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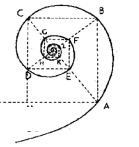

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Mechanisms Involved in the Lack of Immune Reconstitution during Antiretroviral Therapy in HIV-Infected Individuals

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“The important thing is to not stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day.”

—"Old Man's Advice to Youth: 'Never Lose a Holy Curiosity.'" LIFE Magazine (2 May 1955) p. 64”

— Albert Einstein

Abstract

Introduction: *HIV infection is characterized by $CD4^+$ T cell immunodeficiency and chronic inflammation. Antiretroviral therapy (ART) leads to the recovery of $CD4^+$ T cells by suppressing viral replication. However 15-30% of HIV-infected ART-treated patients fail to restore $CD4^+$ T cells despite full viral suppression and they are known as "Immunological Non-Responders (INR)". INRs are characterized by higher risk of AIDS progression and non-AIDS-related morbidity compared to HIV-infected ART-treated immunological responder (IR) patients. Several mechanisms have been involved in immune failure, however none of them provides a full explanation for the lack of immune reconstitution observed in INRs. Inflammasomes are multimeric protein platforms involved in the regulation of inflammatory responses, Th17 activity and in a high inflammatory form of programmed cell-death called "pyroptosis". Pyroptosis was recently shown to play a major role in $CD4^+$ T cell loss and to contribute to immune activation in HIV infection. The possible role of inflammasomes and pyroptosis in the lack of immune reconstitution has nevertheless not been investigated. We analyzed possible associations between inflammasome and Th17 activity, caspase-1 activation, pyroptosis and immune reconstitution in HIV-infected ART treated patients.*

Methods: *39 HIV-infected patients on antiretroviral therapy for ≥24 months and plasma HIV-RNA<50cp/mL for ≥12 months, matched for nadir $CD4^+$ T cell count were enrolled. Exclusion criteria: presence of actual opportunistic AIDS-related diseases, HBV or HCV co-infection, chronic inflammatory disorders, ongoing immunosuppressive therapy. Patients were classified as IRs or INRs if $CD4^+$ T cell count was ≥500 or ≤350 cells/μL, respectively. Immune activation markers (HLA-DRII and CD38) and Th17 activity were evaluated by flow cytometry. Expression of genes involved in the inflammasome pathway and in pyroptosis were measured in unstimulated or LPS- and AT2 treated-HIV-1-stimulated cells. Pro-inflammatory cytokines, caspase-1 expression and microbial translocation markers (sCD14 and LPS) were quantified in plasma samples of all IRs and INRs.*

Results: *INR patients were older and had a higher prevalence of past AIDS-defining illnesses. HLADRII/CD4 were significantly increased in INRs. Higher median levels of Th17 T cells (CD4/IL17A/RORγT) were also seen in INRs in unstimulated, as well as in LPS- and AT2 treated-HIV-1 stimulated conditions. LPS-stimulated inflammasome (NLRP3) and pro-inflammatory cytokines gene expression (IL-1β, IL-18, TNFα, type-I IFNs, CCL3, IL-6) were significantly increased in INR patients. AT2-HIV-1 stimulation induced NLRP3 gene expression in both IRs and INRs; NLRP3 and IL-18 expression were nevertheless significantly increased in INRs compared to IRs. Higher caspase-1gene expression was seen in both unstimulated and AT2-HIV-1 stimulated cells of INRs, whereas caspase 3, 4 and 5 expression was similar in both groups. Plasma concentration of caspase-1 and IL-1βwere higher in INR compared to IR patients. No differences in microbial translocation markers could be detected between the two groups.*

Conclusions: *Increased immune activation levels and percentage of Th17 T cells and higher levels of inflammasome and caspase-1 expression are observed in INR patients. The upregulation of these pro-inflammatory mechanisms plausibly contributes to the persistent immune activation that characterize INRs. Notably,*

caspase-1 activation is likely to induce $CD4^+$ T cell loss via pyroptosis, contributing to the unsatisfactory $CD4^+$ T cell recovery seen in INRs.

Sommario

Introduzione: *La caratterista principale dell'infezione da HIV è lo sviluppo di una immunodeficienza dovuta alla perdita dei linfociti T CD4$^+$. Inoltre, l'infezione da HIV si caratterizza anche per marcati livelli di infiammazione cronica e immuno-attivazione. La terapia antiretrovirale (ART) impedisce la replicazione virale permettendo così il recupero dei linfociti T CD4$^+$. Nonostante ciò, in una percentuale variabile fra il 15 e il 30% dei pazienti HIV infetti e sotto terapia antiretrovirale, non si osserva il recupero delle cellule T CD4$^+$, nonostante la completa soppressione della carica virale. Proprio per questa caratteristica, questi pazienti sono chiamati "Immunological Non-Responders (Immunologicamente non-rispondenti" (INRs). Gli INRs sono pazienti soggetti a un maggior rischio di progressione della malattia verso AIDS conclamata e allo sviluppo di morbidità legate all'AIDS rispetto a pazienti HIV infetti e in terapia antiretrovirale che però presentano un recupero immunologico. Diversi meccanismi biologici sono stati associati al fallimento immunologico della ART, tuttavia nessuno di questi è in grado di spiegare in maniera esaustiva i motivi del mancato recupero immunologico che si osserva negli INRs. Gli inflammasomi sono piattaforme proteiche coinvolte nella regolazione delle risposte infiammatorie, nell'attività dei linfociti Th17 e in una forma altamente infiammatoria di morte cellulare programmata chiamata "piroptosi". Recentemente, alcuni studi hanno dimostrato che la piroptosi gioca un ruolo importante nella morte dei linfociti CD4$^+$ e che potrebbe anche contribuire all'attivazione immunitaria associata all'infezione da HIV. Il ruolo degli inflammasomi e della piroptosi nel mancato recupero immunologico non sono stati, tuttavia, ancora stati studiati. Per questo motivo abbiamo analizzato le possibili associazioni fra l'attivazione dell'inflammasoma, della caspasi 1, della piroptosi e dell'attività dei Th17 e la ricostituzione immunitaria in pazienti sieropositivi e sotto terapia antiretrovirale.*

Metodi: *Sono stati arruolati 39 pazienti HIV infetti e in terapia antiretrovirale da più di 24 mesi, con viremia soppressa (HIV-RNA<50cp/mL da più di 12 mesi) e con comparabile nadir dei linfociti CD4+. I criteri di esclusione sono stati i seguenti: presenza di infezioni opportunistiche correlate ad AIDS, confezioni da HBV o da HCV, malattie infiammatorie croniche e terapia immunosoppressiva in corso. I pazienti sono stati classificati come "Immunological Responders" (IR) con conta di linfociti T CD4+ ≥500 cellule/µl o come "Immunological Non-Responders" con conta dei linfociti ≤350 cellule/µl. I marker di attivazione immunitaria (HLA-DRII e CD38) e l'attività dei linfociti Th17 sono stati valutati tramite citofluorimetria a flusso. L'espressione di geni coinvolti nel pathway degli inflammasomi e della piroptosi è stata valutata in cellule non-stimolate o stimolate rispettivamente con LPS o HIV-1 inattivato da AT2 (AT2-HIV). A livello plasmatico sono stati quantificati i livelli di citochine pro infiammatorie, della caspasi 1 e della traslocazione microbica (sCD14 e LPS).*

Risultati: *I pazienti INRs erano più anziani e avevano una maggiore prevalenza di malattie legate all'AIDS. I linfociti CD4$^+$ positivi per HLADRII sono significativamente aumentati negli INRs. Inoltre, negli INRs sono stati osservati anche maggiori livelli di espressione dei linfociti Th17 (CD4/IL17A/RORγT) sia in assenza di stimolazione, sia in seguito a stimolazione con LPS o con HIV. Negli*

INRs è stato osservato un aumento dell'espressione dell'inflammosoma (NLRP3) e di diverse citochine pro-infiammatorie (IL-1β, IL-18, TNFα, type-I IFNs, CCL3, IL-6) in seguito a stimolazione con LPS. La stimolazione con AT2-HIV è in grado di indurre l'espressione di NLRP3 sia negli IRs sia negli INRs, tuttavia in quest'ultimi, l'espressione di NLRP3 e dell'IL-18 risulta significativamente aumentata rispetto agli IRs. Negli INRs si è osservato anche un aumento dell'espressione genica della caspasi-1 sia in assenza di stimolazione sia in seguito a stimolazione con AT2-HIV, mentre invece l'espressione delle caspasi 3, 4 e 5 è simile in entrambi i gruppi. Le concentrazioni plasmatiche della caspasi-1 e della IL-1β sono più alte negli INRs rispetto agli IRs. Non abbiamo riscontrato nessuna differenza nell'espressione di marcatori di traslocazione microbica fra i due gruppi di pazienti.

Conclusioni: *Nei pazienti INRs abbiamo osservato un aumento dell'immuno-attivazione, delle percentuali di linfociti Th17 periferici e dell'espressione della caspasi-1 e di NLRP3. E' plausibile ipotizzare che l'aumento di espressione di queste meccanismi pro-infiammatori contribuisca in maniera significativa all'immuno-attivazione che caratterizza gli INRs. La caspasi-1 è in grado di indurre la morte dei linfociti T $CD4^+$ tramite piroptosi e, pertanto, è possibile ipotizzare che l'aumentata espressione negli INRs contribuisca al mancato recupero immunologico osservato in questi pazienti.*

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List of Abbreviations

3TC: Lamivudine
ABC: Abacavir
AIDS: Acquired Immunodeficiency Syndrome
ART: Antiretroviral therapy
ART: Antiretroviral Therapy
ASC: Apoptosis-associated speck-like protein
AT2: Aldrithiol 2
AZT: Atazanavir,
CARD: Caspase recruitment domain
CCR: C-C Chemokine Receptor
CD: Cluster of Differentiation
CTL: cytotoxic lymphocyte
CXCR: C-X-C Chemokine Receptor
d4T: Stavudina
DAMP: Danger-associated molecular pattern
DC: Dedritic cell
DRV: Darunavir
dsRNA: Double-strand RNA
EFV: Efavirenz
ENF: Enfuvirtide
FIs: Fusion Inhibitors
FPV: Fosamprenavir
FTC: Emtricitabine
GALT: Gut-Associated Lymphoid Tissue
GBP: Guanylate-binding proteins
GM-CSF: Granulocyte-macrophage colony-stimulating factor also known as colony stimulating factor
GSDMD: Gasdermin D protein
HAART: Highly Active Antiretroviral Therapy
HIV: Human Immunodeficiency Virus
HLA: human leukocyte antigen
HLAC: Human lymphoid aggregate culture
HLA-DR: human leukocyte antigen class II
IA: Immune Activation
IDV: Indinavir
IFN: Interferon
IL: Interleukin
INR: Immunological Non-responder
INSTI: Integrase strand transfer inhibitors
IR: Immunological responder
ISG: Interferon-stimulated genes

LPAC: Lamina propria aggregate culture
LPS: Lipopolysaccharide
LPV: Lopinavir
LRR: Leucine-rich repeat domain
LTR: Long Terminal Repeat
MDM: Monocyte-derived-macrophages
MHC: Major histocompatibility complex
MT: Microbial Translocation
MVC: Maraviroc
NFV: Nelfinavir
NK: Natural Killer cells
NLR: NOD-like receptors
NNRTIs: Non-Nucleoside Reverse Transcriptase Inhibitors
NOD: Nucleotide binding and oligomerization domain also known as NATCH or NBD
NRTIs: Nucleoside Reverse Transcriptase Inhibitors
NVP: Nevirapine
PAMP: Pathogen-associated molecular pattern
PBMCs: Peripheral Blood Mononuclear Cell
PBS: Phosphate-Buffered Saline
PCR: Polymerase chain reaction
PIs: Protease inhibitors
PRR: Pattern-recognition receptor
PYD: Pyrin domain
RAL: Raltegravir
RNA: Ribonucleic Acid
RTE: Recent Thymic Emigrant
RTV: Ritonavir
SIV: Simian immunodeficiency virus
SQV: Saquinavir
ssRNA: Single-strand ribonucleic acid
TCR: T-cell Receptor
TDF: Tenofovir
TGF: Transforming growth factor also known as tumor growth factor
Th: T helper cell
TLR: Toll-like receptor
TMC125: Etravirine
TMC278: Rilpivirine
TNF: Tumor-necrosis factor
TPV: Tipranavir.
TREC: T-cell receptor excision circles
Tregs: Regulatory T Cells
ZDV: Zidovudine

Introduction

1. HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

HIV epidemic was first identified in the 1980s. HIV as a new retroviral virus was first identified by Fracoise Barré-Sinoussi and Luc Montagnier in 1983 and later, in 1984, Robert C. Gallo and his colleagues demonstrated that HIV was the causative agent of acquired immunodeficiency syndrome (AIDS) [1]–[3]. Nowadays the degree of morbidity and mortality caused by HIV infection is still enormous despite the availability of quite effective antiretroviral drugs. It is estimated that HIV has infected 50 to 60 million people, causing death of more than 25 million individuals. 35 million people are living with HIV infection, of which 70% live in Africa, and 1-2 million people die of the disease every year [4].

HIV is able to infect many different immune cells, especially $CD4^+$ T cells, leading to their loss. For this reason, HIV infection is characterized by dramatic immune suppression associated with opportunistic infections, malignant tumors and neurological degeneration. If not treated, HIV infection leads to AIDS and ultimately to death.

1.1 HIV-1Virology

1.1.1 HIV Classification

HIV is a retrovirus belonging to viral *Retroviridae* family and to the genus *Lentivirus*. Two types of HIV have been identified: HIV-1 and HIV-2. HIV-1 raised from cross-species transmission of a chimpanzee virus to humans [5] and it is by far the most common cause of AIDS and new infections globally. It is possible to distinguish three groups of HIV-1 based on their

genome differences: M, N and O [6]. In 2009 a new HIV-1 sequence was isolated from a single individual and analyzed. This newly analyzed sequence was more similar to a simian immunodeficiency virus (SIV) found in gorillas, than to SIVs found in chimpanzee. The sequence has been placed in group P "pending the identification of further human cases" [7]. Group M can be divided into 9 subtypes known as clades (A-D, F-H, J and K) and they are responsible for most HIV infections [8]. HIV-2 stemmed from cross-species transmission of a sooty mangabey virus and its genomic structure and antigenicity is different from that of HIV-1 [9], [10], [11]. HIV-2 has lower infectivity and causes a form of AIDS with slower progression than HIV-1 [12]. HIV-1 is more common in Europe, America and Central Africa, while HIV-2 is more frequent in west Africa and in Asia [13].

1.1.2 HIV Genome and Structure

HIV-1 genome is composed by two identical copies of single strand RNA and is approximately 9.2 kb long. The whole genome is flanked by long terminal repeats (LTRs), which regulate viral gene expression, viral integration into the host genome and viral replication. In the viral genome, there are structural genes like *gag*, *env* and *pol* and regulatory genes like *tat*, *rev*, *vif*, *nef*, *vpr* and *vpu. Gag* sequence encodes core structural proteins, while the *env* sequence encodes the envelope glycoproteins gp120 and gp41, both required for infection. The *pol* sequence encodes for reverse transcriptase, integrases and other viral enzymes necessary for viral replication. *Nef* is a virion associated protein and its deletion resulted in an asymptomatic infection in macques [14], [15]. *Vif* is "virion infectivity factor" and it promotes viral infectivity. *Vpu* is present only in HIV-1 and shares similar functions to *nef* and *env* [16] (Figure 1).

