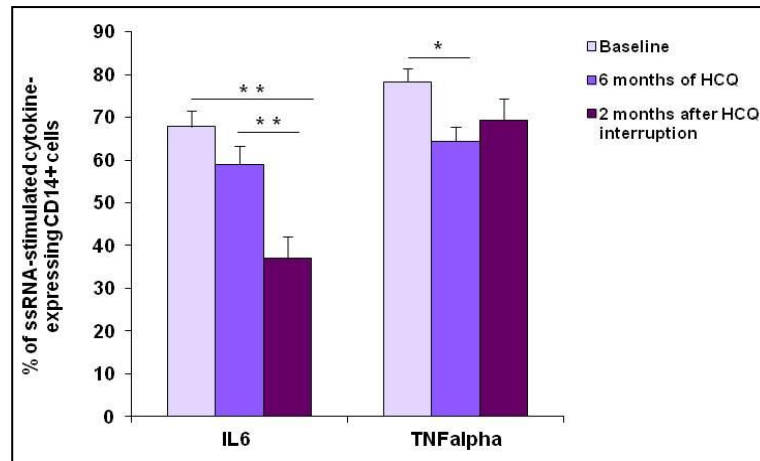
#### 2.4.2. Proinflammatory cytokines

Inflammatory state was evaluated both with flow cytometry and immune enzymatic assays.

IL6 and TNF $\alpha$  production by CD14<sup>+</sup> was analyzed in LPS- and ssRNA-stimulated conditions and IL6 and TNF $\alpha$  production by CD4<sup>+</sup> T cells was investigated env/gag-stimulated condition; the plasma concentration of these cytokines was evaluated as well. Results showed IL6 and TNF $\alpha$ -secreting CD14<sup>+</sup> (figure 33 panel A and panel B) and CD4<sup>+</sup> cells (figure 33 panel C) to be diminished after HCQ treatment, reaching statistical significance only for ssRNA-stimulated CD14<sup>+</sup> cells (2 months after HCQ vs 6 months of therapy, IL6:  $p = 0.0074$ , 2 months after treatment suspension vs baseline IL6:  $p = 0.006$ ; 6 months of therapy vs baseline, TNF $\alpha$ :  $p = 0.038$ ).

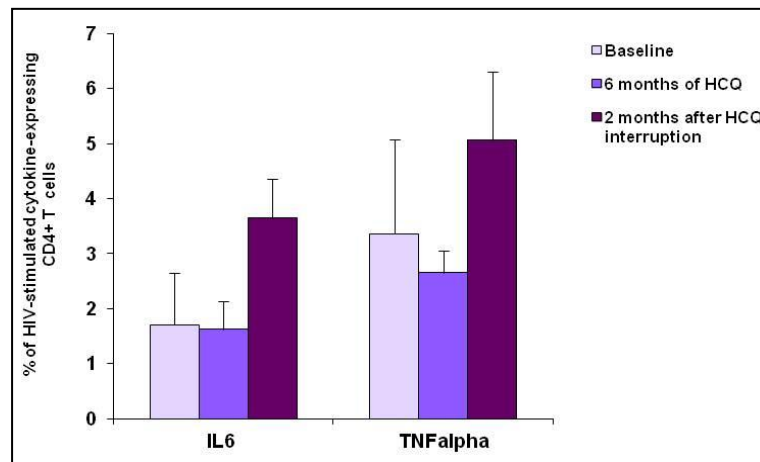


**A**  
Figure 33, panel A. Percentage of proinflammatory cytokines-expressing CD14<sup>+</sup> monocytes following LPS stimulation. Mean values and SE are shown.



B

Figure 33, panel B. Percentage of proinflammatory cytokines-expressing CD14<sup>+</sup> monocytes following ssRNA specific stimulation in HIV-infected INR patients enrolled in the study. Mean values, SE and P are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).



C

Figure 33, panel C. Percentage of proinflammatory cytokines-expressing CD4<sup>+</sup> T cells in HIV-specific stimulated condition in HIV-infected INR patients enrolled in the study. Mean values and SE.

These data from antigen stimulated conditions, showed an increase in the percentage of pro-inflammatory cytokines expressed by monocytes at baseline but once more HCQ administration was able to reduce the pro-inflammatory status. Moreover the suspension of HCQ treatment lead to a rebound in pro-inflammatory cytokines expressions suggesting once more the need for a prolonged HCQ treatment.

Immunoenzymatic assay (figure 34 panel A and panel B) showed that plasma IL6 concentration was significantly reduced after HCQ treatment (baseline vs 2 months of therapy,  $p=0.048$ ), these results are consistent with those previously obtained with flow cytometry analyses. In contrast, no changes in plasmatic TNF $\alpha$  concentrations were detected.



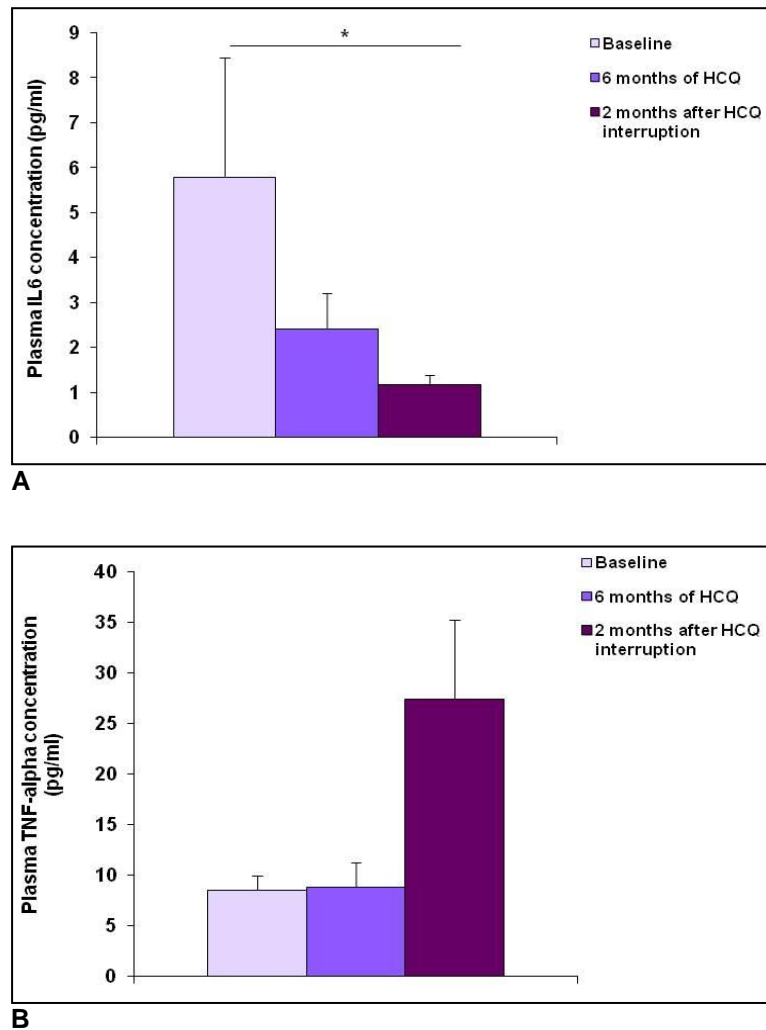


Figure 34, panel A and panel B. Plasma IL6 (A) and TNF $\alpha$  (B) concentration in HIV-infected INR patients enrolled in the study. Mean values and *P* are shown (\*  $p < 0.05$ ).