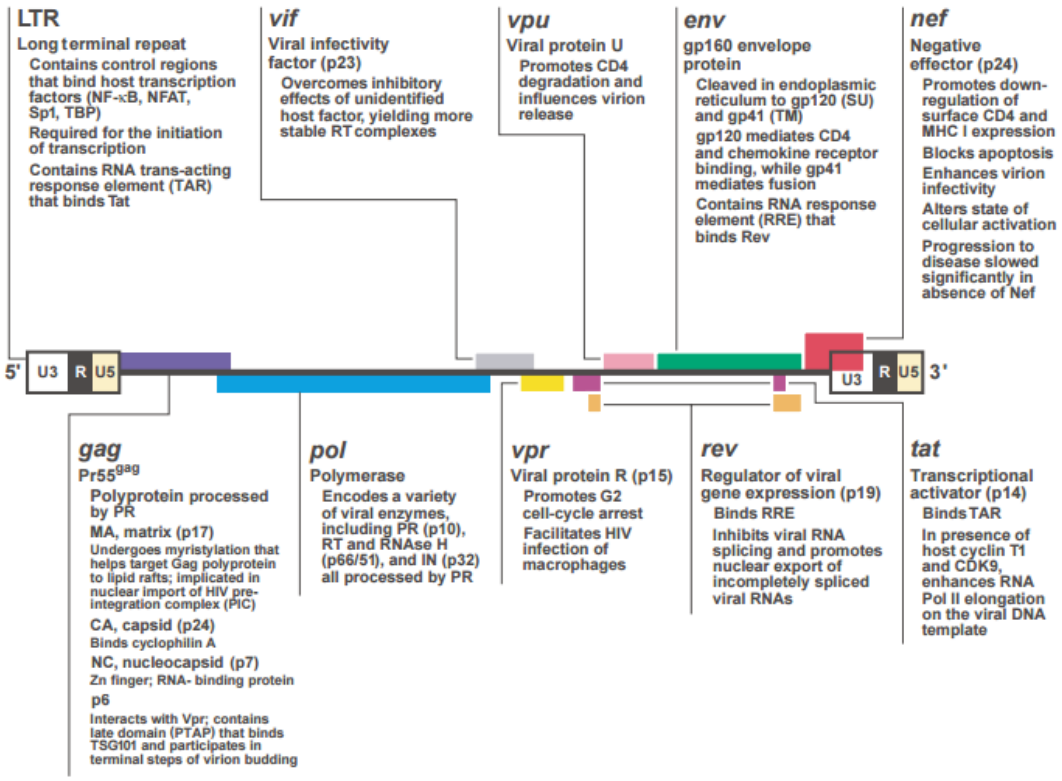


Figure 1: ***HIV-1 genome.*** *Each gene is indicated with a different colored block. Genes using the same sequences of other genes are shown by overlapping blocks. Colored blocks separated by lines indicate genes whose coding sequences are separated in the genome and require RNA splicing to produce mRNA. Image from* [17].

An HIV particle has a spherical shape and it is about 100 nm. HIV-1 consists of two identical positive single strands of RNA (ssRNA), which are enclosed by an inner capsid of p24 viral proteins, which is, in turn, confined by p17 matrix proteins. Core and matrix are surrounded by a phospholipid bilayer envelope derived from the host cell membrane that includes also viral membrane proteins such as gp120 and gp41. gp120 and gp41 are embedded into the envelope in a trimeric form (Figure 2).

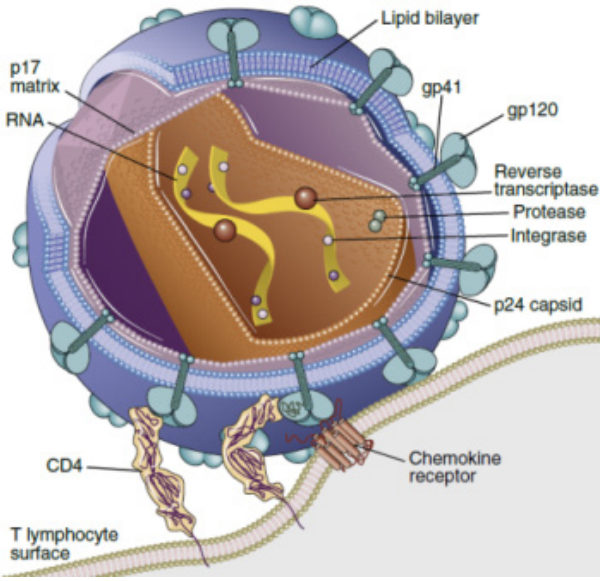


Figure 2: ***HIV-1 Structure.*** *HIV-1 virion binding to a T cell through the CD4 and other chemokine receptors on the target cell surface. Image from* [18]

1.1.3 HIV Life Cycle

The binding of gp120 subunits to CD4 molecules on the host target cell is the first step of HIV infection and it induces a conformational change that allows a secondary gp120 subunit to bind to a chemokine coreceptor (CCR5 or CXCR4). This latter binding induces, in turn, a conformational change in gp41 which allows the exposure of a hydrophobic region, called fusion peptide, which inserts into the host cell membrane and allows the viral membrane to fuse with the target cell membrane. Once a cell is infected, it displays on its cell membrane gp120 and gp41, which may mediate cell-cell fusion with an uninfected cell that expresses CD4 and co-receptors, leading to a new infection (Figure 3) [19]. The most important

chemokine co-receptors for HIV are CXCR4 and CCR5. CCR5 is expressed on macrophages and activated T-cells, while CXCR4's expression is ubiquitous. HIV variants are described as X4 for CXCR4 and R5 for CCR5 binding viruses, those variants who are able to bind both chemokine receptors are called R5X4 [17]. After the fusion, the viral nucleoprotein core is disrupted and the viral enzymes become active: the viral RNA genome is reverse-transcribed into a single stranded DNA copy by the reverse transcriptase, which is then replicated to produce double stranded DNA. Then, the viral DNA enters the host cell nucleus together with the viral integrase. The reverse transcription process is error-prone and it leads to extensive diversity of progeny genomes. Once in the host cell nucleus, the viral integrase catalyzes the integration of viral DNA into the host cell genome. The integrated HIV DNA is called provirus [18], [20], [21]. The provirus may remain transcriptionally inactive for months or years, which is the reason why HIV infection may be latent for long periods in infected individuals. The transcription of the provirus is regulated by the LTR sequence upstream of the viral structural genes and by cytokines that activate T cells and macrophages. LTRs regions contain polyadenylation signal sequences (TATA box promoter sequence) and binding sites for host cell transcription factors - such as NF-kB, SP1, NFAT, AP-1 and STAT- which are active in activated immune cells. Cytokines such IL-2 and tumor necrosis factor (TNF) stimulate HIV gene expression in infected T cells, while other cytokines like interleukin (IL) IL-1, IL-3, IL-6, TNF, interferon γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulate HIV gene expression in infected macrophages [22]–[27]. The fact that host factors activate HIV replication is very important to the pathogenesis of AIDS because the normal response of latently infected T cells to a microbe may lead to virus replication and spread. Therefore, multiple infections that HIV-infected patients acquire in their life stimulate HIV replication and infections of new cells. HIV gene expression may be

divided into an early stage, during which regulatory genes are expressed and in a late stage, in which structural genes are expressed. Also, the mRNAs, which encode for all the different HIV proteins, are derived from a single full genome length transcript by alternative splicing. After transcription, viral proteins are translated in the host cell cytoplasm. Soon after, the assembly of infectious viral particles begins: full-length RNA transcripts of the proviral genome are packaged within a nucleoprotein complex including *gag* core proteins and *pol* encoded enzymes. The nucleoprotein complex then buds from the plasma membrane and during this process *env* and host glycoproteins are included in the viral envelope (Figure 4). Some host factors expressed in certain cell types, such as tetherin protein, may prevent virion release. Another important restriction factor is APOBEC3 (Apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3), which is able to interfere with viral replication [18]. TRIM5α causes premature viral uncoating and proteosomal degradation of viral reverse transcriptase in non-human primates [28].

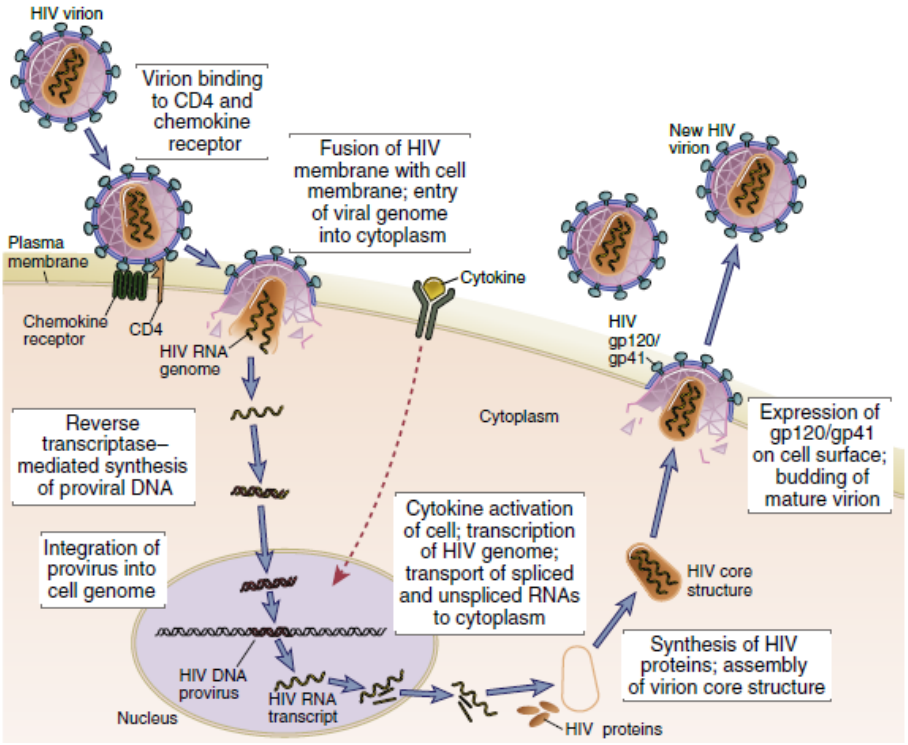


***Figure 4*: Schematic representation of HIV life cycle.** *Image from* [18].

1.2 HIV Pathogenesis

HIV can be transmitted from one individual to another by different routes. Sexual contact is the most common mode of transmission (horizontal transmission), followed by transmission through needle sticks among drug abusers. Infection via blood transfusions have happened in the past when little was known about transmission modes of HIV, but nowadays routine laboratory screenings on blood transfusions have greatly reduced the risk. Another mode of transmission is mother-to-child (vertical transmission), which accounts for the majority of pediatric cases of AIDS. Nowadays it is

possible to prevent this type of transmission by treating the mother with antiretroviral drugs during pregnancy [18].

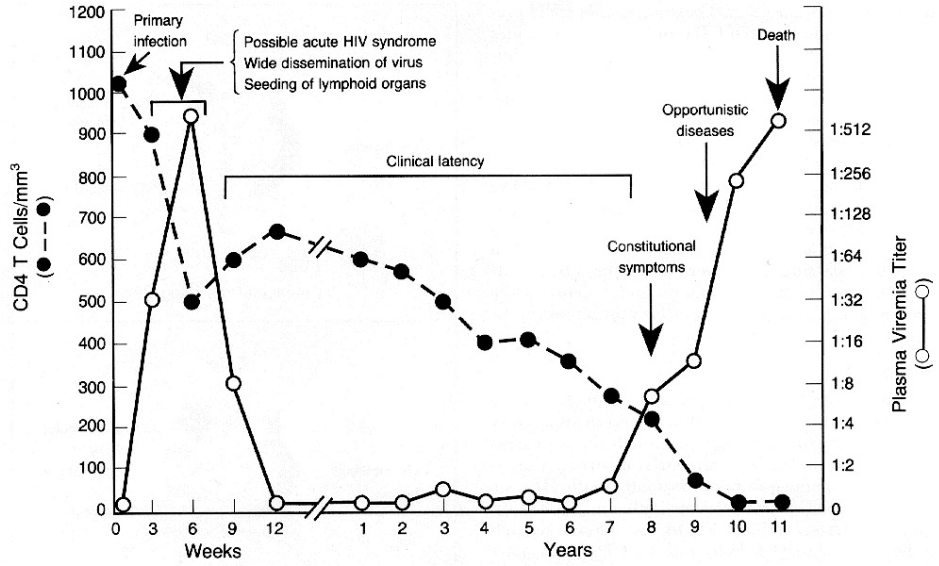


***Figure 5:* Natural course of HIV infection.** *Image from* [29].

Untreated HIV infection is characterized by three different phases: the acute phase, the chronic phase and AIDS (Figure 5). The acute phase is characterized by acute infection symptoms, even though the infected individual may show non- specific symptoms of infection: it is estimated that 50-70% of individuals with HIV primary infection develop an acute mononucleosis-like syndrome [20]. At this stage the host immune system is not able to control the infection and therefore viral loads in blood are high. Usually, the virus enter the host via mucosal epithelia where it infects memory $CD4^+$ T cells in mucosal lymphoid tissues causing a rapid and severe depletion of lymphocytes. Dendritic cells in epithelia are also able to capture the virus and then migrate into the lymph nodes, where they can infect $CD4^+$ T cells by direct cell-cell contact, causing high levels of viremia.

Because of high viremia levels, HIV can then spread throughout the body and infect helper T cells, macrophages and dendritic cells. At the same time, the adaptive immune system mounts humoral and cell-mediated immune responses that partially control the infection and the viral spread, which are reflected by a drop in viremia 12 weeks after the primary infection. During the chronic phase the immune system is somewhat competent at managing various infections, even though HIV replication is still present in lymph nodes and spleen, but there is nonetheless a steady decline of $CD4^+$ T cells. Yet, at this stage there are no clinical manifestation of HIV infection and for this reason this phase is considered a latency period. As the disease progresses, the host is more susceptible to new infections. The immune response to these opportunistic infections, in turn, stimulates HIV production. Therefore, as the immune system is attempting to cope with new infections, it facilitates also HIV replication and its own destruction. When $CD4^+$ T cells level drops below a certain threshold (usually 200 cells/mm^3) there is the onset of AIDS, which is the final and lethal phase of the disease. During this phase the host is unable to cope with any new opportunistic infections because the immune system is severely depleted and thus patients are affected with new opportunistic pathogens, neoplasms, central nervous system degeneration and even kidney failure, which lead to death [29], [30].

It is important to take into consideration that the progression of the disease is highly variable and that some infected individuals are long-term non-progressors. In addition, advent of highly antiretroviral therapy has greatly improved the course of disease and thanks to therapy patients are now able to survive for longer periods.

2. HIV IMMUNOLOGY

2.1 Immune responses to HIV

The early immune response to HIV infection is similar to the immune response to other viruses, however it provides limited protection and it fails to eradicate the infection.

2.1.1 Innate Immune Response

The first line of defense against pathogens is provided by the innate immune system. The innate immune system is able to identify pathogens by pattern recognition receptors (PPR), which are able to recognize evolutionary conserved structures known as pathogen-associated molecular patterns (PAMPs). The most well characterized PPRs are the family of Toll-like receptors (TLRs). Viral infections are detected by TLR9, which recognizes DNA, by TLR7, 8 and 3, which are able to recognize either single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). When TLRs recognize the viral pathogens a cascade of cytokine production begins. At first there is a rapid increase in the levels of many cytokines, which is promoted by DCs, infected $CD4^+$ $CCR5^+$ T cells, monocytes, macrophages and natural killer (NK) cells. In particular, there is a rapid increase of interferon α (IFNα), which is followed by a transient increase in IL-15, IL-18, IL-22, IL-10, IFNγ, TNFα, and CXCL10 levels [31]. IFNα inhibits HIV replication at different stages of the viral life cycle and through mechanisms mediated by the “interferon stimulated genes” (ISG) [32]. An expansion of NK cells has been observed during acute HIV-1 infection and it is probably due to high levels of pro-inflammatory cytokines secreted by

monocytes and DCs, which is then followed by a decrease of NK cells as the disease progress.

2.1.2 Cellular Immune Response

Another important aspect of the immune response against HIV is characterized by adaptive immune system, which consists of humoral and cellular immune responses. T lymphocytes play a central role in the cellular immune response and they can be divided into two main clusters: T helper (Th) $CD4^+$T cells and cytotoxic $CD8^+$ T cells (CTL). $CD4^+$Th cells can then differentiate into different T cells subsets such as Th1, Th2, Regulatory T cells (Tregs) and Th17. Th1 cells produce type 1 cytokines such as IFNγ, TNFα and IL-2, which are necessary for the proliferation of T cells, activation and differentiation of CTLs cells into memory or terminally differentiated CTLs. Th2 cells activate the humoral immune response by producing IL-4, IL-5 and IL-13. CTLs main function is to detect and eliminate virus-infected cells. CTLs recognize viral antigens associated with MHC I molecules on cell surface and, once activated, they produce and release perforines and granzymes that can directly kill infected cells [33]. CTLs may also induce apoptosis of infected cells by the interaction between Fas ligand, expressed on CTLs, and Fas receptor expressed on infected cells [34]. During HIV infection the first adaptive immune response is characterized by the appearance of HIV-specific $CD8^+$ T-cells and it has been established that they play a key role in controlling viral replication [35]. These $CD8^+$ T cells are usually specific for *env* and *nef*, while $CD8^+$ T cells for other viral proteins appear later during infection [36]. Cellular immune responses are critical in HIV control as demonstrated by observing the viral evolution under immune pressure. Indeed, the evolution of the virus resulted in viral isolates that are able to escape to CTLs and $CD4^+$ T cells because they have lost their original CTL and $CD4^+$ T cells epitopes [37].