These data are in concert with the ones reported about immune activation and strengthen the possibility to exploit the immune modulating properties of HCQ in the treatment of HIV infection. The down modulation of TLR expression and the decrease production of IL6 put in evidence even more the significant effect on immune activation displayed by HCQ.

## 2.5. Evaluation of microbial translocation

The plasma LPS concentration is an index of microbial translocation; an augmented LPS concentration is associated with alterations of the gut permeability. As shown in figure 35, HCQ treatment induced a reduction in LPS plasma concentration that was maintained after interruption of therapy (baseline vs 2 months after interruption,  $p=0.05$ ; 6 months HCQ vs 2 months after interruption,  $p=0.001$ ).

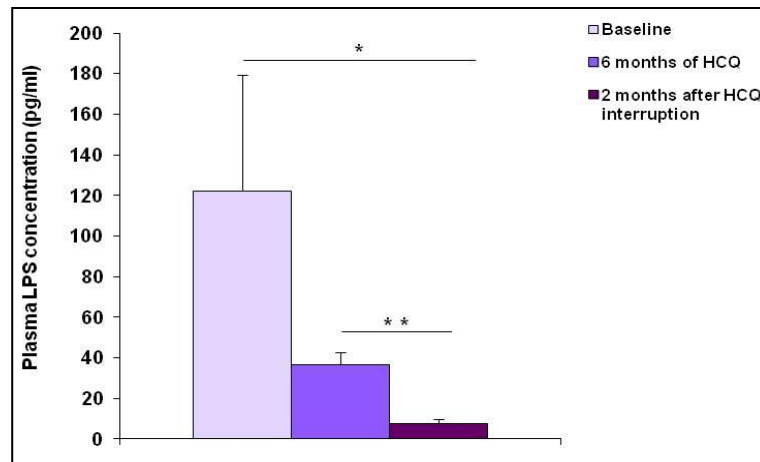


Figure 35. Plasma LPS concentration in HIV-infected INR patients enrolled in this study. Mean values, SE and P are shown (\*  $p<0.05$ , \*\*  $p<0.01$ ).

The reduction of LPS concentration suggests an improvement in gut mucosa permeability. It will be interesting to determine whether this is a direct consequence of HCQ or rather a consequence of the reduced peripheral immune activation seen in these patients. These considerations notwithstanding, because LPS directly triggers TLR4-expressing immune cells, the HCQ-associated reduction of plasmatic LPS probably plays a major role in the effect on immune activation by HCQ described herein.

All the data presented herein regarding immune activation and antiviral responses, indicate that HCQ has a significant effect on immune activation, as shown by the reduction of circulating activated immune cells, the down-modulation of TLR-mediated signal transduction and the decreased production of IL6. This effect is probably associated with the increased concentration of Treg lymphocytes and the reduced quantities of serum LPS observed in HCQ-treated individuals and is accompanied by significantly augmented percentages of circulating pDCs.

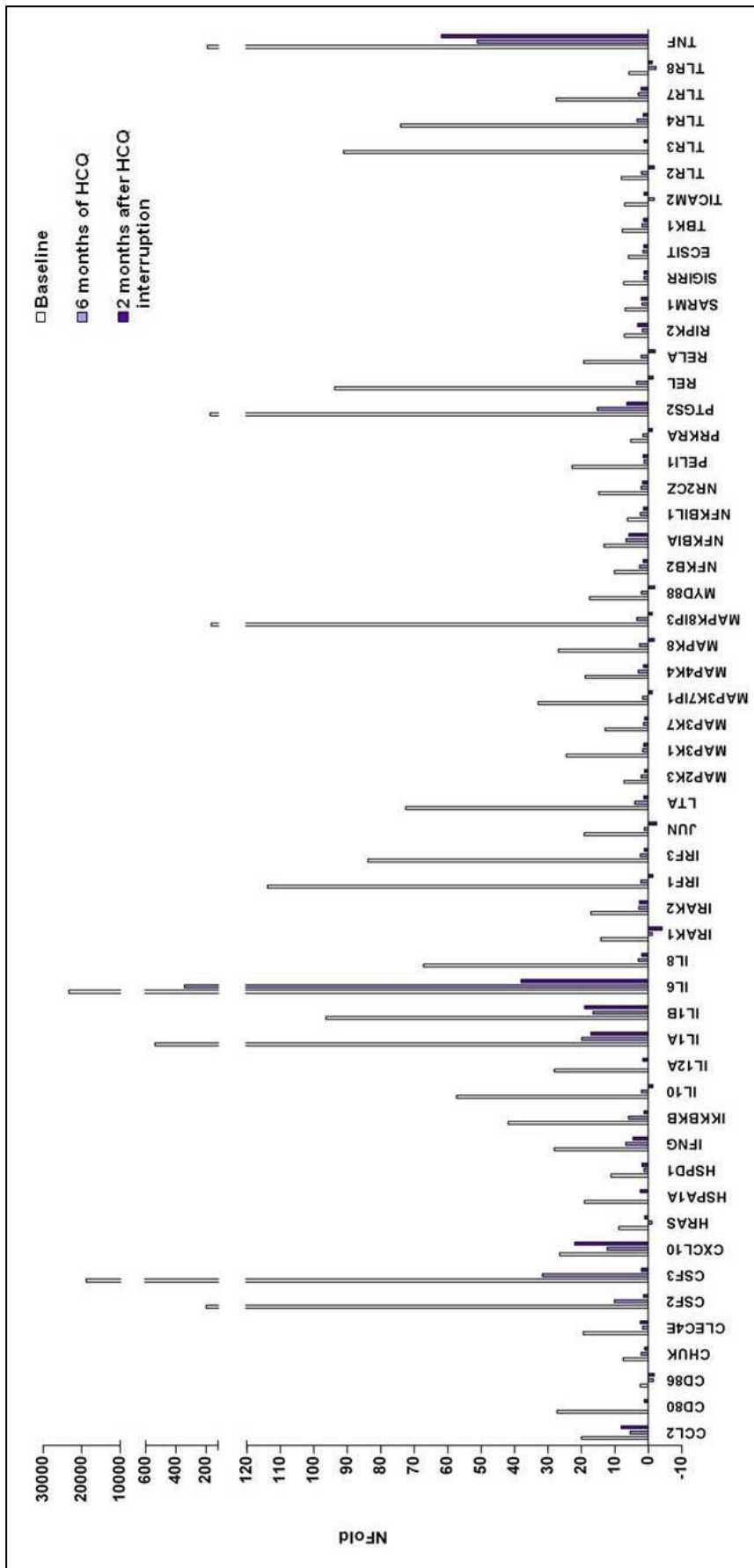
## 2.6. Evaluation of TLR signaling pathway

Beside having an effect on TLR expression, HCQ reduces TLR-mediated signal transduction in vitro and in the murine model. To evaluate whether a similar effect could be seen in vivo in HIV-infected patients. TLR-mediated signaling pathways were evaluated using an RT-PCR array screening for the expression of 84 genes involved in such pathway. Engagement of TLR7/8 with specific agonists at baseline resulted in the activation of TLR-signaling pathways as demonstrated by increased expression of mediators involved in the transduction pathway (MAP2K3, MAP2K4, MAP3K1, MAP3K7, MAP3K7IP1, MAP4K4, MAPK8, MAPK8IP3, MYD88, NFKB1, NFKB2, NFKBIA, NFKBIL1, NFRKB, NR2C2, PELI1, PPARA, PRKRA, REL, RELA, RIPK2, SARM1, SIGIRR, ECSIT, TBK1, TICAM2 and TIRAP) and of effector molecules (CSF2, CSF3, IL1, IL6, IL10, IL12 and PTGS2). After 6 months of HCQ therapy, however TLR7/8 responsiveness was markedly down-modulated, and it was not restored even after treatment suspension. Of note, TLR7 and TLR8 mRNA specific expression progressively decreased from baseline to suspension of HCQ treatment, suggesting a possible explanation for the decreased responsiveness observed after ssRNA stimulation (figure 36, panel A).

Data collected after TLR4 stimulation resembled the immunologic profile observed in TLR7/8-stimulated PBMCs. Thus, at baseline PBMCs were highly responsive to LPS, and HCQ therapy resulted in a marked inhibition of TLR4 pathway that persisted after treatment suspension. Similarly to what observed for TLR7/8, the gradual decrease in cytokine/chemokine expression after LPS stimulation was accompanied by reduced expression of TLR4 mRNA (figure 36, panel B).

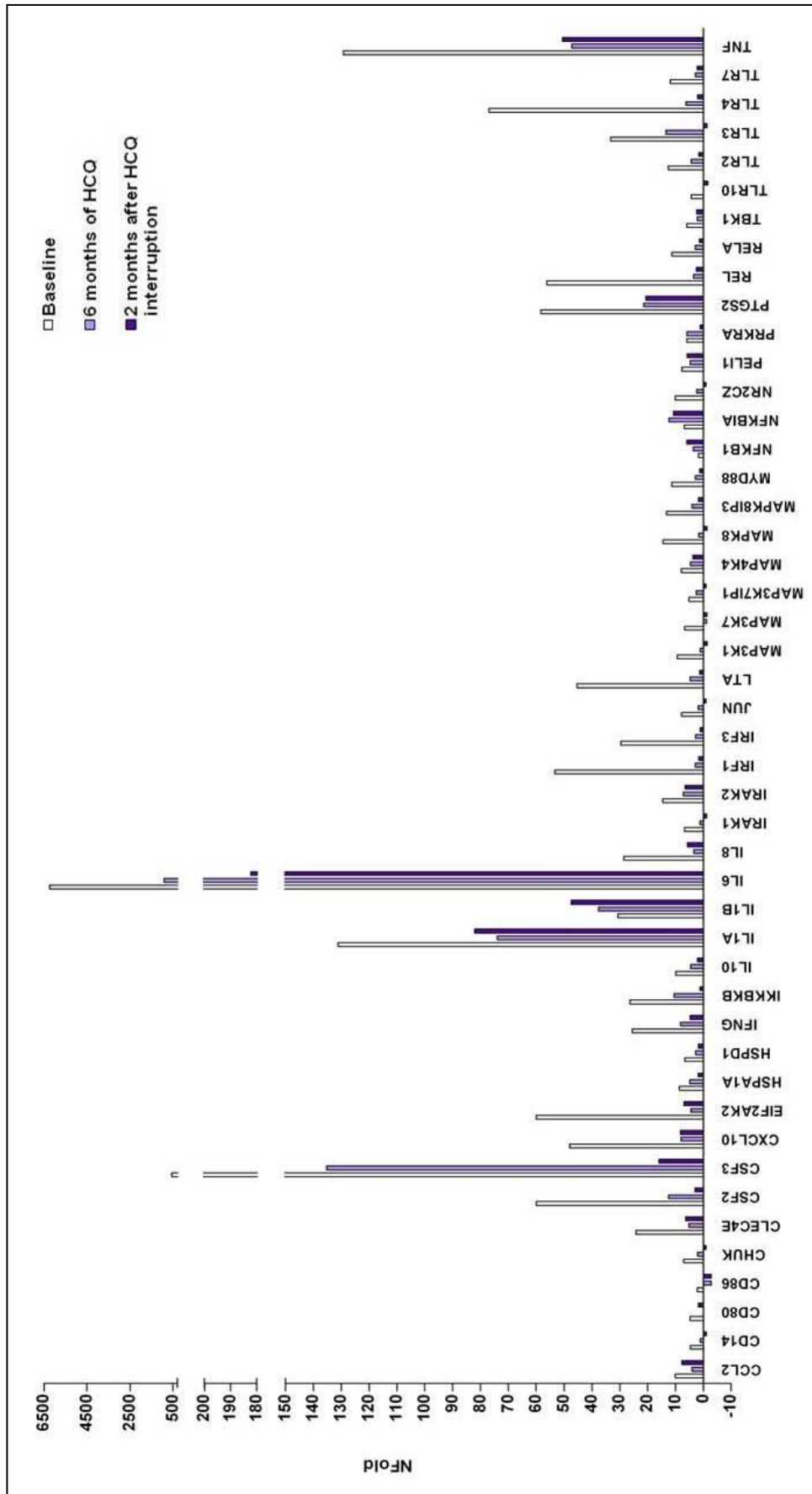
Considering the previous data regarding immune activation and antiviral responses, HCQ reduced IFN $\alpha$  production by pDCs is dependent on TLR9 engagement [118]. We did not directly examined the TLR9 pathway but rather concentrated on TLR4 and TLR7/8. Nevertheless, because we saw decreased mRNA synthesis for these 3 TLRs, as well as down-regulation of TLR4 and TLR7/8 signaling pathways, it is

possible to speculate that reduced expression of TLR9 on pDCs and/or diminished efficacy of the TLR9-mediated signaling could justify the reduced IFN $\alpha$  production seen in pDCs of HCQ-treated individuals. In-depth analyses will be needed to clarify the exact mechanisms.



A

Figure 36, panel A. TLR7/8 signaling pathway after ssRNA stimulation in HIV-infected INR patients enrolled in the study. NFold values are shown.