Persistent viremia leads to a functional exhaustion of HIV-specific T cells. T cell exhaustion is defined as a condition during which T cells lose proliferative and cytotoxic abilities and die by apoptosis [38]. This phenomenon has been extensively studied in HIV infection and many markers of T-cell exhaustion have been identified such as programmed death 1 (PD-1), cytotoxic T-Lymphocyte Antigen (CTLA)-4, lymphocyte activation gene (LAG)-3, T cell immunoglobulin domain and mucim domain (Tim)-3 [38]. Tregs cells function is to maintain peripheral tolerance and preventing autoimmune diseases and chronic inflammation by inhibiting immune functions. They suppress antigen-specific-T-cell responses and control exaggerate immune activation [39]. In HIV infection, Tregs have been shown to play different roles because by inhibiting immune responses, they help to suppress immune activation thereby slowing disease progression. Conversely, they inhibit antiviral immune responses contributing to pathogen persistence and disease progression. Tregs levels decline with disease progression, while their frequency increases on HAART [40]–[43]. A lot of effort has also been put in describing the ratio between Tregs and other pro-inflammatory subsets, such as Th17 T cells and imbalances in Th17/Tregs ratios have been reported in HIV-infected individuals [44]–[46]. Th17 cells produce IL-17 promoting inflammation and the clearance of pathogens, especially at mucosal sites [47]. They have also been implicated in autoimmunity diseases, indicating how these cells can be both protective and harmful. Human Th17 T cells express CCR6, CCR5 and α4β7 integrin, which direct homing to mucosal sites. The role of Th17 T cells in HIV infection is not yet fully understood and investigations on the role of these cells in HIV infection have reached different and opposing conclusions. Recently, HIV-specific Th17 T cells have been reported, however another report did not support this finding (reviewed in [48]). It has been observed that during HIV infection there is a selective depletion of Th17 T cells at mucosal tissues, such as the gastro-intestinal

(GI)-tract. The loss of these cells at mucosal GI-tract could result in loss of the physical and immunological integrity of the mucosal barrier and in leakage of natural flora and thus in immune activation, as it will be further explained in next chapter. Less is known about the role of peripheral Th17 T cells during HIV infection. Maek-a-nantawat *et al*., stimulated peripheral blood mononuclear cells (PBMCs) with phorbolmyristate acetate and ionomycin and reported that HIV infected individuals had a significant increase in Th17 T cells compared to healthy control. Nonetheless, other studies have reached different conclusions and the role of peripheral Th17 T cells during HIV infection is still a matter of debate [49]–[56].

2.1.3 Humoral Immune Response

Another important arm of the adaptive immune response is characterized by the humoral response. Uptake of viral proteins by DCs and the subsequent processing into small peptides activate humoral responses. DCs present antigen associated with Major Histocompatibility Complex (MHC) II molecules to $CD4^{+}$Th cells, which then stimulate naïve B cells by producing specific cytokines. Subsequently, B cells recognize specific epitopes by surface antibodies and differentiate into plasma cells that will be able to produce IgG, IgA, IgE and into memory B cells.

Antibody responses to HIV can be detected within 6 to 9 weeks after infection and this process is commonly referred to as seroconversion [57]. The most immunogenic proteins of HIV are the envelope glycoproteins, as high titers of anti-gp120 and anti-gp41 can be detected in HIV-infected patients. The early antibodies usually are not neutralizing and are not efficient against the infection. Neutralizing antibodies have been found after 3 months from primary infection however they are not able to cope with the evolving virus. Interestingly, broadly neutralizing antibodies have been found in patients who have been infected with HIV-1 for a few years.

Broadly neutralizing antibodies bind to epitopes that the virus cannot afford to mutate as they are necessary for its own survival, like, for example, the CD4 binding site of gp140. Interestingly, these broadly neutralizing antibodies which are effective in clearing the infection have been selected after extensive somatic hypermutation, which implies that naïve B cells bind weakly to antigenic epitopes. Many vaccine strategies are based on the production of these broadly neutralizing antibodies, even though so far very little has been achieved.

2.2 Immune Activation in chronic HIV infection

Immune activation (IA) is defined as a condition characterized by higher frequencies of activated T-cells and B-cells, higher rates of lymphocyte turnover and higher plasma and serum levels of pro-inflammatory cytokines and chemokines. Persistent immune activation is one of the key features of HIV infection and it has led HIV infection to be considered also as a chronic inflammatory disease. Indeed, immune activation is considered today one of the main drivers of $CD4^+$ T cell depletion and progression to AIDS. Several markers have been associated with IA during HIV infection such as CD38 expression on $CD8^+$ T cells and HLA-DRII expression in T cells. Interestingly, high levels of activated $CD8^+$ T cells were shown to be associated with AIDS progression. Higher rates of proliferation, defined by Ki-67 expression, have also been associated with IA. In addition, increased numbers of HLA-DR^+, $CD38^+$ $CD8^+$ T cells were linked to a decrease in CD4+ T cell counts and AIDS [58]. It has observed that in each patient the level of T cell activation tend to stabilize for a period of time after 6 months post-acute infection. This period is thus called “set-point”. T cell activation at set-point is predictive of the rate at which $CD4^+$ T cells are lost [59]–[61]. Persistent IA is associated with inflammation, microbial translocation (MT)

and increased levels of plasma coagulation markers, such as β2-microglobulin (β2M), TNFα, soluble TNF receptor II (TNFRII), soluble IL-2 receptor and IFNγ, IL-10 and TGF-β1 [62]–[65].

Monocyte and macrophage activation has been investigated too. Thieblemont N. *et al*., and Dutertre *et al*., have investigated monocyte and macrophage activation in relation to disease progression and both studies indicated higher numbers of pro-inflammatory $CD14^+CD16^+$ monocytes in viremic and untreated patients [66], [67]. In addition soluble CD14 (sCD14), a marker of monocyte activation and indirect marker of microbial translocation, is negatively correlated with $CD4^+$ T cell counts in HIV-2 infected patients [68].

2.2.1 Mechanisms of Immune Activation and Microbial Translocation

The mechanisms underlying IA in HIV infection are not yet clear, even though many have been proposed. Indeed, understanding the molecular mechanisms behind chronic IA is not a simple task. There are many multiple mechanisms that have been proposed and it is likely that they may synergistically contribute to cause IA (Figure 6). Paiardini *et al*., suggested that it is also very likely that the relative contribution of the different mechanisms changes in each HIV-infected individual, during the different phases of infection and in naïve or HAART-treated patients [48].

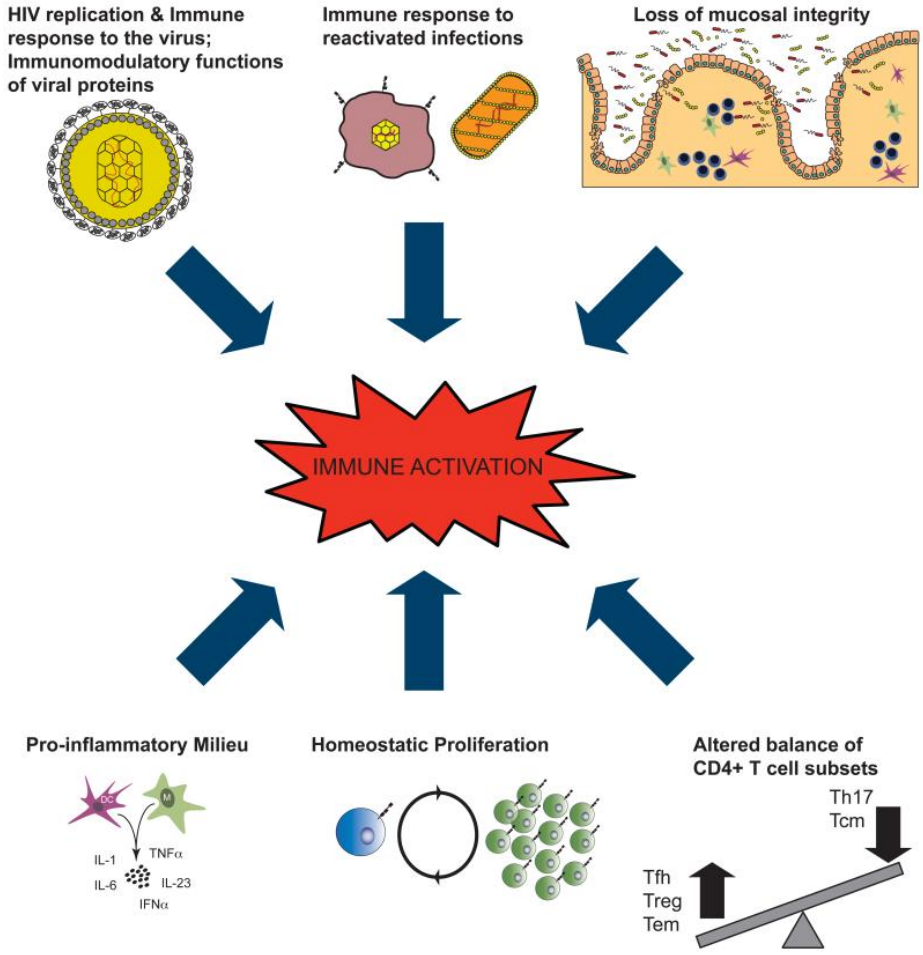


Figure 6: ***Mechanisms of Immune Activation during HIV infection.*** *HIV replication, immune response to reactivated pathogens, microbial translocation, increased production of pro-inflammatory cytokines, increased homeostatic proliferation and altered balance of CD4+ T cell subsets may all contribute to immune activation. Image from* [48].

One of the proposed mechanisms of IA is the innate and adaptive immune response against HIV. As mentioned before, HIV replicates in active immune cells and therefore it promotes T cell activation. Despite this, several evidences indicate that high viremia is neither sufficient nor

necessary to induce pathological IA levels. Frequency of activated T cells is higher than the one of HIV-infected $CD4^+$ T cells and other cell types show increased levels of activation [69]–[74]. In addition, even in patients treated with HAART, immune activation is still higher compared to healthy individuals [75], [76]. HIV proteins have been proposed to be involved in IA [77] as well as opportunistic infections, especially in late phase. It has been clinically observed that in HIV-infected patients latent viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivate more frequently than in healthy controls due to $CD4^+$ T cell loss and the consequent inability to mount a proper immune response. CMV reactivation has been found to promote immune activation and inflammation [78], [79].

Another important aspect involved in persistent immune activation is the loss of mucosal integrity of the GI-tract and the consequent microbial translocation (Figure 7). It has been established that during the acute infection many $CD4^+$ $CCR5^+$ T cells are lost in the GI-tract [80]–[82]. The massive loss of $CD4^+$ T cells in this area compromises the integrity of the mucosal intestinal barrier leading to microbial translocation. Microbial translocation implies the translocation of microbial and fungal products (peptidoglycan, lipoteichoic acid, LPS, flagellin) from the intestinal lumen to the system circulation [83], [84]. These microbial products would then be able to stimulate many different TLRs and induce the production of many pro-inflammatory cytokines such TNFα, IL-6, IL-1β and type I interferons, which would, in turn, contribute to persistent immune activation in HIV infection [64], [85], [86]. Despite these evidences, the relative contribution of microbial translocation to IA is not yet fully elucidated and is still a matter of discussion. Indeed, immune activation has been observed also in absence of increased levels of LPS in plasma [87]. As mentioned before, in SIV/HIV infection, intestinal Th17 T cells loss compromises the physical and immunological mucosal intestinal barrier. For these reasons the loss of

these cells has been proposed as one of the main causes of microbial translocation [52], [88]–[90].

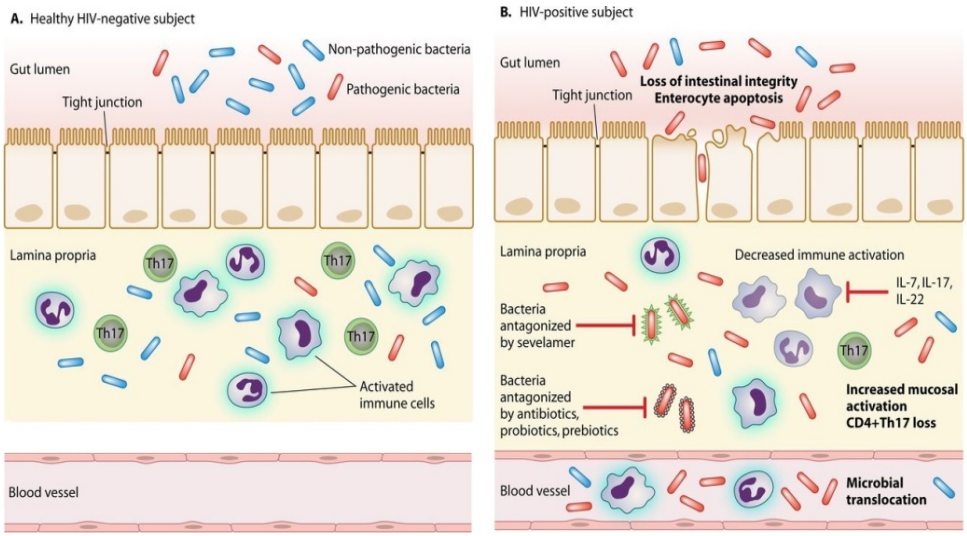


Figure 7: ***Microbial Translocation in HIV-infected individuals.*** *In healthy subjects the mucosal barrier is preserved thus limiting the leakage of bacteria into blood. However in HIV-infected patients, the mucosal barrier is damaged due to CD4+ T cell loss causing bacterial products leakage in blood stream and IA. Image modified from* [91].

Central memory (CM) $CD4^+$ T cells ($CD62L^+$ and $CCR7^+$) reside in the lymph nodes and, while not displaying strong effector functions, they are able to proliferate in response to antigenic re-stimulation and are thus important in maintaining $CD4^+$ T cells homeostasis. These cells represent the largest reservoir of HIV-1 infected $CD4^+$ T cells [81], [92]. CM $CD4^+$ T cells have been proposed to be important in the establishment of chronic immune activation. Indeed, depletion of CM $CD4^+$ T cell during infection may lead to T-cell homeostatic proliferation and, considering that they

harbor high levels of infection and that they reside in lymph nodes, they could also contribute to higher viral replication and thus immune activation.

Many studies have also focused on Tregs as they are deeply perturbed in HIV/SIV infection. Tregs are usually defined as CD25 high, CD127 low and Fork head Box P3 protein $(FoxP3)^+$and their main function is to lessen the effector activity of other immune cells by secreting anti-inflammatory cytokines such as IL-10 and TGF-β. Data on Tregs during HIV infection are somehow conflicting, even though there have been clear evidences that Tregs percentage in blood is increased, while their absolute counts are reduced [93]. Interestingly, the ratio of Th17 to Tregs cells is decreased in blood and GI-tract of HIV-infected patients and positively correlates with increased plasma markers of microbial translocation [94]. How Tregs could impact chronic immune activation is not yet clear. On one hand, these cells are considered harmful because they are able to suppress HIV-specific immune response, on the other they are considered beneficial as they could reduce inflammation. Interestingly, it has been demonstrated that depletion of Tregs correlates with $CD4^+$ and $CD8^+$ T cell activation in HIV infection [44], [95], [96].

Type I interferon have also been proposed as inflammatory mediators that could contribute to IA. They are released by innate immune cells upon recognition of HIV products by PRRs. Physiologically, type I interferon induces the expression of hundreds of genes known as interferon-stimulated genes (ISGs), which restrict intracellular pathogens. In the context of HIV infection the persistent expression of ISGs could actually be harmful by inducing the chronic production of pro-inflammatory chemokines and thus immune activation [97]. On the other hand, a large clinical study in which IFNα was administered for 12 weeks to HIV infected individuals not receiving HAART revealed that IFNα was well tolerated, increased some immune activation markers on $CD8^+$ T cells, reduced plasma viral load and

no signs of AIDS progression were observed [98], [99], confirming previous data by Lane H.C. and colleagues [100], [101].

2.2.2 Immune Activation and HIV Pathogenesis

Even if the causes of IA have not yet been fully elucidated, it has become clear that it plays a critical role in the pathogenesis of HIV infection.

Considering that HIV replicates in activated immune cells, persistent immune activation may provide available targets for HIV virus replication. This situation may trigger a vicious cycle in which new rounds of infections stimulate immune activation, which is able in turn to stimulate new infections leading to $CD4^+$ T cell loss. In addition, $CD4^+$ T cell loss may trigger a homeostatic response able to induce activation and proliferation of immune cells in order to replenish the lost cells. This situation, while trying to preserve the immune system, also provides new substrates for HIV replication. It should also be mentioned that persistent IA deregulates the architecture of immune tissues, such as bone marrow, thymus and lymph nodes.