B

Figure 36, panel B. TLR4 signaling pathway after LPS stimulation in HIV-infected INR patients enrolled in the study. NFold values are shown.

***Conclusions***

Antiretroviral therapy changed the classical course of HIV infection. HAART therapy is highly effective in increasing survival and life quality of HIV-infected patients through the reduction of HIV replication to undetectable level and the increase of CD4<sup>+</sup> T cell count. Nevertheless 15-30% of HIV-infected HAART-treated patients are immunological non responder (INR); in fact, despite optimal suppression of viral replication, restoration of CD4<sup>+</sup> T cell counts is not always achieved.

Several factors seem to be involved in the lack of CD4<sup>+</sup> T cell recovery: residual viral replication, altered thymic function, older age, immune activation, apoptosis and viral coinfections were all proposed to play a significant role in this phenomenon. Moreover it has been demonstrated that CD4<sup>+</sup> T cell nadir influences not only the possibility to normalize cell counts in response to therapy but also affects the time to reach this goal. The time between HAART initiation and achievement of CD4<sup>+</sup> T cell count more than 500cells/ $\mu$ l is significantly longer in individuals with lower baseline lymphocyte counts. Recent evidences, coming from clinical studies, assess that CD4<sup>+</sup> T cell count greater than 500 cells/ $\mu$ l and long-term combination of antiretroviral therapy might be positive factors for a better outcome of the disease. In particular, the presence of 500 cells/ $\mu$ l has been associated with the same rates of mortality as among the general population after 6 years of HAART.

Recent evidences indicate that immune activation plays a key role in the pathogenesis of HIV infection. The main cause of immune activation is the destruction of CD4<sup>+</sup> T cells in the gastrointestinal mucosa characterizing the earliest phase of the disease. Loss of mucosal barrier integrity results in impaired local cellular immunity and translocation of microbial products, including LPS, which contribute to persistent inflammation and immune activation through TLR activation. Given the deleterious effects of sustained inflammatory responses, the immune system tries to restore homeostasis with immune suppressive activity displayed by Treg cells. Treg cells are divided in two subpopulations, naïve and activated, and they act mainly through the secretion of immune suppressive cytokines and the induction of apoptosis. It has been demonstrated that Treg cells can, however, display opposite effects: a positive effect in preventing chronic immune activation or worsen the course of infection by blocking the development of antiviral immune responses. Moreover it has been elucidated that Treg cells express TLRs, above all TLR2 and TLR4, and their TLR-mediated activation increase the immune suppressive activity.

Aim of the first part of this project was to analyze possible relationships between CD4<sup>+</sup> T cell nadir and the lack of CD4<sup>+</sup> T cell recovery observed in HIV-infected INR patients treated with HAART therapy and to identify immune mechanisms involved in these interactions.

In literature conflicting results regarding the role of Tregs in the immunopathogenesis of HIV disease have been reported. Our results suggest that both subpopulation of Treg cells were augmented in patients with CD4 cell count less than 500cells/ $\mu$ l and they were fully competent in mediating immune suppression. In particular, activated Treg cells could prevent full CD4<sup>+</sup> T cells reconstitution via both direct (immune suppressive cytokines) and direct (apoptosis) mechanisms. Our data put in evidence that, following HIV specific stimulation, PBMCs of patients with CD4<sup>+</sup> cell count less than 500cells/ $\mu$ l were characterized by an increase in the expressions of immune suppressive cytokines, caspase 8/9 activation and apoptotic cells than PBMCs of patients with CD4<sup>+</sup> cell count more than 500cells/ $\mu$ l. Furthermore we observed that blockage of PD1/PDL1 pathway with a neutralizing antibody against PD1 induces an increase in viable cells and a decrease in early and late apoptotic cells in patients with CD4<sup>+</sup> cell count less than 500cells/ $\mu$ l but patients with CD4<sup>+</sup> T lymphocytes counts more than 500cells/ $\mu$ l. The addition of a neutralizing antibody against PDL1 didn't influence the percentage of both viable and apoptotic cells.

As previously explained, acute HIV infection is characterized by depletion of cellular population localized in the gut-associated lymphoid tissue. This massive destruction of CD4<sup>+</sup> T cells enhances the pathogenesis of HIV infection and our experiments were designated to verify the immune activation status and the presence of microbial translocation. Data herein presented showed that patients with CD4<sup>+</sup> cell count less than 500cells/ $\mu$ l were characterized by an increase of activated CD4<sup>+</sup> T cells and of TLR2 and TLR4 expression on Treg cells. The augmented expression of TLR expression on multiple cell types is well correlated with the observed increased concentration of plasmatic LPS, index for microbial translocation. Thus, altered gut permeability resulting in the increase of plasmatic LPS concentration would trigger TLR4-mediated immune activation of several cell types like CD4<sup>+</sup> T cells, monocytes and Treg cells.

Correlation analysis was conducted and indicated that defective CD4<sup>+</sup> T cell count recovery in HAART-treated individuals with suppressed viremia was associated with lower CD4<sup>+</sup> T cell nadir, increased microbial translocation, immune activation, immune suppression and higher susceptibility to apoptosis. The observation that all immune parameters analyzed are altered upon HIV-specific but not CMV-specific stimulation deserves further analyses and underlines the strong expansion of HIV-specific lymphocytes in this infection. Data presented herein are in agreement with earlier studies reporting a correlation between CD4<sup>+</sup> T cell nadir and increases in CD4<sup>+</sup> T cells during HAART, and identify novel mechanisms possibly responsible for lack of CD4<sup>+</sup> T cell recovery.

Aim of the second part of this study was to verify the immune modulating and antiviral activity displayed by HCQ. HCQ is endowed with immune modulating effects including the reduction of inflammatory cytokine

production and a down modulation of natural killer cell activity. In vitro experiments have shown that HCQ is also able to modulate the intracellular TLR pathway as it reduces TLR9 and TLR4 expression and subsequent activation of NF- $\kappa$ B and AP-1. In addition, HCQ is characterized by antiviral activity in preventing the entry of virus into target cells and, regarding HIV, in blocking HIV-1 replication and Tat-mediated transactivation of HIV-1 LTR, leading to a reduction in virion production. Based on the assumption that HCQ down regulates TLR signaling and the production of inflammatory cytokines, we decided to verify whether this compound might modulate immune activation. Results herein presented, indicate that HCQ reduces the activation status of both lymphocyte and monocyte population and down modulates TLR expression on monocyte's population. HCQ was effective in reducing the percentage of proliferating CD4<sup>+</sup> T lymphocytes and of activated monocytes, whereas its effect on CD8<sup>+</sup> T cells was reduced.

The "immune activation hypothesis", proposed by Brenchley and Douek, asserts that during HIV infection, a state of chronic immune activation is determinant for CD4<sup>+</sup> T cell depletion and progression to AIDS and that an increased microbial translocation drives systemic immune activation through the persistence presence of microbial products such as LPS. According to this hypothesis, we dosed plasma LPS concentration and data obtained showed that HCQ was effective in reducing its quantities, this effect might suggest a HCQ-driven improvement in gut mucosa permeability.

The immune system tries to counterbalance immune activation with the immune suppression activity displayed by Treg cells and, as previously observed, these processes are strictly correlated. Analysis conducted to evaluate Treg cells percentages showed that, in both unstimulated and HIV-stimulated conditions, HCQ induced an increase of Treg cells subpopulation all throughout the entire study. Moreover it has been observed a strong increase in TLR expression on Treg cells, these subpopulation are endowed with a strong immune suppressive activity and probably play a pivotal role in the reduction of immune activation.

An impairment of pDC function was described in HIV infection and pDCs were shown to inversely correlate with CD4<sup>+</sup> T counts and viral loads so it has been supposed that pDCs may play a fundamental role in the protection against the progression of HIV infection. pDCs are the main producer of IFN $\alpha$  whose level has been correlated with the prognosis of the infection: presence of elevated level of this cytokine in acute infection reduces viral burden while in the advanced phase of the disease it is associated with increased microbial translocation and immune activation. Results herein presented demonstrate that HCQ is effective in augmenting the number of pDCs and in reducing the production of IFN $\alpha$ . Furthermore analysis conducted to evaluate plasmatic concentration of pro inflammatory cytokines, put in evidence the ability of HCQ in reducing IL6 production throughout the entire study. This effect might be associated with the increased percentage of Treg cells, with the reduced immune activation and concentration of plasma LPS previously observed.

On the basis of the effect played by HCQ on TLR signaling pathway and consequently on gene expression, we decided to verify the functionality of the main TLR involved in HIV infection. Data herein presented, showed a decrease in mRNA expression specific for TLR4 and TLR7/8, as well as the down regulation of their signaling pathway under specific stimulation.

In conclusion, data presented herein suggest a multi factorial model explaining failed CD4<sup>+</sup> T cell recovery in successfully HAART-treated patients. Moreover HCQ exerts a beneficial effect on TLR/LPS-mediated immune activation, which is responsible for the lack of CD4<sup>+</sup> T-cell recovery in INR patients. HCQ could be considered to be a useful immune modulating drug in particular groups of HIV-infected patients. The observation that lower nadir results in a more difficult increase of CD4<sup>+</sup> T cells supports the hypothesis that the CD4<sup>+</sup> T cell threshold actually used to start HAART should be reassessed and supports even more the need to introduce an immune modulator agent in the therapy of HIV infection.



***Appendix***

## PRINCIPLES OF FLOW CYTOMETRY

The flow cytometry allows automatic analysis of cell suspensions, by measuring the physical and/or biochemical in a laminar flow that is hit by a beam of monochromatic light. By cytometry you can capture and store multiple parameters for each measured cell, such as volume, granularity and fluorescence, using different windows electronic analysis on specific populations, discriminated according to different parameters brands.

The principle underlying the cytometry is the possibility to analyze each individual event of the test sample, thanks to a fluidic system that generates a single line of cells. While proceeding, the individual cells through a measuring point where interacting with the beam of excitation. The fluorescent emission signal generated (due to the phenomena of diffraction, refraction and reflection), is collected by a system of lenses, dichroic mirrors and optical filters and sent to the sensors (photomultiplier) that measures the amplitude of signal. The signals from each sensor are digitized, combined with each other and sent to a computer that provides the presentation of data and their statistical definition.

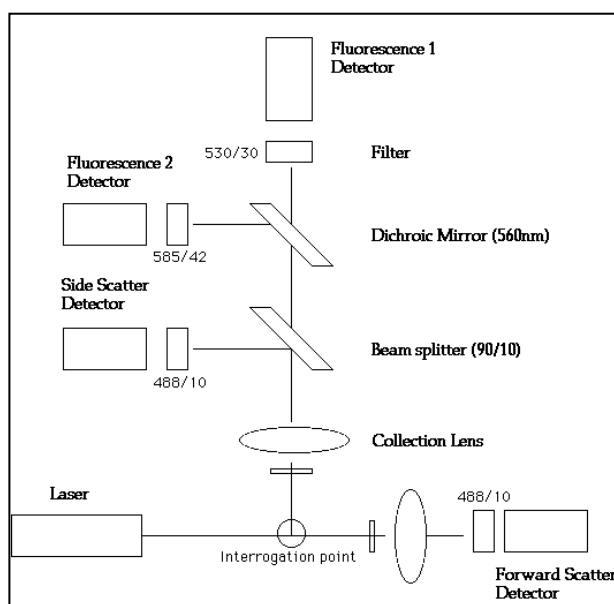


Figure 1. Schematic representation of a cytometer: laser, dichroic filters, band pass filter and photomultiplier are in evidence.

The cytometer consists of four main components (fig. 1), detailed explaining in the following paragraphs:

- fluidics system, that allows to obtain a monodisperse flux of cells;
- optics and detectors, that allow the excitation of the fluorochrome and the detection of fluorescent signal and of the physical and/or biochemical characteristic of cells;
- signal processing, that allows the conversion of light in analogical signals, that can be received by a computer;
- data analysis, that allows to display data acquired in different graphic representations.

### Fluidic System

When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system.

Essentially, the fluidics system (fig. 2) consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This flow is characterized by two coaxial flows: the internal flow (flow core) contains the cells, while the outside retains the ideal flow along the axis. By controlling the difference between the internal and the external flow, adjust the speed of flow cell (flow rate), usually measured as the number of events per second. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its centre and zero velocity at the wall. The effect creates a single line of particles and is called hydrodynamic focusing. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid. Without hydrodynamic focusing the nozzle of the instrument (typically 70  $\mu\text{M}$ ) would become blocked, and it would not be possible to analyze one cell at a time.

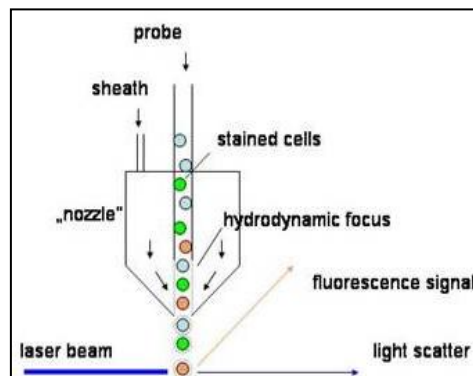


Figure 2. Hydrodynamic focusing produces a single stream of particles.

### Optics and detection of fluorescence

After hydrodynamic focusing, each particle passes through one or more beams of light. Light scattering or fluorescence emission (if the particle is labelled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry. Lasers produce a single wavelength of the light at one or more discrete frequencies. Arc lamps tend to be less expensive than lasers and exploit the color emissions of an ignited gas within a sealed tube.