It has also been observed that IA may inhibit the normal functions of B cells, NK cells, DCs and monocytes leading to less viral control and even more immune activation [48], [102].

Persistent activation of monocytes and increased plasma levels of pro-inflammatory cytokines (IL-6, sCD14, D-dimer) may induce damages in the vasculature which result in cardiovascular diseases [103], [104].

3. HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) AND IMMUNE RECONSTITUTION

Nowadays management of HIV infection includes the use of many different antiretroviral drugs, which are able to inhibit different steps of HIV replication cycle. The first drug to be approved for HIV infection treatment was zidovudine (AZT) in 1987 by the US Federal and Drug Administration and it was soon followed by the introduction of new antiviral drugs during the next decade. Major breakthroughs in the treatment of HIV infection using antiretroviral therapies began in 1995-1996 with the introduction of 3-drug therapy into clinical practice and the so called "combinational antiretroviral therapy", which lead to 80% decline in rates of AIDS, death and hospitalization of HIV-infected patients. ART transformed HIV infection from being a fatal condition leading to progressive immunodeficiency and ultimately to premature death into a chronic and non-fatal condition [105].
Antiretroviral drugs can be classified according to the viral life-cycle phase that they inhibit. In particular, there are 6 different classes:

- Non-nucleoside reverse transcriptase inhibitors (NNRTIs): NNRTIs are able to bind to and block HIV reverse transcriptase, thus preventing HIV from replicating. They interfere with the reverse transcriptase activity by blocking the hydrophobic pocket of the p66 subunit, which in turn leads to a conformational change that alters the catalytic site of the reverse transcription enzyme. Commercial drugs are Efavirenz (EFV) and Nevirapine (NVP), which are also considered first generation NNRTIs. Etravirine (TMC125) and Rilpivirine (TMC278) are considered second generation NNRTIs. HIV-2 is naturally resistant to this type of drugs.

- Nucleotide reverse transcriptase inhibitors (NRTIs): NRTIs are able to block HIV reverse transcriptase, however their mechanism of action is different from that of NNRTIs. NRTIs act as competitive inhibitors by blocking nucleosides addition to the DNA chain during the reverse transcription of HIV. Some currently used NRTIs are Abacavir (ABC), Emtricitabine (FTC), Lamivudine (3TC), Stavudina (d4T), Tenofovir (TDF) and Zidovudine (ZDV).

- Protease inhibitors (PIs): PIs are able to block HIV protease enzyme preventing new immature HIV virions from becoming infective viruses. Drugs belonging to this class are Atazanavir (AZT), Darunavir (DRV), Fosamprenavir (FPV), Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV) and Tipranavir (TPV).

- Fusion Inhibitors (FIs): FIs block HIV fusion with the target cell. They work in competition with gp41, blocking entry process of the virus into the host cell. Enfuvirtide (ENF) is a FI.

- CCR5 antagonists: They are able to block HIV entry into host cells by binding to CCR5 receptor. Maraviroc (MVC) belongs to this class. When administering this drug, some caution should be used as it may be possible a shift in tropism which allows HIV to target other co-receptors such as CXCR4.

- Integrase strand transfer inhibitors (INSTI): These drugs work by blocking HIV integrase from incorporating HIV DNA into the host cell's genome. Drugs belonging to this class are Raltegravir (RAL) and Elvitegravir.

Nowadays the therapeutic approach to treat HIV infection is based on a combined therapy in which drugs belonging to different classes are administered to achieve a synergistic effect. This type of therapy is called Highly Active Antiretroviral Therapy (HAART). A standard antiretroviral regimen usually consists of two NRTIs plus one NNRTIs or a combination of two NRTIs plus one PI. Mono-therapy has been shown to be unsuccessful: the use of a single inhibitor allows the emergence of new mutant viruses which are resistant to the drug due to both the extreme HIV genetic variability and the selective pressure applied by the drug itself. The combination therapy allows a drastic and stable viral load suppression and a rapidly increase in $CD4^+$ T cell counts and delays in time the emergence of new mutant viruses. In addition, current guidelines suggest testing genotyping drug resistance at the time of HIV diagnosis in order to choose the right combination of antiretroviral drugs. In general, HAART efficacy is achieved in 4-6 months after initiation with suppression of viral replication (< 50 copies/ml) and an increase of 100-150 cells/mm^3/year of $CD4^+$ T cell count [106], [107], [108]. It needs to be mentioned that the success of antiretroviral therapy greatly depends on the patient's adherence to the treatment regimen and one of the most common causes of suboptimal adherence is poor tolerability of ART treatment. In fact, adverse events have been associated with antiviral drugs and they can be classified as being short-term (such as anemia, dizziness, diarrhea, fatigue, nausea and vomiting, headaches, pain and nerve problems as well as coetaneous rashes) and long-term. Some of the most common long-term effects are lipodystrophy, insulin resistance which may lead to diabetes, lipid abnormalities characterized by increases in cholesterol and triglycerides, decrease in bone density and lactic acidosis. In order to avoid long-term complications of HAART, it is possible to change the drug regimen. Recently, the medical and scientific community has also looked into the possibility of suspending HAART regimen for short periods in order to

reduce toxic effects of long-term therapy and to improve patient's compliance. One of these largest studies was the SMART trial funded by the National Institute of Health. However the SMART study demonstrated that the benefits of continuous antiretroviral therapy outweighed the risks of long-term side effects. Another important aspect of HAART therapy is when it should be started. Current guidelines suggest starting HAART upon HIV diagnosis, especially if CD4 count is <350 cells/mm^3. Data from two large, randomized controlled clinical trials ART-START (Strategic Timing of Antiretroviral Therapy) and TEMPRANO demonstrated a 50% reduction in both morbidity and mortality among HIV-infected patients with CD4 counts >500 cells/mm^3 randomized to receive ART immediately versus delaying initiation of therapy [109]–[114]. Interestingly, more recently, there have been many evidences that HAART may prevent HIV infection and transmission. Indeed, two studies demonstrated that lower plasma HIV RNA levels are associated with a decrease in the virus concentration in genital secretions and studies of HIV-serodiscordant heterosexual couples showed that lower HIV plasma levels are associated with lower transmission rates [115]–[117].

3.1 Immune reconstitution in HAART

HAART reconstitutes $CD4^+$ T cell counts in a tri-phasic process: the first phase is characterized by an initial high increase in $CD4^+$ T cells due to reduced apoptosis and redistribution of memory $CD4^+$ T cells from lymphoid tissues to the blood compartment; the second phase is characterized by a slower continuous increase in part caused by generation of naïve $CD4^+$ T cells, while the third phase is characterized by a normalization of naïve and memory $CD4^+$ T cell compartments (Figure 8). Moreover, upon HAART

initiation there is a decrease of previously expanded HIV-specific T cells and a re-expansion of previously depleted pathogen-specific T cells [118].

More specifically, during the first 3 months of therapy there is a rapid reduction in viral load, which is accompanied by an increase of $CD4^+$ T cells (+200 cells mm^{-3} $year^{-1}$), especially of peripheral memory $CD4^+$ T cells. There is also a reduction of circulating inflammatory cytokines and chemokines such as TNF-α, IL-6 and MCP-1, which is accompanied by a decrease in activation markers on $CD4^+$ and $CD8^+$ T cells and in activation-induced cell death [119].

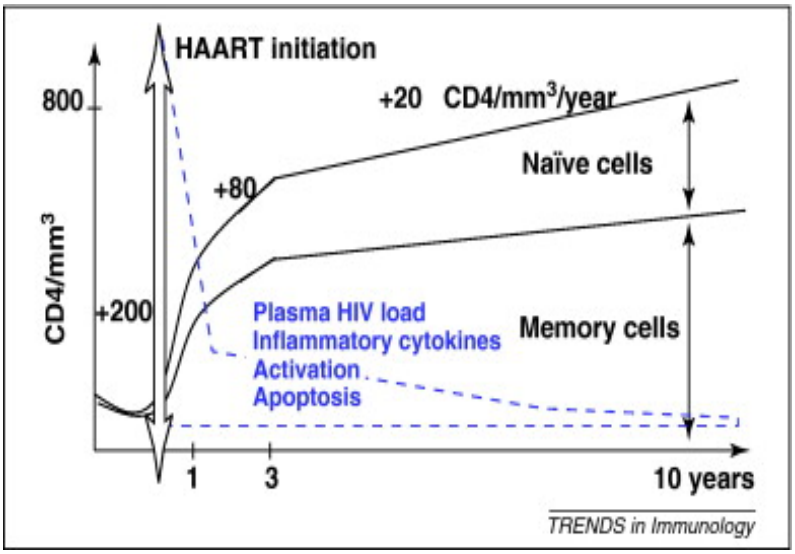


Figure 8: ***Dynamics of $CD4^+$ T Cell reconstitution during HAART.*** *In the first months after HAART initiation, the inhibition of virus replication leads to a rapid increase in $CD4^+$T cells(+200 cells mm^{-3} $year^{-1}$).Subsequently there is another increase (+80 cells mm^{-3} $year^{-1}$) due to regeneration of naïve $CD4^+$ T cells. This is then followed by another slope (+20 cells mm^{-3} $year^{-1}$) a normalization of naïve and memory $CD4^+$ T cell compartments and cellular activation parameters. Image from* [118] *and* [120].

The initial reconstitution of $CD4^+$ T cells during the first phase is a consequence of a redistribution of memory $CD4^+$ T cells from the lymphoid tissues towards the blood: immunophenotype analysis showed an up-regulation of CD45RO expression (marker of memory T cells) and a down-regulation of adhesion molecules like VCAM-1, ICAM-1, CCR7 and CD62L, which are necessary to maintain $CD4^+$ T cells into secondary lymphoid organs. Therefore the down-regulation of these adhesion molecules suggests that $CD4^+$ T cells are released from lymphoid organs into the periphery. During the first 3 months of HAART there is also an increase of $CD8^+$ T cells, NK cells and B cells in the peripheral blood, which seems to depend on cell redistribution [121], [122] (Figure 9). During this phase there is also a decrease of previously expanded HIV-specific T cells and a re-expansion of previously depleted pathogen-specific T cells recovered from pre-existing residual memory T cells [123], [118]. This recovery is strictly dependent on the control of HIV replication and it seems to be independent of $CD4^+$ T cell count and disease stage at HAART initiation [124], [125].

The second phase occurs after one year of HAART, when there is an increase (+80 cells mm^{-3} $year^{-1}$ slope, in Figure 1) in $CD4^+$ T cells due to the regeneration of naïve $CD4^+$ T cells [123], which is fundamental in restoring the diversity of $CD4^+$ T cell receptor (TCR) repertoire [126]. It has been demonstrated that the second phase of HAART immune recovery depends on the magnitude of viral replication control and it is not correlated on $CD4^+$ T cell depletion before treatment [127], [128]. After the second phase, there is a normalization of naïve and memory $CD4^+$ T cell compartments and cellular activation parameters (+20 cells mm^{-3} $year^{-1}$slope, in Figure 8).

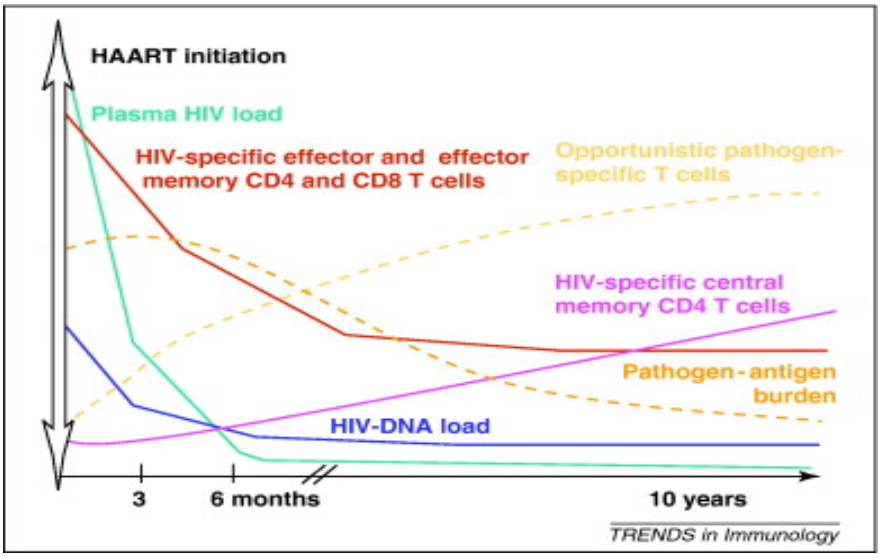


***Figure 9:* Recovery of pathogen and HIV-specific T cells by HAART.** *Broken yellow line: reconstitution of memory T cells specific for opportunistic pathogens; broken orange line: burden of pathogens, which decreases upon restoration of specific memory T cells (broken yellow line). HIV-specific effector and effector memory CD4$^+$ and CD8$^+$T cells (red line) decreases upon reduction of plasma HIV viral load (turquoise line). Pink line: HIV-specific central memory CD4$^+$ T cells: their restoration is possible thanks to the persistence of some HIV antigens from HIV reservoirs (blue line) and IL-7 dependent T cell homeostasis. Image from* [118].

The increase of naïve CD4$^+$ T cell during the second phase depends on restoration of thymic production on naïve cells and not to conversion of memory CD45RO$^+$ CD4$^+$ T cells. This observation was demonstrated by analyzing the TCR rearrangement excision circles (TREC). TRECs are episomal DNA fragments that are excised during TCR gene rearrangement in the maturing T cells in the thymus: Naïve T cells with higher levels of TRECs were observed after HAART, reflecting the output of recent thymic

emigrants [129]. It should be noted that that the high concentration of TRECs content could be due IL-7 level normalization. Indeed, before HAART treatment, IL-7 levels are high due to $CD4^+$ T cells homeostatic proliferation to compensate the cell loss; after HAART, IL-7 levels tend to normalize, thus reducing the homeostatic proliferation and enriching the CD4 TRECs content without increasing thymic output [130]. Nonetheless, the rise in $CD4^+$ T cells depends also on the homeostatic proliferation of the residual $CD4^+$ T cells and on the extension of $CD4^+$ T cells half life.

The mechanisms of immune reconstitution are not qualitatively comparables. Healthy T cells are able to recognize many different pathogens because of their repertoire, which allows them to recognize many different antigens. During HIV infection there is a reduction in this repertoire due to $CD4^+$ T cells loss. If the increase in CD4 counts stem from de novo T cell production there will be a restoration of this repertoire, however if this increase stems from homeostatic proliferation or extension of $CD4^+$ T cells half life, the T cell repertoire will remain truncated.

3.2 Immune Failure in HAART: Immunological Non-Responders (INRs)

Achieving a good virological and immunological response is important during HAART. A good virological response is reached when HIV replication is inhibited and can be monitored by evaluating plasma HIV viral loads in the affected individual. It is also important to achieve a good immunological response because it reduces morbidity and mortality among HIV-infected individuals [131]–[135]. A good immunological response is reached when $CD4^+$ T cell counts are normalized and generally above 500 cells/μl because most HIV-infected individuals with these levels of immune

reconstitution have morbidity and mortality rates close to uninfected individuals [136].

Nevertheless, 15-30% of HAART treated patients fail to restore $CD4^+$ T cell counts despite fully suppressed viral replication [119], [123], [137]. These individuals are referred to as "Immunological Non Responders" (INRs).

There is a certain lack of consensus regarding the definition of INR. Usually, INRs are defined as having $CD4^+$ T cell counts <350 cells/µl, however some studies consider INRs only those with $CD4^+$ T cell counts <200 cells/µl and treatment duration necessary to define patients as INR is variable. Some other groups have defined INRs by the percentages of $CD4^+$ T cell increase, commonly <20% increase from baseline [124], [138]–[140]. Recent data showed that $CD4^+$ T cell counts <350 cells/µl are associated with the risk of non-AIDS-clinical events, therefore nowadays Guidelines of the Department of Health and Human Services define immunological failure as the lack of increase in the $CD4^+$ T cell count to more than 350-500 cells/µl after 4-7 years of effective HAART [141], [142].

There is a clinical concern regarding the long-term risks of subclinical immunodeficiency and many studies have tried to elucidate the immunological dynamics that may cause this immune failure. Many factors have been associated with immunological failures, such as older age [143], probably due to a decrease of thymic function and other functional impairments [131], [134], [144]–[146], [147], longer duration of HIV infection prior to HAART initiation, co-infections with viral hepatitis viruses and lower nadir $CD4^+$ T cell counts [131], [144], [145], [148]–[152]. None of these factors provide a comprehensive explanation for the immune failure in INRs. For this reason many studies have tried to elucidate the mechanisms that lead to immunological failure (Figure 10).