The interaction of the light with the cell gives rise to the phenomena of light scattering and fluorescence. Light scattering allows to discriminate physical characteristics of the cell based on two factors: forward scatter (FS) and side scatter (SS). Light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis, is collected by a lens known as the forward scatter channel (FSC) and it defines cell dimension while when the light is scattered at a 90° angle to the excitation line it is collected by a side scatter channel (SSC) and it provides information about the cellular roughness, ratio nucleus/cytoplasm and cell surface irregularity. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell populations in a heterogeneous sample.

Once the radiation was deflected by the cell, depending on the parameter taken into account, it collides against the detector, usually consisting of photodiodes, or material that, when stimulated by a radiation to generate electricity. The signals from the sensors, proportional to the size of continuously measured parameter, are transformed into electrical signals by photomultiplier which are then interpreted by appropriate software, into numerical values. The combination of the two types of signals (SS and FS) is obtained by a two-dimensional diagram known as "cytogram", in which it is possible to detect different cell populations based solely on physical characteristics.

Fluorescence is the phenomenon whereby a molecule hit by a light radiation of defined wavelength ( $\lambda$ ),  $\lambda$  emits another at higher and lower energy, and enables quantification of a macromolecule of interest within the cell. Many biological molecules can be identified with fluorescent ligands or with specific monoclonal antibodies (mAb) labeled with fluorochromes (FITC, PE, ECD, PC5, APC, PC7). Initially, the dye absorbs laser energy, then releases the energy consumption for both vibration and heat dissipation, and the emission of photons of a wavelength longer than in all directions of space, which are then collected by a photomultiplier and processed into an electrical signal (fig.3).

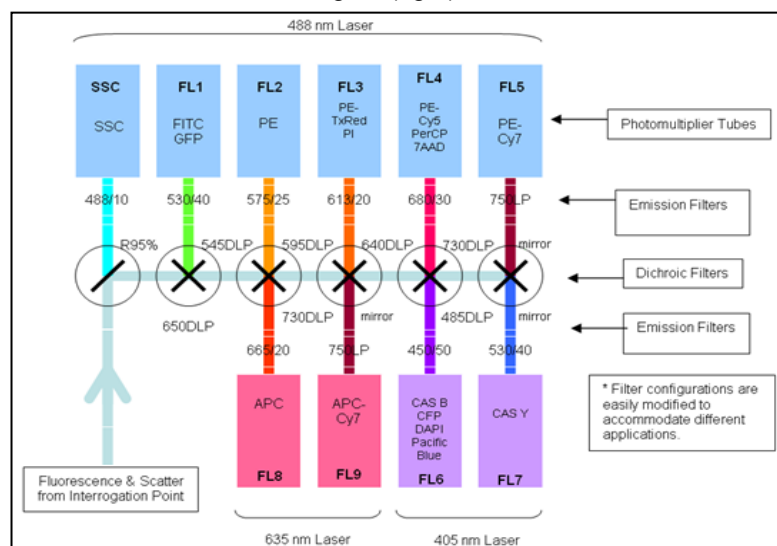


Figure 3. Schematic overview of cytometer setup. FL channel and optical filters are shown.

The photomultiplier is preceded by an optical system characterized by filters to select the characteristic wavelength of the fluorochrome in question. The intensity of fluorescence emitted is directly proportional to the binding sites for the fluorochrome present on the cell. Each dye has a characteristic band of  $\lambda$  for excitation and emission and when using more fluorochromes in the same protocol, it's important to minimize the possible signal interference by setting the right compensation (for detail see data analysis section). Flow cytometers use separate fluorescence (FL-) channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings. The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types (fig. 4). 'Long pass' filters allow through light above a cut-off wavelength, 'short pass' permit light below a cut-off wavelength and 'band pass' transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption. When a filter is placed at a 45 degrees angle to the oncoming light it becomes a dichroic filter/mirror. As the name suggests, this type of filter performs two functions, first, to pass specified wavelengths in the forward direction and, second, to deflect blocked light at a 90 degrees angle. To detect multiple signals simultaneously, the precise choice and order of optical filters will be an important consideration.

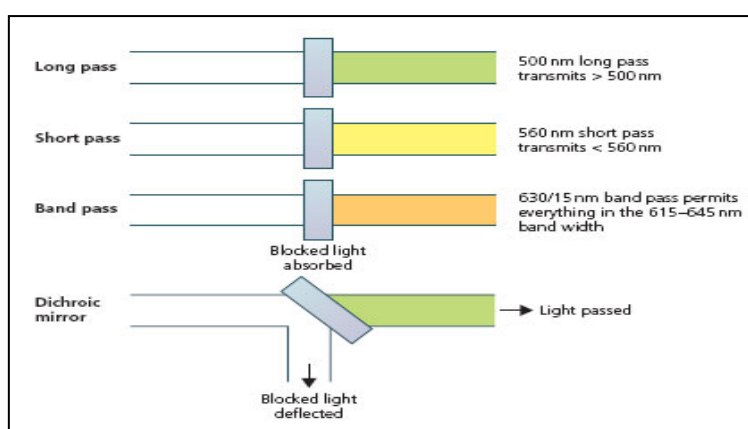


Figure 4. Different types of optical filters.

### **Signal processing**

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis.

The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

### **Data analysis**

The data analysis software permits to analyse the data acquired also in presence of different colour (multicolour) of fluorescence and to represent the data themselves in different graphic formats. One consideration to be aware of when performing multicolor fluorescence studies is the possibility of spectral overlap. As previously said, when two or more dye are used in the same protocol, fluorescence compensation is applied during data analysis.

**Fluorescence compensation** calculates how much interference (as a %) a fluorochrome will have in a channel that was not assigned specifically to measure it. So, this mean that when the two fluorochromes are used for a dual colour experiment, the true reading for fluorochrome A is (total fluorescence measured in FL1) minus (5% of fluorochrome B's total fluorescence). Similarly, for B, the true signal is (total fluorescence measured in FL2) minus (17% of fluorochrome A's total fluorescence).

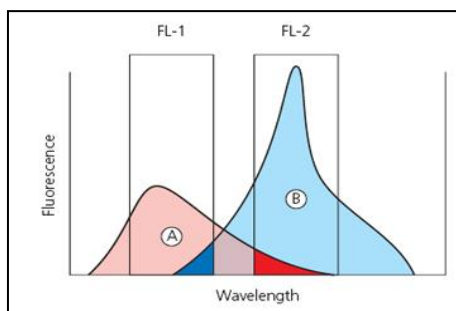


Figure 5. Fluorescence compensation, spectral overlap is shown.

**Gating** is the procedure by which cells of interest can be selective visualize while eliminating results from unwanted particles (dead cells and debris). Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells. The different physical properties of granulocytes, monocytes and lymphocytes allow them to be discriminated.

To represent data deriving from fluorochrome labelling, two different ways are used: the single-parameters histograms and two-parameters histograms.