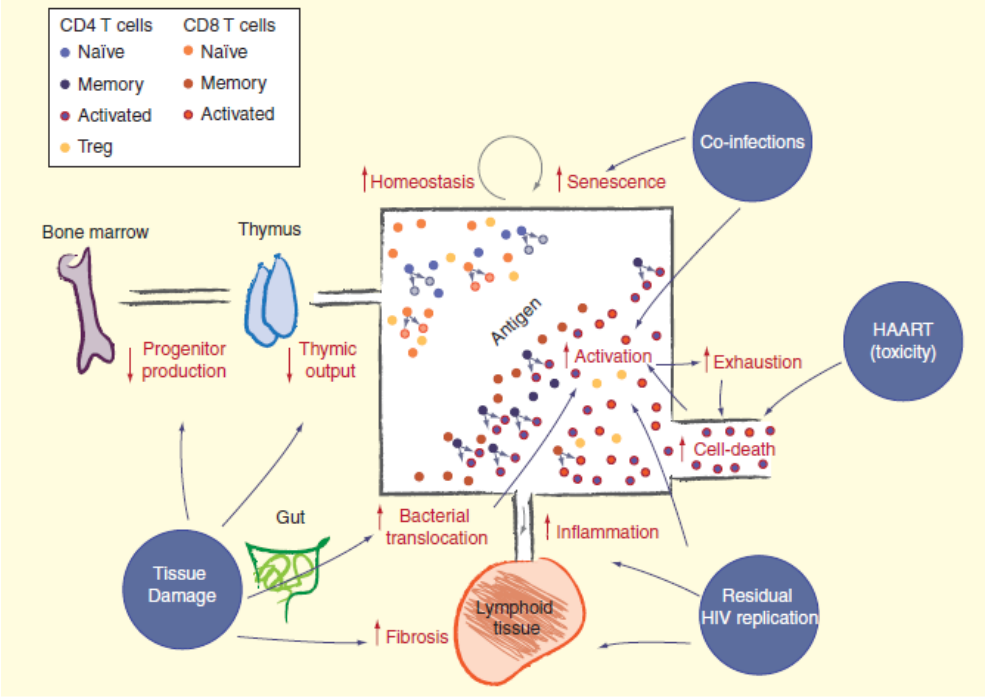


Figure 10: ***Dynamics of Immune Failure during HAART.*** *INRs have reduced bone marrow progenitors production and thymic output, which leads to a decreased pool of naïve T cells. INRs show increased homeostatic proliferation. In the gut, CD4+ T cell depletion leads to microbial translocation, which contributes to IA. Lymphoid tissue is damaged impairing de novo T cell production. Reactivation of latent viruses and co-infections may contribute to IA. Residual HIV replication may play a role in immunological failure too. Image from* [153].

Before analyzing the immunological dynamics at the basis of immune failure, we should consider that the $CD4^+$ T cell count in a given individual at any time is the result of production, destruction and traffic between lymphatic tissues and blood. Therefore INRs may have alterations either in the production or destruction of $CD4^+$ T cells leading respectively to a reduction of output or to an increased turnover. Furthermore, the

distribution of $CD4^+$ T cells between blood and lymphatic tissues may be different in INRs compared to immunological responders (IR) individuals.

3.2.1 Failures in the production of $CD4^+$ T Cells in INRs

Thymus function has been demonstrated to be particularly important in immune restoration. $CD4^+$ T cells generate by proliferation from already existing $CD4^+$ T cells or they are produced *de novo* in the thymus. Only the $CD4^+$ T cells produced in thymus display a full immunological repertoire because the genesis of TCR takes place only in this organ [154], [155]. Interestingly, it has been reported that HIV-infected patients with larger thymuses have a better immune recovery and broader immunological repertoire than patients with a small thymus [155], [156]. Different studies have been conducted in order to assess thymic function in HIV-infected individuals. Some groups have visualized thymic tissue in HIV-infected adults on computed tomography (TC) and the size of the thymus has been positively associated with both naïve and total $CD4^+$ T cell counts [155]–[158], others have assessed thymic function by looking at TRECs, recent thymic emigrants (RTEs) and naïve $CD4^+$ T cells. In fact, during the maturation process, T cells migrate from the thymus to the periphery and during this process they are classified as RTEs. They will then mature into naïve T cells. However, it has to be taken into consideration that thymic output is reduced with age and therefore, in such conditions, naïve cells are going to be increasingly generated by proliferation [159]. Many studies have demonstrated that the naïve T cell compartments in INRs seems to be more perturbed than the one in IRs and that INRs have a hypofunctional thymus [139], [160]. In particular, it has been suggested an altered thymopoiesis in INRs, which is supported by the finding of decreased levels of T cells expressing CD28 and by higher percentages of peripheral proliferation marker Ki-67 observed in INRs [161]. Immunological failure

has been also associated to lower thymopoietin levels in INRs [138], [162]–[164].

Impairments in bone marrow progenitor cell growth have been described in HIV-infected patients [165]–[167] and it is known that many hematopoietic progenitor cells (HPCs) express receptors CD4, CCR5 and CXCR4 and that could be therefore susceptible to HIV infection [168], [169]. Recent observations have hypothesized that immune failure in INRs could be in part explained by impaired bone marrow functionality [160], [170]–[173].

Another important role in T cell homeostasis is played by cytokines. As mentioned in the previous paragraph IL-7/IL-7R pathway is important in maintaining a correct T cell balance and HIV-infected individuals show higher serum levels of IL-7 and reduced levels of IL7R compared to healthy controls (HC) [174]. This pathway has been studied also in INRs and one study found that INRs' reduction of naïve $CD4^+$ T cells was associated with a reduced expression of IL-7R and increased levels of IL-7 [138] and other studies confirmed higher levels of IL-7 in INRs compared to IRs [160], [161], [175]. Higher levels of IL-7 in INRs are somewhat considered to be expected given the physiological role of IL-7 (promoting $CD4^+$ T cell production) and it may suggest that immunological signals are not impaired in INRs and that the lack of immune recovery might be attributed to the source of $CD4^+$ T cells.

Others cytokines have been found to be altered in HIV-infected individuals: IL-2 and IL-15 levels seem to be down-regulated [176], [177]. Production of IL-2 has been shown to be decreased in INRs compared to IRs [139], which is highly interesting considering IL-2 role in T cell proliferation.

3.2.2 Excessive destruction of $CD4^+$ T Cells in INRs

Immunological failure in INRs may also depend on excessive $CD4^+$Tcellsdestruction. One of the drivers of excessive destruction may be immune activation (IA). IA, as mentioned previously, is a key feature of HIV infection and HIV-infected patients, even on therapy, show elevated levels of activation markers [178], [179]. Hazenberg and colleagues proposed that IA may lead to $CD4^+$ T cell depletion by eroding the naïve T cell pool in untreated HIV infection [180]. Interestingly, many researchers have shown that INRs have higher levels of IA compared to HIV-infected individuals even though there are still no clear evidences that associate IA to excessive destruction of $CD4^+$ T-cells [138], [139], [161], [164], [181], [182]. Mavigner *et al*., have reported that increased residual viremia can be detected in INRs and it seemed to be linked to increased IA in these patients [183]. INRs have also shown to have higher levels of proviral DNA in different subsets of $CD4^+$ T cells even during viral suppression and this condition could lead to increased $CD4^+$T cell activation and loss [164], [184], [185]. Other studies reported higher frequencies of CXCR4 virus in INR: this is an interesting observation as X4 viruses appear later during HIV infection and they have been associated with increased thymic destruction, suggesting that X4 viruses may contribute to the depletion of naïve $CD4^+$ T cells [186].

Recently, Marchetti *et al*., reported a consisted trend of high levels of plasma LPS in INRs, compared to HIV-infected individuals with full immunological recovery. Higher levels of plasma LPS in INRs are also correlated with the proportion of activated $Ki67^+$, $CD4^+$ and $CD8^+$ T cells [187]. This observation is of particular interest because recent works - in particular by Brenchley *et al*., associated increased microbial translocation products from the gastrointestinal lumen, such as LPS, to a continuous trigger of immune activation in HIV-infected patients [83], [91], [188].

Indeed, according to Marchetti *et al*., microbial translocation may trigger continuous peripheral $CD4^+$ T cell activation, which in turn may lead to an inefficient immunological recovery in INR patients [91]. Despite these evidences, the role of microbial translocation in cART-treated individuals is still not clear [189].

A role for increased destruction of $CD4^+$ T cells may also depend on increased senescence and apoptosis of immune cells in HIV infection. It has been demonstrated that HIV infected patients have shorter telomere length and irregular telomerase activity, which is associated with cellular senescence and apoptosis [190], [191]. Many reports have shown that in INRs there are elevated levels of apoptosis of $CD4^+$ T cells [192]–[196]. Activation-associated T cell molecule programmed death-1 (PD-1) is a negative regulator of T cells and is selectively up-regulated in exhausted T cells during chronic viral infections [197]. Interestingly, Nakanjako *et al*., and Grabmeier-Pfisterhammer *et al*., have shown elevated levels of PD-1 in INRs compared to IRs [181], [198]. In our lab we have observed the same phenomenon too, suggesting that in INRs there is a certain degree of immune exhaustion [196].

In more recent studies, scientific focus has been on the discovery of new T cell subsets with pro- and anti-inflammatory properties. In particular, as mentioned previously, two subsets of $CD4^+$ T cells have been gained attention: Th17 cells with pro-inflammatory properties and regulatory T cells (Tregs) with anti-inflammatory properties. It has been shown that Tregs levels decline with disease progression, while their frequency increases on HAART [40]–[43]. However, in a prospective study Tregs were measured in HIV-infected patients before and after HAART and in healthy controls. In this study, Tregs levels were found to be higher in patients than in controls [41]. Other studies have reported increased fractions of Tregs in INR compared with immunological responders, in particular, activated Tregs appear to be more frequent, while resting Tregs are reduced [196], [199]–

[202]. Thus it is still unclear whether Tregs are beneficial or harmful by down-regulating HIV-specific responses and their role in immune reconstitution is still debated.

Th17 T-cells undergo a rapid depletion during infection and a dysregulated ratio of Th17 cells and Tregs have been associated with high IA, microbial translocation and disease progression in HIV infection [94], [203]. Recently, Gaardbo *et al*. reported disturbances in the Tregs and Th17 cell compartments in INRs, suggesting a potential role of these two subsets on immune reconstitution [200]. In another recent report, Girard and colleagues have found increased frequency of peripheral $\beta7^+$ Th17 and Treg cells in INRs compared to IRs and healthy controls probably to replenish the lost cells in the gut. However, once in the gut these $CCR6^+\beta7^+$ Th17 are more sensitive to infection than Tregs and could disturb the mucosal Th17/Tregs ratio [204].

4. INFLAMMASOMES AND HIV PATHOGENESIS

4.1 Inflammasomes

Inflammasomes were first discovered by Tschopp and colleagues in 2002. They are multimeric protein complexes that are able to recognize many different stimuli. They are a key component of the innate immune system and are fundamental in order to eliminate pathogens and host infected cells. Many different types of inflammasomes have been discovered since their first description, however they are characterized by a similar core structure. It usually consists of a unique patter-recognition receptor (PRR), which is activated in response to pathogen-associated molecular patterns (PAMPs) or other endogenous danger signals in the cytosol. PRR activation leads to oligomerization and recruitment of an adaptor protein known as ASC, which is composed of two death-fold domains: a pyrin domain (PYD) and a caspase recruitment domain (CARD). The adaptor protein ASC bridges the inflammasome to caspase-1: it binds the PRR through its PYD domain and it binds to caspase-1thanks to its CARD domain. Caspase-1 is then processed into active protease caspase 1, which, in turn, is able to initiate downstream responses by releasing IL-1β and IL-18 and by inducing pyroptosis, which is a form of programmed cell death characterized by high levels of inflammation [205], [206]. Some PPRs have a CARD domain and they are able to recruit caspase-1 without ASC protein. Indeed, inflammasome's oligomerization occurs through homotypic PYD-PYD or CARD-CARD interactions [207], [208]. Inflammasomes containing PYD domains, such as NLRP3, AIM2 and pyrin, recruit ASC for the complete assembly, whereas the ones containing CARD domains, such as NLRC4, are able to directly bind caspase-1. All inflammasomes are able

to induce pyroptosis however ASC-mediated complex assembly is necessary for cytokine processing [209]–[212].

Inflammasomes can be distinguished according to their structural features by their unique PRR and they are usually grouped into nucleotide-binding domain-like receptors (NLRs), absent in melanoma 2-like receptors (AIM) and pyrin (Figure 11). Recently “non canonical” inflammasomes have reported and they are able to target caspase 11 in mice and caspase 4 or 5 in humans. Other receptors have been confirmed to promote caspase 1 activation and they are NLRP6, NLRP7, NLRP12, retinoic acid-inducible gene I (RIG-I) and IFNγ-inducible protein 16 (IFI16).

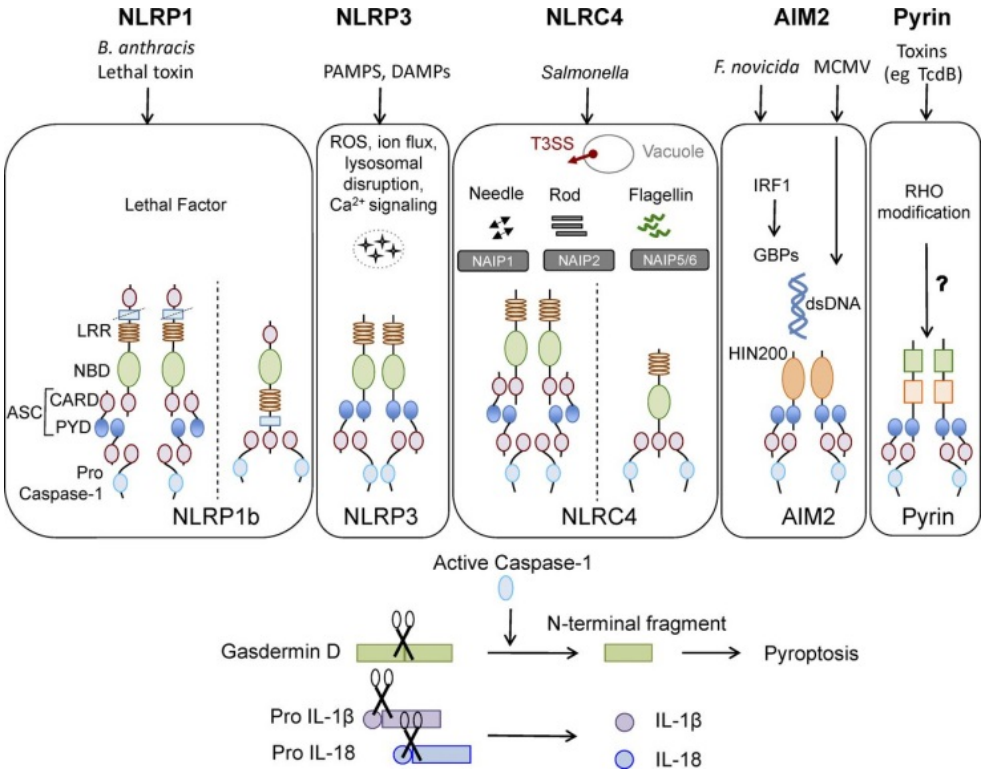


Figure 11: ***Structure of canonical inflammasomes NLRP1, NLRP3, NLRC4, AIM2 and Pyrin.*** *NBD is also known as NOD and NATCH domain. Image from* [213].

ASC-dependent NLRP3 and AIM2 activation has been studied extensively and it has been demonstrated that it is a process that requires two nucleation events. Upon sensing a stimulus inflammasomes are able to interact with ASC pyrin domain. This interaction leads to the assembly of ASC into a large protein specks (known as “ASC specks”), which consist of multimers of ASC dimmers. Thanks to CARD domain, ASC is able to cluster pro-caspase-1 so that NLRP3 sensor, ASC adaptor and pro-caspase-1 are present at increasing concentrations allowing signal amplification. This step allows caspase-1 self cleavage and the formation of homotetrameric caspase-1. Caspase-1 is then able to process IL-1β and IL-18 into their active forms. Activation of inflammasomes is an all-or-none phenomenon. It has been observed that inflammasomes activation leads also to release of ASC specks, which are phagocytosed by adjacent cells. This process seems to drive ASC nucleation and new rounds of inflammasome activation [214].

It has been demonstrated that exists differences in inflammasome expression between cell types. In a mice study, it was observed that splenic neutrophils, macrophages, monocytes and conventional dendritic cells had high expression levels of NLRP3, whereas lymphoid subsets, eosinophils and plasmacytoid dendtric cells had lower levels of NLRP3 expression [215]. There have been also reports of NLRP3 expression in ganulocytes and in human T and B lymphocytes [216], [217].

4.1.1 NLR Family

NLR cytosolic receptors are involved in recognition of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). NLRs have a common structure consisting of a C-terminal leucine-rich repeat (LRR), a central nucleotide-binding and oligomerization domain (NOD, also known as NATCH or NBD) and a N-terminal domain

which may contain either a pyrin domain (PYD), which is typical of NLRP subfamily, or a caspase recruitment domain (CARD), which is typical of NLRC subfamily (Figure 12).

NOD1, NOD2, NLRC4 and proteins with a related but not yet characterized domain (known as X) such as NLRX1 belong to the NLRC subfamily, while the NLRP subfamily consists of 14 different proteins. When these receptors recognize either DAMPs or PAMPs, they are able to trigger the NF-kB and MAP kinase signaling cascade and the formation of inflammasomes. NLRP1, NLRP3 and NLRC4 are known to be able to form inflammasomes, while for others, such as NLRP6 and NLRP12, it has only been established their ability to activate caspase-1 [218].

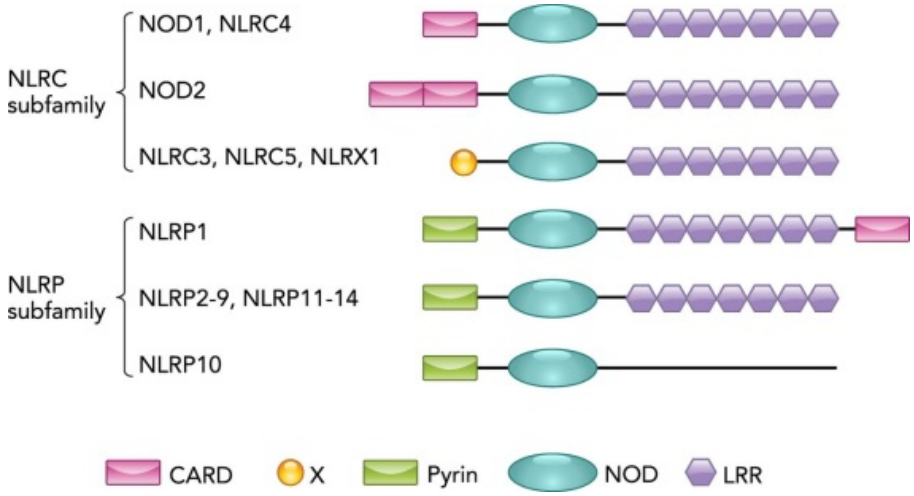


Figure 12: ***Schematic structure of NLRs subfamilies.*** *Image from* [219].

4.1.1.1 NLRP1

NLRP1 was the first NLRs to be recognized as being able to form an inflammasome complex. Humans have only one NLRP1, which consists of a NOD and LRR domains, a pyrin domain, a function to find domain and a C-terminal CARD domain. In mice, three different NLRP1 have been indentified such NLRP1A, NLRP1B and NLRP1C, with a similar domain organization but lacking PYD domain [220]. The specific triggers of NLRP1 inflammasome activation have not yet been fully understood. It has been demonstrated that two of the five mice *Nlrp1b* alleles are activated by anthrax lethal toxin, which comprises a protective antigen and a lethal factor. The protective antigen is able to generate pores in the host cell membrane, whereas the lethal factor is able to promote the cleavage of NLRP1 leading to the activation of inflammasomes. Toxoplasma has been shown to be able to induce NLRP1 inflammasome activation [221], [218]. Interestingly, it has been shown that mice harboring a mutation in the *Nlrp1a* gene (Q593P) develop a systemic inflammatory disease, which is mediated by caspase-1 and IL1β. In addition, these mice have a significant loss of hematopoietic progenitor cells suggesting an involvement of inflammasome activation and pyroptosis in the altered myelopoiesis. In humans NLRP1 mutations have been linked to autoinflammatory disease, Addison's disease, rheumatoid arthritis, systemic sclerosis and Crohn's disease [222].

4.1.1.2 NLRP3: canonical and non canonical inflammasome

NLRP3 is the most well characterized inflammasome as it has been associated with auto inflammatory conditions [223], [224] and its constitutive active form has been linked with many different human diseases. NLRP3 can be activated by many different PAMPs and DAMPs, such as microbial cell wall components, nucleic acids, toxins, pollutants, ATP, serum amyloid A and uric acid crystals [225]. It has been demonstrated that NLRP3 basal expression is not sufficient for inflammasome activation in resting cells. In most cell types, NLRP3 requires a priming step and a second activation steps that can be induced by different stimuli. Given the diversity of triggers that are able to activate NLRP3, it has been assumed that a common upstream signal is required for its activation. Many factors have been proposed as upstream signals such as, reactive oxygen species (ROS) production, potassium efflux, calcium signaling and lysosomal disruption [226]–[233]. The typical example of priming stimuli for NLRP3 is the binding of LPS to TLR4. Despite this, a unifying mechanism for NLRP3 activation has not yet been established and many different activation mechanisms have been proposed. It has been suggested that potassium release is associated with all NLRP3 activation mechanisms. Recent studies have shown that under resting conditions ASC is localized in the mitochondria, cytosol and nucleus, while NLRP3 can be found associated with the endoplasmatic reticulum [230]. Upon the correct stimulus, the subcellular localization of these molecules changes: mitochondria are dynein-transported toward the endoplasmatic reticulum allowing ASC to be close to NLRP3. Mitochondrial dysfunction are then able to induce microtubule alterations that allow NLRP3 to interact with ASC [234]. Interestingly, very recently NLRP3 activation has been associated with cell-cycle progression. The serine/threonine kinase NEK7 is necessary for progression through mitosis

and it has been observed that is also able to interact with LRR domain of NLRP3 and that this interaction is independent of its kinase activity and necessary for NLRP3 inflammasome activation. NEK is able to directly bind NLRP3 and to control the oligomerization process [235], [236].

Recently, it has been shown that inflammasome NLRP3 can also be activated thanks to caspase-11 in mice or caspase-4/5 in humans (Figure 13). This pathway is called the “non canonical inflammasome”. Caspase-11 has been shown to induce pyroptosis independent of capsase-1 and that it is necessary for NLRP3 inflammasome activation, especially in response to G ram-negative bacteria [237], [140]. In “Non canonical inflammasome” pathway a bacterial toll-like receptor (TLR), such as TLR4, leads to cellular priming and consequent up-regulation of NLRP3 and pro-IL1β expression. Subsequently, bacterial mRNA activates NLRP3 and LPS stimulates TLR4 and TRIF (TIR domain-containg protein inducing IFNβ) signaling, which leads to secretion of type I IFNs and caspase-11 induction[235], [239].In addition, caspase-11 detects intracellular LPS by directly binding it. This is facilitated by guanylate-binding proteins (GBPs). The LPS binding results in activation of caspases, which then cleaves Gasdermin D to induce pyroptosis. This process is able to induce the assembly of NLRP3 and the consequent activation of caspase-1 [206], [220], [240]. In humans, active caspase-4 can promote the activation the primed NLRP3 inflammasome without a canonical NLRP3 activating stimulus [241]. Notably, caspase-11 and caspase-4/5 are not able to cleave pro-IL1β and pro-IL18, although by activating NLRP3 it is still possible to observe cytokine release [238], [242].

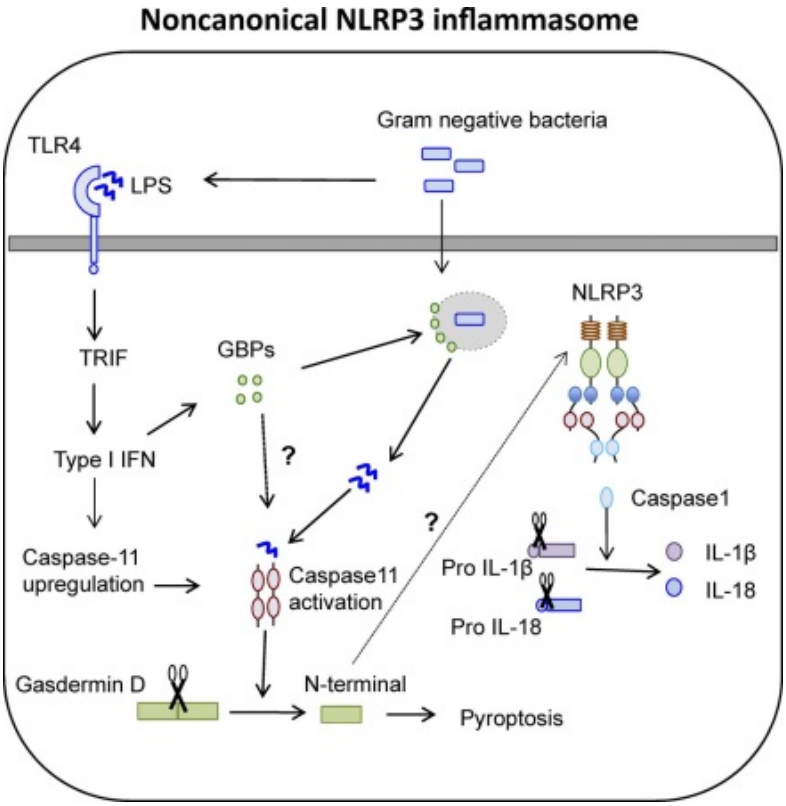


Figure 13: ***Caspase 11-non canonical NLRP3 inflammasome.****LPS is recognized by TLR4, which then mediates the up-regulation of caspase-11 and induction of pyroptosis and NLRP3 inflammasome assembly. Caspase-11 is also able to bind intracellular LPS facilitated by GBPs proteins. The binding directly activates caspase-11 which induces the cleavage of Gasdermin D inducing NLRP3 activation and pyroptosis. Image from* [213].

4.1.1.3 NLRC4

Salmonella infection, bacterial flagellin and multiple components of the bacterial type III secretion system are able to active NLRC4[243], [244]. NAIP proteins act as a sensors that recognize the ligands and induce NLRC4 activation [244]–[246]. In addition to IL-1β/IL-18 secretion and induction of pyroptosis, NLRC4 activation upon *Salmonella* infection may induce actin polymerization response, which prevents bacterial uptake and increases ROS production to enhance bacterial intracellular clearing [247].

4.1.1.4 AIM2 and Pyrin

AIM2 is the main member of the AIM family. It is involved in the immune response against many different types of viral and bacterial infections[248], [249]. It has been established that Type I IFN signaling is necessary for AIM2 activation during bacterial infections only [225], [250]. Pyrin was first discovered as an inflammasome in a mouse model of Mediterranean fever [222]. AIM2 has been associated with many human diseases such as psoriasis, abdominal aortic aneurysm and systemic lupus erythematosus.

4.2 Inflammasomes and T Cell Responses

The release of IL-1β and IL-18 by inflammasome activation has many different effects on the T helper cell polarization [251], [252] (Figure 14).

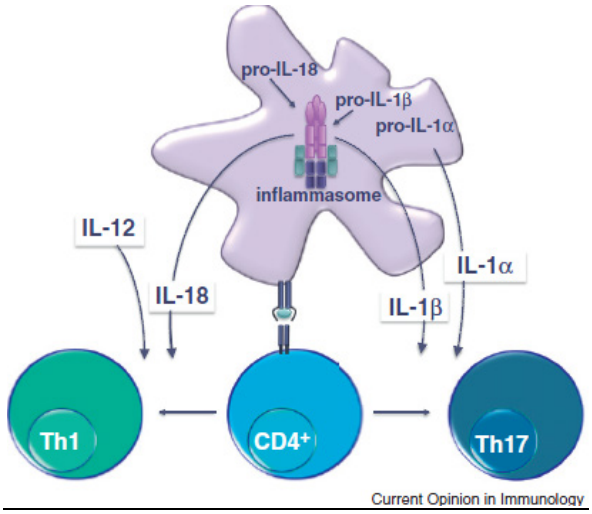


Figure 14: ***Inflammasome activation and T Cell Responses.*** *IL-18 stimulates differentiation in Th1 subset in synergy with IL-12, while IL-1β favours Th17 differentiation. Image from* [253].

It has been demonstrated that IL-1β stimulates T helper differentiation into Th17 T cells [254]. It was observed that certain DAMPs, known to activate inflammasomes, such as uric acid crystals, were able to promote Th17 responses in an ASC/caspase-1 dependent manner [255]–[257]. The role of inflammasome in Th17 polarization has been extensively studied in multiple sclerosis. IL-1β and inflammasomes have been implicated in the development of multiple sclerosis, which is driven by Th17 T cells [258]–[260]. In animal models during *Candida albicans* infection, it was reported a crucial role of caspase-1 and ASC in Th17 polarization [261]. In another

study, using *Asc*-deficient and *Nlrp3*-deficient mice challenged with *Schistosoma mansoni*, there was a reduction of IL-17 and IFNγ production [262]. On the other hand, IL-18 is able to stimulate the differentiation towards the Th1 subset by amplifying the production of IFNγ in many different infection models of infection [263].

4.3 Pyroptosis

Inflammasome activation leads to pyroptosis (from the Greek “pyro”, for fire and fever, and “ptosis”, which means falling), which is a form of programmed cell death dependent on activation of caspase-1 (and also caspase-4 and caspase-5). Pyroptosis, contrary to what happens during apoptosis, is characterized by plasma-membrane rupture, cell swelling and the release of proinflammatory cytokines and intracellular content [264], [265].The defining feature of pyroptosis is its dependence on caspase-1. Interestingly, caspase-1 is not involved in apoptosis and typical apoptosis related caspases, such as caspase-3, caspase-6 and caspase-8 are not involved in pyroptosis. Cell membrane rupture during pyroptosis is mediated by caspase-1 dependent plasma membrane pores: these pores dissipate cellular ionic gradients thus increasing osmotic pressure, water influx, cell swelling and finally osmotic lysis and relase of inflammatory cytokines and cellular contents [265]. DNA damage has been observed during pyroptosis, as well as destruction of the actin cytoskeleton [264], [266]–[269]. Pyroptosis has been described in macrophages, monocytes and dendritic cells where it contributes to defense against pathogens [270]. Contrary to other types of cell death, like necroptosis, in pyroptosis extra cytoplasmatic ligands, such as TNF or those that bind to TLRs, do not trigger directly pyroptosis but only “prime” the cell to pyroptosis by inducing the synthesis of proteins that signal it. The triggering event is the

recognition of intracellular pathogen components by PPRs. Caspase-4 and Caspase-5 are PPRs themselves and they can be activated by LPS and then trigger pyroptosis (non canonical pathway). Caspase-1 is dependent on PRRs like NLRs as mentioned in the previous chapter. Despite this, clear markers of pyroptosis are still missing. This is mainly due to the fact that many molecules involved in cell death are also involved in other functions in living cells. Recent studies have suggested that gasdermin-D (GSDMD) may be a good marker of pyroptosis. GSDMD is cleaved by caspases and it seems to contribute to death induction. Knock-out (KO) cells for GSDMD are resistant to the induction of pyroptosis by caspase-11 (or caspase-4/5 in humans) [206], [240].

4.4 Inflammasomes, pyroptosis and HIV pathogenesis

Many viruses are able to activate inflammasomes, caspase-1 and the production of IL-1β. Therefore, recently the association between inflammasomes and HIV has been elucidated by several research teams.