The first type display a single measurement parameter (relative fluorescence or light scatter intensity) on the x-axis and the number of events (cell count) on the y-axis: the histogram is a very basic representation but is useful for evaluating the total number of cells in a sample that possess the physical proprieties selected for or which express the marker of interest. Cells with the desired characteristics are known as the positive dataset. Ideally, flow cytometry will produce a single distinct peak that can be interpreted as the positive dataset. However, in many situations, flow analysis is performed on a mixed population of cells resulting in several peaks on the histogram. In order to identify the positive dataset, flow cytometry acquisition should be repeated in the presence of an appropriate negative control.

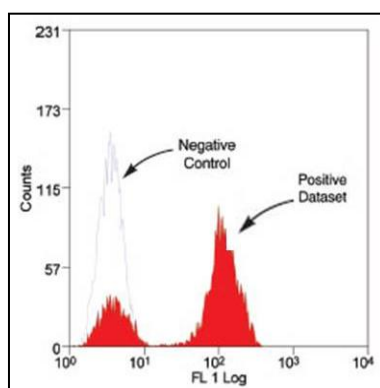


Figure 6. Schematic representation of a single parameter histogram

The two-parameter histograms are graphs that display two measurement parameters, one on the x-axis and one on the y-axis. The parameters could be SSC, FSC or fluorescence. This graph permits to evaluate the correlation between two data set acquired.

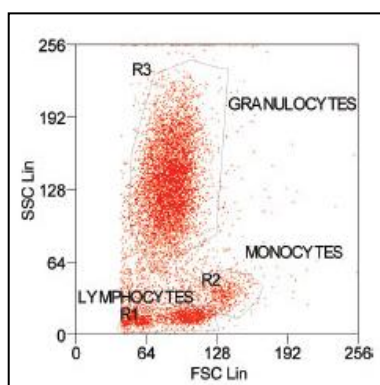


Figure 7. Schematic representation of a two parameter dot plot.

## PRINCIPLES OF REAL-TIME PCR

Real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”.

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle.

Real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for post amplification manipulation is eliminated.

Realtime PCR is based on the monitoring and detection of fluorescence emitted during the reaction, by a fluorescent dye used as an indicator of production of new amplified. This is possible because the fluorescence signal emitted by the fluorophores increases in direct proportion to the amount of product that is being formed that is, the higher the initial number of copies of target, before you will see a significant increase in fluorescence.

The fluorescence emitted in response to a specific part of the radiation from a light source of the instrument is measured in real time by a detector (photomultiplier or CCD camera) and all operations related to the measurement is carried out under the control of a software controlled by a computer.

### Detection systems

Detection system for real-time PCR is based on generating a fluorescent signal that can be obtained using three different strategies: non-specific detection system, detection system based on probes and on primer/oligo.

**Non-specific detection system** is based on the use of intercalating dyes that emit fluorescence when they bind to the double helix of dsDNA. The most common dye used is the SYBR Green I, it exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds to minor groove of dsDNA. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified. SYBR Green I fluorescence depends on the amount of dsDNA present, but this system is not specific because it binds indiscriminately to all dsDNA and therefore also to nonspecific products or primer dimers that can be formed during the PCR reaction. The specificity of these dyes can still be monitored by analyzing the melting curve with which you can highlight the presence of undesirable products. The advantages of using dsDNA-binding dyes include simple assay design (only two primers are needed; probe design is not necessary), ability to test multiple genes quickly without designing multiple probes (e.g., for validation of gene expression data from many genes in a microarray experiment), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

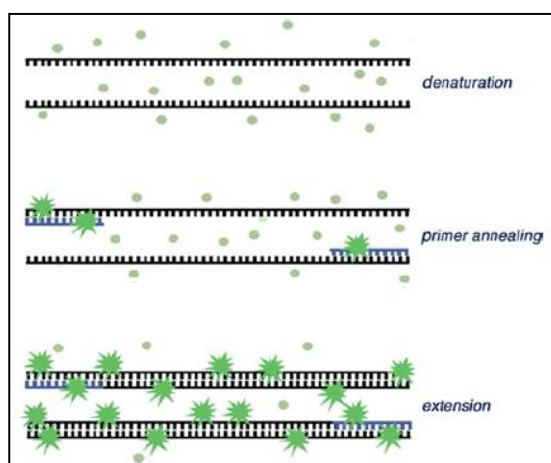


Figure 8. SYBR green during PCR amplification, the fluorescence of this dye increases when bound to the minor groove of double stranded DNA. [132]

**Detection system based on probes:** it allows a high sensible and specific detection for a single target sequence. This system requires a double tag of the probes. The most common probes are: TaqMan and molecular beacons.

TaqMan probe contains a fluorescent reporter at the 5' end and a quencher at the 3' end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridizes to the target and the dsDNA-specific 5'→3' exonuclease activity of Taq cleaves off the reporter. As a result, the reporter is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample. One commonly used fluorescent reporter quencher pair is fluorescein (FAM, which emits green fluorescence) and Black Hole Quencher 1. The main advantages of using TaqMan probes include high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. The disadvantages are that the initial cost of the probe may be expensive.

Molecular beacon probe employs a sequence-specific, fluorescently labeled oligonucleotide probe, which is a dye-labeled oligonucleotide (25–40 nucleotides) that forms a hairpin structure with a stem and a loop. A fluorescent reporter is attached to the 5' end of the molecular beacon and a quencher is attached to the 3' end. The loop is designed to hybridize specifically to a 15–30 nucleotide section of the target sequence. On either side of the loop are 5–6 nucleotides that are complementary to each other, and form a stem structure that serves to bring the reporter and quencher together. In the hairpin structure, no fluorescence is detected from the reporter due to its physical proximity to the quencher. During the annealing step of the amplification reaction, the molecular beacon binds to its target sequence, separating the reporter and quencher such that the reporter is no longer quenched.

**Detection system based on primer/oligo:** it allows a high sensitive and specific detection of DNA and RNA, in this case the fluorophore is directly bound to the primer.

The two probes are designed to bind to adjacent sequences in the target. The first probe carries a donor dye at its 3' end, while the second carries an acceptor dye at its 5' end. The donor and the acceptor dyes are selected such that the emission spectrum of the donor dye overlaps significantly with the excitation spectrum of the acceptor dye, while the emission spectrum of the donor dye is spectrally separated from the emission spectrum of the acceptor dye. Excitation is performed at a wavelength specific to the donor dye, and the reaction is monitored at the emission wavelength of the acceptor dye. During the annealing step of PCR, the probes hybridize to their target sequences in a head-to-tail arrangement. This brings the fluorescent molecules into proximity, allowing fluorescence resonance energy transfer from donor to acceptor. The increasing amount of acceptor fluorescence is proportional to the amount of amplicon present. The most common are Amplifluor primers characterized by both a fluorophore and a quencher. In the hairpin conformation, the reporter fluorescence is quenched due to its proximity to the quencher.

Several components are requested to set up a Real-Time PCR assay. DNA polymerase and design of the primers are two important factors.