4.4.1 Inflammasomes and HIV-1

One of the first reports about a possible relation between inflammasomes and HIV comes from the work of Pontillo A. and colleagues: they reported that a 3' UTR SNP in *NLRP3* gene is associated with susceptibility to HIV-1 infection [271]. The same group observed that HIV-1 was able to induce the expression of NLPR3 and IL-1β secretion in alditrithiol-2-inactivated HIV-1 treated dendritic cells from healthy individuals but not from HIV-infected patients, suggesting that NLPR3 inflammasome could play a role in the first steps of HIV-1 infection. Dendritic cells from HIV-infected patients, although chronically activated, seemed to be unresponsive against pathogens [272],

[273]. More recently, it was demonstrated that HIV-1 is able to induce the first signal to activate NLRP3 inflammasome in monocyte-derived macrophages (MDM). MDMs were primed with LPS or different types of HIV-1 (HIV-1_{VSV-G} or HIV-$1_{\Delta envVSV-G}$) and subsequently treated with activators of the second signal for NLRP3 inflammasomes (ATP, nigericin, silica, alum and MSU) and then IL-1β production was evaluated. It was observed that MDMs primed with either types of HIV-1 were able to induce the secretion of IL-1β comparable to the one induced by LPS. In addition, they demonstrated that HIV-1 initiates the priming signal for NLRP3 inflammasome activation through the NF-kB-associated pathway [274]. Guo H. *et al*., investigated how HIV-1 is able to induce IL-1β production and they observed that HIV-1 was able to induce the expression of pro-IL-1β via TLR8 and that NLRP3 inflammasome was required for IL-1β maturation and secretion. In particular, they observed that HIV-1 infection induced caspase-1 activation and that NLRP3 inflammasome activation in response to HIV-1 is dependent on ROS production and Cathepsin B. Interestingly, they also observed that transfection of HIV-1 derived single-stranded RNA into THP-1 cells induced caspase-1 activation and IL-1β production, indicating that HIV-1 activates NLRP3 inflammasome thanks to its RNA [275]. Inflammasome activation has also been studied in the brains of HIV-infected individuals: in particular, it has been reported that IL-1β, IL-18 and capsase-1 were induced in the brains of HIV-infected patients and were detected in microglial cells upon HIV-1 infection [276]. More recently, vpr HIV protein has been reported to be able to cause caspase-1 cleavage and IL-1β release with reduced cell viability dependent on NLRP3 stimulation. It was suggested that Vpr may act independently as either or both of the inflammasome signals [277]. In addition, it has been demonstrated that upon primary HIV/SIV infection, there is a rapid activation of NLRX1 inflammasome [278]–[280]. The upregulation of NLRX1 is of particular interest as it inhibits the antiviral IFN responses, thus promoting HIV-1

infection and facilitating HIV-1 reservoirs. Interestingly, Nasi M. *et al*. reported a decreased expression of NLRX1 in ART-treated-HIV infected patients, suggesting that antiretroviral therapy may be able to inhibit this inflammasome [281].

4.4.2 Pyroptosis and HIV

Very recently, Doitsh G. *et al*., demonstrated that the majority of T cells dies via pyroptosis during HIV infection [282]. This work has been a huge breakthrough in the field because it demonstrated for the first time a key role of inflammasomes and pyroptosis in the pathogenesis of HIV. Many studies have tried to elucidate how $CD4^+$ T cells are lost during HIV infection and many mechanisms have been proposed. At first, it was suggested that productively infected and activated T cells were the ones dying and that depletion was due to a cytopathic effect of the viral life cycle [283]. However, it became clear that the frequency of activated $CD4^+$ T cells was very limited and could not explain the massive depletion of this cell subset [284]. Therefore, it was suggested that actually most of the dying cells were bystander $CD4^+$ T cells in the lymph nodes who were not actively infected [70]. Yet, how $CD4^+$ T cells are actually lost during infection is still a matter of debate. It was proposed that some host factors, such as TNFα, Fas ligand and TRAIL, and viral factors, such as Tat, Vpr and Nef, could contribute to cell loss [77], [285]–[287]. Doitsh G. and colleagues used an *ex vivo* human lymphoid aggregate culture (HLAC) system using fresh human tonsil or spleen tissues [288] to better understand how bystander T cells could die upon HIV infection. They observed that infection of HLACs with X4-tropic HIV-1 resulted in almost a complete depletion of $CD4^+$ T cells, even if only approximately 5% of these $CD4^+$ T cells became productively infected with the virus. Thus, indicating that 95% of the dying CD4 T cells were resting $CD4^+$ T cells. Subsequently,

they treated these cell cultures with antiviral drugs and discovered that bystander $CD4^+$ T cell death was prevented by entry inhibitors and fusion inhibitors, but not by NRTIs. Interestingly, the use of NNRTIs blocked $CD4^+$ T cell death in HIV-infected HLACs, suggesting that a certain degree of viral DNA is required to induce cell death [289], [290]. One of the hypotheses was then that cell death observed in these HLACs cultures involved abortive viral infection of resting $CD4^+$ T cells. According to this model, HIV binds and fuses to bystander $CD4^+$ T cells, however due to their resting state, the viral life cycle attenuates during reverse transcription giving rise to incomplete cytosolic viral DNA transcripts [290]. These viral DNA transcripts are then sensed by IFI16, which is known to induce caspase-1 activation [291]. Indeed, treatment of HIV-infected HLACs with caspase-1 inhibitor or with shRNA-mediated knockdown of caspase-1 prevented $CD4^+$ T cells loss. Of note, inhibitor of caspase-3 or shRNA-mediated knockdown of caspase-3 did not prevent cell loss. Given these data, Doitsh G. *et al*., suggested that death of abortively infected $CD4^+$ T cells in HLACs cultures was mediated by pyroptosis. In subsequent studies, it was shown that blood cells were highly resistant to pyroptosis and this is probably due to their deeper state of cellular rest, which is associated with fewer viral DNA and lower expression of innate immune sensors like IFI16. It should be noted that when pyroptosis-resistant peripheral blood $CD4^+$ T cells were co-coltured with either $CD4^+$ or $CD8^+$ T cells or B cells from lymphoid tissues, they readily died by caspase-1 activated pyroptosis induced by caspase-1 activation [292]. Galloway and colleagues observed that in HLACs models, the mode of viral spread affects cellular death: according to them, infection with free HIV-1 particles is unable to trigger innate immune recognition and pyroptotic cell death, thus infected $CD4^+$ T cells die by apoptosis. On the other hand, cell-to-cell spread of HIV-1 gives rise to massive abortive infection, which leads to pyroptosis [293]. These

observations are interesting considering that it has always been assumed that circulating free virions are the main cause of $CD4^+$ T cell loss [290].

Doitsh G. and colleagues have also suggested a model in which pyroptosis could contribute to sustain chronic inflammation and immune activation in HIV infection. In their studies they observed that tissue-derived $CD4^+$ T cells are primed to mount inflammatory responses as they produce in high quantities pro-IL1β, ASC and NLRP3 inflammasome [282]. In addition, the secretion of proinflammatory cellular contents during pyropototic $CD4^+$ T cell death may provide a second inflammatory stimulus which could lead to the activation of NLRP3 inflammmasome and caspase-1 in surrounding $CD4^+$ T cells and thus to new round of pyroptosis, cell death, inflammation and immune activation, even when viral replication is suppressed by HAART (Figure 15).

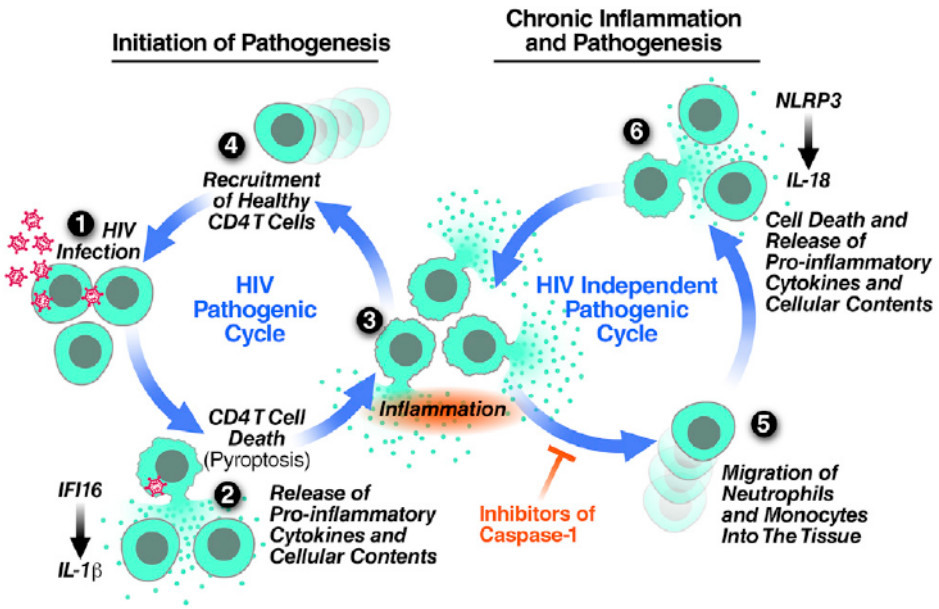


Figure 15: ***Pyroptosis and HIV pathogenesis in lymphoid tissue.*** *Abortive HIV infection of CD4+ T cells leads to cytosolic DNA products sensed by IFI16 (1), which leads to pyroptosis and inflammation (2 and 3). Inflammation promotes the migration of new circulating central memory CD4 T cells into the lymph nodes (4) establishing a vicious cyle of HIV infection, cell loss and inflammation. Inflammation also promotes the recruitment of neutrophils and monocytes in the lymphoid organs (5). These cells constitutively express high levels of pro-IL18, ASC and NLRP3 inflammasome. The release of pro-inflammatory content by pyroptotic cells may activate NLRP3 inflammasome leading to new rounds of pyroptosis and cell death even during antiretroviral therapy (6). Image from* [290].

Therefore, according to this new interesting model, abortive infections of resting $CD4^+$ T cells would lead to HIV DNA sensing by IFI16 and to the subsequent activation of caspase-1 and pyroptosis. The release of pyroptotic pro-inflammatory content would then activate NLRP3

inflammasome, which – in turn – would lead to new rounds of pyroptosis and cell death, thus sustaining chronic immune activation in HIV infection.

Of note, most of the experiments about pyroptosis in HLACs model were conducted with X4-tropic HIV isolates, so it still remains to be elucidated if R5-tropic HIV viruses induce the same effects. It is established that X4 isolates may emerge later during infection and have been associated with extensive $CD4^+$ T cell loss, however most HIV viruses found in HIV infected individuals are actually R5. For this reason, Steele A. and colleagues evaluated if pyroptosis occurs in $CD4^+$ T cells after exposure to CCR5-tropic HIV. They created a model from gut cells called Lamina Propria Aggregate Culture (LPAC) and infected them with a R5-tropic HIV isolate. They found that the magnitude of cell loss correlated with the productivity of HIV infection, however the majority of dying $CD4^+$ T cells was not infected and expressed caspase-1, suggesting pyroptosis. Treatment with a caspase-1 inhibitor was able to reduce T cell loss, suggesting that pyroptosis was one of the major reasons of cell death.

Given the possible role of microbial translocation in HIV pathogenesis, Steele and colleagues performed similar experiments with the addition of *Escherichia coli*. They observed that *E. coli* enhanced $CD4^+$ T cell depletion and caused a shift in the type of cell death from pyroptosis to apoptosis. Indeed, in this model, caspase-1 inhibitor was not able to rescue $CD4^+$ T cells, however caspase-3 inhibitor was. The authors suggested a model in which pyroptosis predominates in the early phases of HIV infection contributing to breakdown of gut wall epithelial integrity. Once microbial translocation is established, T cells and APCs would be exposed to commensal bacteria resulting in enhanced HIV-1 replication. This would lead to a shift in the cell death pathway to apoptosis [294].

Interestingly, in another work, the expression of IFI16 was significantly higher in untreated HIV infected patients. IFI16 expression levels correlated positively with viral load and CD38 expression level, thus with immune

activation and inversely correlated with $CD4^+$ T cell counts [295].Another report suggesting a possible role of caspase-1 in HIV pathogenesis and inflammation comes from Song J. and colleagues. They analyzed the dynamics of caspase-1 and caspase-3 in the peripheral blood of HIV infected untreated patients with $CD4^+$ T cell counts <250 cells/µl ($CD4_{low}$) and in HIV-infected untreated patients with $CD4^+$ T cell counts >450 cells/µl ($CD4_{high}$) within 2 years after primary infection. They observed that caspase-1 and caspase-3 plasma levels underwent a rapid increase, followed by a decrease after a short time during early infection in the $CD4_{high}$ group, probably due to the typical cytokine storm of early HIV infection. On the other hand, caspase-1 and caspase-3 levels were not increased during acute HIV-1 infection in the $CD4_{low}$ group, which is probably due to a weak immune response. However, one year after infection, caspase-1 and caspase-3 levels were increased in the $CD4_{low}$ group but not in the $CD4_{high}$ group, suggesting that that the rapid disease progression of the $CD4_{low}$ group could be associated with cell death later in the course of infection [296].

Aim of the Study

The determinants of immune failure under HAART are incompletely understood, even if many factors have been shown to be associated with this condition, such as older age, residual HIV replication, impairments of $CD4^+$ T cell *de novo* production and excessive destruction. A suboptimal immunological response to HAART may have dramatic clinical consequences as INRs are at increased risk of clinical progression to AIDS and of non-AIDS related diseases. Indeed, these patients show higher levels of immune activation and chronic inflammation compared to other HIV-infected individuals under medical treatment. Thus, it is important to find new factors associated with immunological failure in order to find new possible therapeutic approaches that could help to manage this clinical condition and improve HIV-positive subjects' health.

Recent discoveries on the role of inflammasomes in immunity and in HIV pathogenesis have shed new interesting perspectives on $CD4^+$ T cell loss during HIV infection and new possible immunological pathways leading to immune activation and chronic inflammation that characterize HIV infection.

Therefore, the aim of this study is to analyze and evaluate possible associations between inflammasomes activation, T cell activation and the degree of immune reconstitution in HIV infected HAART-treated patients.

Materials and Methods

1. SAMPLE COLLECTION

1.1 Study Population

39 HIV- infected ART-treated patients were enrolled at the Unit of Infectious Diseases, San Gerardo Hospital, Monza, Italy. 22 patients were immunological responders (IR) and 17 were immunological non responders (INRs). The inclusion criteria were: men and women >18 of age, HIV positivity tested with ELISA and confirmed by Western Blot, combined antiretroviral therapy, duration of ART > 24 months, plasma HIV-RNA < 50 cp/mL for at least 12 months, $CD4^+$ T cell count <350/mmc if immunological non responder (INR) or >500/mmc if full responder (IR).The exclusion criteria were: presence of actual opportunistic AIDS-related diseases, chronic inflammatory disorders, immunosuppressive therapy ongoing. Written informed consent was obtained from each patient before enrollment in accordance with the declaration of Helsinki.

1.2 PBMCs Separation

Peripheral whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) (BD, Rutherford, NJ, USA). Samples were centrifuged at 1500 rpm for 10 minutes at room temperature to obtain plasma, which was then collected and stored at -80°C for subsequent analyses. The blood sample was then diluted in phosphate buffered saline (PBS) (Euroclone, Milano, Italy) and PBMCs were separated by density gradient centrifugation on lymphocyte separation medium (Ficoll-Hypaque) (Cedarlane Laboratories Limited) at

2300 rpm, without brake, for 25 minutes at room temperature. Ficoll-Hypaque solution was layered slowly underneath the blood/PBS mixture by placing the tip of the pipet containing the Ficoll-Hypaque solution at the bottom of the sample tube. 10 ml of Ficoll-Hypaque solution were used for 35 ml of blood/PBS mixture. PBMCs separation by Ficoll-Hypaque takes advantage of the density differences between the mononuclear cells and other cellular elements in the blood samples. After centrifugation, the low density mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer, while the high density red blood cells (RBC) and granulocytes collect at the bottom. PBCMs are then collected and washed in PBS and centrifuged at 1750 rpm for 10 minutes at room temperature. Cellular pellets were dissolved in PBS and cell count and vitality were evaluated.

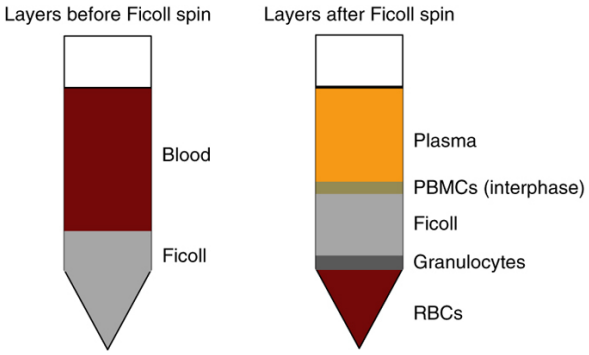


Figure 16: ***PBMCs separation.*** *RBC: red blood cells. Image from* [297].

1.2.1 Cell Count

The number and viability of the cell were determined by automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). Two sensitive fluorescence dye staining solutions, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium Iodide/PBS) are

used to determine cell count. Solution T measures total cell concentration by disrupting plasma membrane and nucleus staining. AccuStain Solution N stains non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

1.3 PBMCs Cell Culture and Stimulation

PBMCs were subsequently resuspended in fresh completed medium composed of RPMI 1640 (Euroclone, Milano, Italy), 1% L-glutamine (Sigma-Aldrich), 1% Pen/strep (Sigma-Aldrich) and 10% Human AB Serum (Euroclone, Milano, Italy). For flow cytometry experiments, PBMCs were then cultured ($1x10^6$ cells/ml) for 18 hours either in complete medium (basal condition) or in complete medium with LPS (1 µg/mL) (Sigma-Aldrich) or in complete medium with aldrithiol-2 (AT2 treated)-HIV-1_{BaL}virions (300 ng/mL) (a kind gift from Julian Bess, Frederick Laboratory for Cancer Research, National Institute of Health, Frederick, MD, USA). Treatment with aldrithiol-2 suppresses the infectivity of the viral particles, while maintaining the conformation and functional integrity of virion surface proteins [298]. In cell cultures stimulated with LPS or AT2-treated HIV-1_{BaL}virions, anti-CD28 monoclonal antibody (2µg/ml) (R&D Systems) was added to facilitate co-stimulation. For cytokine analysis, 1 µg/mL of Brefeldin A (Sigma-Aldrich) was added to the cell cultures 6 hours before flow cytometry cell staining to block protein secretion. For gene expression analyses, PBMCs were cultured as described before, however they were stimulated for 3 hours either with LPS or with medium alone (negative control) or for 24 hours with AT2 treated-HIV-1_{BaL}virionsor medium alone (negative control).