The performance of a PCR depends largely on the specificity of the enzyme, so the choice of polymerase is a critical parameter. Taq polymerase presents a residual activity even at low temperatures, an activity that could lead to the synthesis of nonspecific products, to overcome this problem nowadays Hot start polymerases are commonly used, the peculiarity of these enzymes is that they are activated only with an incubation of 2 minutes at 95°C. At last optimal primer design is critical for efficient amplification of desired sequences.

### **Real-Time PCR thermal cycler**

Real-time PCR thermal cyclers combine PCR product generation and recognition into one integrated format that allows for the subsequent analysis of the captured data. To accomplish these two tasks, real-time PCR machines incorporate traditional PCR thermal cycler technology with integrated fluorimeters and detectors that provide the ability to both excite a fluorochrome and detect the emitted light.

One fundamental difference between platforms is the ability to detect more than one fluorochrome with the addition of multiple excitation and detection channels. Because real-time PCR reactions are quantified at each cycle by measuring fluorescence, most real-time thermal cyclers need to be calibrated for the particular tube or microtiter plate and microtiter plate seal used, and for the PCR reaction volume.

As shown in figure, the light from the halogen lamp passes through an optical filter that cuts to 485nm. this wavelength is possible to excite both fluorophores SYBR green, which related to various probes. The light is then reflected on a switch and is conveyed through a mirror on a Fresnel lens and then on the samples. The light emitted from the sample is collected by a CCD camera that captures and stores the signal. During each cycle of PCR, multiple images of the samples are captured, which at the end of the reaction are analyzed by a software able to construct an amplification curve.

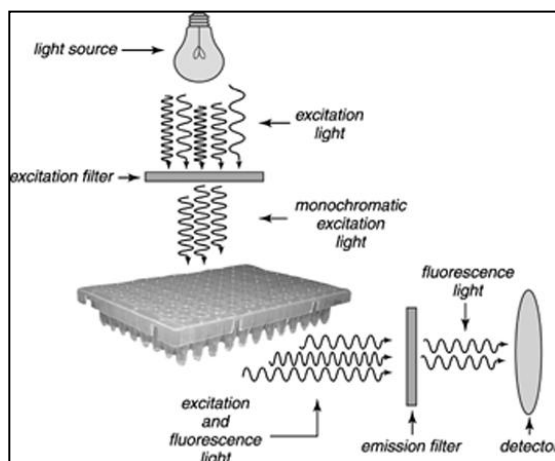


Figure 9 Schematic representation of a Real-Time PCR thermal cycle

### Analyses of the data

Several parameters must be set in an accurate way to perform analysis of the data, these parameters are: baseline, threshold line, threshold cycle and melting curve.

**Baseline**, is the initial cycle of PCR in which we observe a small change in the fluorescence signal. Baseline must be carefully specified to allow an accurate determination of the  $C_T$ , moreover also must be large enough to eliminate the background of the initial cycles of amplification, but must not overlap with the area where the signal begins to increase compared to background noise.

**Threshold cycle ( $C_T$ )** is specific for each sample tested and it identifies the cycle at which the fluorescence signal gets over the threshold.

**Threshold line** is parallel to baseline and it cut the exponential curve at the  $C_T$  point.

**Melting curve** identifies the fluorescence variation observed when there are the reaction temperature augments and so when the dsDNA melts in ssDNA. The analysis of melting curve is an easy way to control the quality of the PCR reaction, it allows to detect primer-dimer formation, contamination and specificity of the reaction. It can be performed only when SYBR green is used.

The amplification plot shows two phases, an exponential phase followed by a non exponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycle 28-40 in fig.10). Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (fig. 10, cycle 1-18) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal, the cycle number at which this occurs is the  $C_T$ .

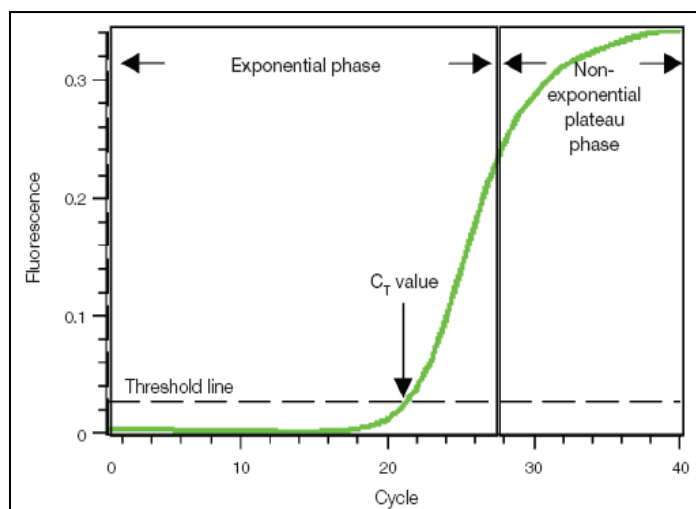


Figure 10 Example of amplification plot.

Since the  $C_T$  value is measured in the exponential phase when reagents are not limited, real-time PCR can be used to reliably and accurately calculate the initial amount of template present in the reaction. The  $C_T$  of a



reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early,  $C_T$ . In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late,  $C_T$ . This relationship forms the basis for the quantitative aspect of real-time PCR.

### **Quantification of the amplicon**

Real-time PCR is a method for the quantification of the amount of nucleic acid in a sample, it can be performed in absolute and relative ways. For both absolute and relative quantification methods, quantities obtained from a PCR experiment must be normalized in such a way that the data become biologically meaningful. This is done through the use of normalizers. Although the normalizer for either quantification method can be the number of cells used for template preparation,  $\mu\text{g}$  of nucleic acid used as PCR template, or the expression level of a reference gene, the first two are more commonly used for absolute quantification, whereas the third is typically used for relative quantification. A reference gene is one whose expression level is constant across all test samples and whose expression is not affected by the experimental treatment under study. The use of a reference gene is advantageous in cases where the precise quantification of input RNA amount is not possible, such as in cases where only a small amount of starting template is available.

**Absolute quantification** is achieved by comparing the  $C_T$  values of the test samples to a standard curve. The result of the analysis is quantity of nucleic acid (copy number,  $\mu\text{g}$ ) per given amount of sample (per cell, per  $\mu\text{g}$  of total RNA). In this case normalizers are used to adjust or standardize the obtained target quantity to the unit amount of sample.

In **relative quantification**, the analysis result is a ratio: the relative amount (fold difference) of a target nucleic acid for equivalent amounts of test and control sample normalized to a reference gene.

The advantage of using a relative quantification approach is that there is no need for generating a standard calibration curve. If amplification efficiencies of the target and control sequences are identical, then one simply compares relative expression values for the target gene in different samples expressed as a ratio. The ratio is then normalized using the housekeeping gene's expression in the same samples. There are several mathematical models available for calculating relative expression. Pfaffl developed a mathematical formula that takes into account the contribution of PCR amplification efficiencies and is widely used for the relative quantification of gene expression in real-time RT-PCR.

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