2. FLOW CYTOMETRY ANALYSIS

2.1 Immunophenotypic analysis

Immediately after separation, $0{,}5x10^6$ PBMCs were stained with fluorochrome-labeled monoclonal antibodies for 15 minutes at room temperature in the dark. Cells were then washed, resuspended in PBS and immediately acquired by the cytofluorimeter. 5 μl of following antibodies conjugates were used: anti-CD4-Phycoerythrin (PE)-Cyanine 7 (Clone RPA-T4, IgG1, kappa mouse, eBioscience), anti-CD8-PE-Cyanine 5 (Clone RPA-T8, IgG1, kappa mouse, eBioscience), anti-CD38-PE (Clone HB-7, IgG1, kappa mouse, BioLegend), anti HLADR-II-fluorescein Isothiocyanate (FITC) (Clone Immu-357, IgG1 Mouse, Beckman-Coulter). Unstained control sample and single simple stained samples were used for color compensation. Cytometric analyses were performed on 200000 events using a Gallios500 flow cytometer (Beckman-CoulterFlow). Data were analyzed by first gating on the lymphocyte population as defined by forward and side light scatters. CD4 or CD8 cells against side scatter were then selected and used to evaluate the percentage of positive cells for the marker analyzed. Data were analyzed using Kaluza 1.3 software (Beckman Coulter).

2.2 Intracellular Cytokine Staining and T cell subsets detection

After 18 hours of stimulation, cells were centrifuged at 1750 rpm for 10 minutes at room temperatures. Cells were then resuspended in PBS and stained with fluorochrome-labeled monoclonal antibodies for 15 minutes, in dark and at room temperature. Cells were then fixed in 1% paraformaldehyde (PFA) (Sigma-Aldrich), permeabilized with 0,5% saponin (Sigma-Aldrich) and stained for intracellular antigens and cytokines for 45 minutes, on ice and in dark. Cells were washed with PBS and then fixed in 1% PFA (Sigma-Aldrich). 5μl of following antibodies conjugates were used: anti-CD4-PE-Cyanine 7 (Clone RPA-T4, IgG1, kappa mouse, eBioscience), anti-RORγT-PE (Clone AFKJS-9, IgG2a Rat, eBioscience) anti-IL-17A-FITC (Clone eBio64DEC17, IgG1, kappa mouse, eBioscience). Unstained control sample and single simple stained samples were used for color compensation. Data were acquired by a Gallios500 flow cytometer (Beckman-Coulter) and analyzed by first gating on the lymphocyte population as defined by forward and side light scatters. CD4 or CD8-positive cells against side scatter were then selected and used to evaluate the percentage of positive cells for the marker analyzed. Data were analyzed using Kaluza 1.3 software (Beckman Coulter).

3. GENE EXPRESSION ANALYSIS

3.1 Total RNA Extraction

Total RNA was extracted by using the acid guanidium thiocyanate-phenol-chloroform method. $1x10^6$ of freshly isolated PBMCs were immediately lysed by 200 µl of RNAzol B reagent (Duotech, Milan, Italy). LPS-stimulated PBMCs ($1x10^6$cells) and their relative non-stimulated control ($1x10^6$ cells) were lysed by 200 µl RNAzol B reagent after 3 hours of stimulation, whereas AT2-treated HIV-$_{1BaL}$-stimulated PBMCs ($1x10^6$cells) and their relative non-stimulated control ($1x10^6$cells) were lysed by 200 µlRNAzol B reagent after 24 hours of stimulation. RNAzol B reagent (Duotech, Milan, Italy) is a monophase solution containing phenol and guanidine thiocyanate. After cellular lysis, chloroform (20% of initial volume of RNAzol B) was added to separate the lysate into aqueous and organic phase. Samples were then centrifuged at 12,000g 15 minutes at 4°C and DNA and proteins were separated from the aqueous phase containing RNA. The aqueous phase containing RNA was collected and transferred to a sterile tube and an equal volume of isopropanol was added. Samples were then stored at -20°C overnight to allow complete precipitation of RNA.

The day after, samples were heated at 4°C for 15 minutes and centrifuged at 13000g for 15 minutes. Supernatant was removed and RNA pellet was washed with 100µl of 75% ethanol twice. Subsequently the sample was centrifuged for 8 minutes at 12000g at 4°C. Supernatant was removed and the pellet was dried and dissolved in RNAse-free water (Sigma-Aldrich). The quality of RNA was tested using Nanodrop Spectrophotometer.

3.2 DNAse treatment

The RNA was treated with DNAse to eliminate contamination of genomic DNA. For each sample a reaction mix was prepared containing 1µg of RNA, 2 µl of DNAse 2U/µl (New England Biolabs), 1.15 µl of 10x DNAse buffer (10mM TRIS-HCl ph 7.5, 25mM $MgCl_2$, 5mM $CaCl_2$), 0.35 µl 1U/µl RNAsi inhibitor (20 U, Promega) and pure water up to 11.5 µl of final volume. Reaction mix was incubated at 37°C for 30 minutes. 1µl of EDTA 60 mM (Ambion Inc., Austin, Texas, USA) was added to each sample to stop DNAsi action at 75°C for 10 minutes.

3.3 Retrotranscription

1 µg of RNA was reverse transcribed into first-strand cDNA in a 20-µl final volume. A reaction mixture, containing 1 µM random hexanucleotide primers, 1 µM oligo dT and the RNA, was heated at 70 °C for 5 minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent secondary structure from reforming. A dNTPs mix, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 U Recombinant RNasi inhibitor and M-MLV 5X reaction buffer were added (Promega, Fitchburg, WI, USA). The reaction mix were incubated 60 minutes at 42 °C and then heated 5 minutes at 95 °C to inactivate the RT. Each cDNA sample was diluted 1:8 in RNAse and DNAse free water (Sigma-Aldrich) and stored at -20°C.

3.4 Real-Time PCR Array

Inflammasome signalling pathways were analyzed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA) according to manufacturer's instructions. This approach permits the monitoring of mRNA expression of 84 genes (Table 1) related to the inflammasome pathway, plus housekeeping genes, following the procedures suggested by the manufacturer. Controls were also included on each array for genomic DNA contamination, RNA quality, and general PCR performance. The results were analyzed by SABioscences online software and GAPDH and β-Actin were used as housekeeping genes. Only targets showing at least a 2-fold modulation were considered significant. The experiments have been run on samples pooled into two distinct groups (INRs and IRs) on the basis of condition (LPS stimulation and relative untreated control and AT2-treated HIV-1_{Bal} stimulation and relative untreated control). Thus, results represent the mean value of the different targets analyzed in each group. The expression of some targets that showed a modulation equal to nfold>2 were confirmed by Real time PCR on each individual sample.

Functional Gene Grouping	Genes
Toll-like Receptor (TLR) Signaling:	Receptors & Cofactors: CD14, LY96 (MD-2), TLR1, TLR2, TLR4, TLR5, TLR6, TLR9. MYD88-Dependent: FADD, IRAK1, IRAK3, IRF5, IRF7, MAP3K7 (TAK1), MYD88, TIRAP, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TOLLIP, TRAF6. TICAM1 (TRIF) Dependent (MYD88-Independent): IRF5, IRF7, MAP3K7 (TAK1), RIPK1, TICAM1 (TRIF), TICAM2 (TRAM), TLR4, TRAF6. Other: AKT1, CASP8 (FLICE), LBP, PIK3CA (p110a), RAC1
NOD-like Receptor (NLR) Signaling:	Receptors: NAIP (BIRC1), NLRC4 (IPAF), NLRP1, NLRP3, NOD1 (CARD4), NOD2. Inflammasomes: CASP1 (ICE), NAIP (BIRC1), NLRC4 (IPAF), NLRP1, NLRP3, PYCARD (TMS1/ASC). Regulation: BIRC3 (c-IAP1), CARD6, CARD9, CASP8 (FLICE), HSP90AA1, MEFV, PSTPIP1,RIPK2, SUGT1, TNF, XIAP
Other Bacterial Pattern Recognition Receptors (PRRs):	APCS, CRP, DMBT1, ZBP1.
Downstream Signal Transduction:	NF?B Pathway: CHUK (IKKa), IKBKB, NFKB1, NFKBIA (IkBa/MAD3), RELA, TNFRSF1A. ERK Pathway: JUN, MAP2K1 (MEK1), MAPK1 (ERK2), MAPK3 (ERK1). p38/JNK Pathway: JUN, MAP2K3 (MEK3), MAP2K4 (JNKK1), MAPK14 (p38 MAPK), MAPK8 (JNK1).
Apoptosis:	AKT1, BIRC3 (c-IAP1), CARD6, CARD9, CASP1 (ICE), CASP8 (FLICE), CD14, FADD, IFNB1, IKBKB, IL12A, IL12B, IL1B, IL6, IRAK1, JUN, MAP3K7 (TAK1), MAPK1 (ERK2), MAPK8 (JNK1), MPO, NFKB1, NFKBIA (IkBa/MAD3), PIK3CA, PYCARD, RAC1, RIPK1, RIPK2, TNF, TNFRSF1A, TRAF6.
InflammatoryResponse:	AKT1, APCS, CCL3 (MIP-1A), CCL5 (RANTES), CD14, CRP, CXCL1, CXCL2, IL1B, IL6, IL8, LBP, LY96 (MD-2), LYZ, MEFV, MYD88, NFKB1, NLRC4, NLRP3, NOD1 (CARD4), RAC1, RELA, RIPK2, SLC11A1, TICAM2 (TRAM), TIRAP, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TNF, TNFRSF1A, TOLLIP.
Cytokines&Chemokines:	CCL3 (MIP-1A), CCL5 (RANTES), CXCL1, CXCL2, IFNA1, IFNB1, IL12A, IL12B, IL18, IL1B, IL6, IL8.
AntimicrobialPeptides:	BPI, CAMP, CTSG, LCN2 (NGAL), LTF, LYZ, MPO, PRTN3, SLPI

***Table 1:* List of genes in the real-time PCR Array.** *(SABioscience)*

3.5 Real-Time PCR

cDNA quantification of genes involved in this study was performed by real-time PCR (CFCX Connect Bio-Rad). Reactions were performed using a SYBR Green PCR mix (iTaq™ Universal SYBR Green Supermix, Bio-Rad). SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. Mixes were prepared according to Table 2:

Component	**Volume per 1 Reaction**
Primer Mix (Forward and Reverse) 10 µM	0.5 µl
iTaqUniversal™ SYBR Green® Supermix	5 µl
cDNA	2 µl
Nuclease-free water	2.5 µl
Final volume	10 µl

Table 2: ***Individual Real-Time PCR reaction setup***

Reactions were performed according with the following thermal profile: an initial activation step (95 °C, 2 minutes) followed by 40 cycles of 5 seconds at 95 °C (denaturation) and 30 seconds at 60 °C (annealing and extension). Melting Curve analysis was performed for each reaction at 65°-95°C (0.5 °C increments) 5 sec/step. By recording the amount of fluorescence emission at each cycle, the PCR reaction was monitored during exponential phase, where the first significant increase in PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The threshold line is the level of the detection or the point at which the reaction reaches a fluorescent intensity above background (the

mean of fluorescence values detected from to third to tenth cycle, when target amplification it is no appreciable yet). The parameter Ct (Threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the initial amount of cDNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct value. GAPDH and β-Actin were used as housekeeping genes and the mean Ct of these two genes was used as housekeeping gene. Results are expressed as fold change $2^{\Delta\Delta Ct}$ and were calculated according to Livak's method [299]. Primers used are shown in Table 3. For detection of NLRP3, Caspase-1 (CASP1), Caspase-3 (CASP3), Caspase-4 (CASP4), Caspase-5 (CASP5) and IFI16 primers were purchased already optimized by Bio-Rad (PrimePCR).

Gene	**Primer Forward**	**Primer Reverse**
IFNγ	GGCGACAGTTCAGCCATCAC	TGTGGAGACCATCAAGGAAGACA
TNFα	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG
IL-6	GGTGTTGCCTGCTGCCTTC	GCCAGTGCCTCTTTGCTGCT
CCL3	ATGGCTCTCTGCAACCAGTTC	AGCACAGACCTGCCGGCTTCG
GAPDH	CGGATTTGGTCGTATTGGG	GCTTCCCGTTCTCAGCCTTG
KI67	TGTGTTGGATTTGTGGAACTGA	CACCTGCTTGTTTGGAAGGG
β ACTINA	ATGCCCAGGAAGGAAGGCTG	GGGAAATCGTGCGTGACATT
IL-1β	TTCTGCTTGAGAGGTGCTGATG	TGTCCTGCGTGTTGAAAGATGA
IL-18	GCTGCTGAACCAGTAGAAGAC	CCGATTTCCTTGGTCAATGAAGA
IFNβ	CAGGCCGCATTGACCATCTA	GACATTAGCCAGGAGGTTCTCA

Table 3: ***Primers sequences used in Real-Time PCR experiments.*** *Primers were purchased from Sigma-Aldrich.*

4. ELISA ASSAYS

4.1 Plasma LPS quantification

LPS concentration was measured on plasma samples from each subject included in the study. LPS was measured by Limulus Amebocyte Lysate (LAL) Chromogenic Endpoint Assay (Hycult Biotechnology, Uden, The Netherlands). The assay is based on Frederick Bang's observation that the endotoxin of bacteria caused intravascular coagulation in the American horseshoe crab (*Limulus polyphemus*) [300]. Levin, Bang and coworkers then found that the coagulation was due to an enzyme in the circulating blood cells of the crab (amoebocytes) and that the reaction was initiated by the endotoxin itself [301]. In the chromogenic LAL assays, LPS activates LAL clotting enzyme, however the original enzyme's substrate - the clotting protein - is removed and replaced by a chromogenic substrate, such as p-nitroaniline, which will cause a yellow colour to develop upon cleavage. The chromophore contains an amino acid sequence which is similar to that present in the clotting protein cleaved by the enzyme. The absorbance will be indirectly a function of the amount of endotoxin present in the sample [302]. Measuring LPS in biological fluids, such as plasma, is challenging as many factors can inhibit endotoxins, nowadays chromogenic LAL assay is considered to be the best option available as it compromises increased sensitivity with relative convenience of operation [303]–[305].

Samples, standards and reagents were prepared according to manufacturer's instructions. Plasma samples were heated at 75°C for 5 minutes in order to neutralize endotoxin inhibiting compounds. Plasma samples were then diluted 1:5 in endotoxin free water. 50µl of each diluted sample and standard were plated in duplicate in a 96-well plate and

immediately 50µl of LAL reagent was added in each well. After 30-minutes incubation at room temperature, reaction was stopped with 50µl of stop solution. Absorbance was measured at 415nm with a spectrophotometer (iMark Bio-Rad). The blank optical density was subtracted from the average of duplicate readings for each standard and sample. 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) using iMark Bio-Rad Software. LPS concentration was calculated relatively to the standard curve, multiplied by the dilution factor, expressed in EU/ml.

4.2 Plasma sCD14 quantification

Soluble CD14 (sCD14) was measured by Quantikine ELISA Kit (R&D), following manufacturer's instructions. Samples were diluted 1:400 in Calibrator Diluent RD5P. 100µl of Assay Diluent RD1W (provided in the kit) were added to each 96-well pre-coated with anti-sCD14 antibody. 100µl of standards and sample were added per well in duplicate. Samples were then incubated for 3 hours at room temperature. Plate was washed4 times using an autowasher. 200µl of sCD14 Conjugate were added to each well and the plate was incubated for 1 hour at room temperature. After 4 washes, 200 µl of substrate solution were added to each well and incubated for 30 minutes at room temperature in dark. Finally, 50 µl of stop solution were added to each well and the plate was read at 450 nm with wavelength correction set to 540 nm. Standard Curve was created by generating a four parameter logistic curve-fit using iMark Bio-Rad software. The blank optical density was subtracted from the average of duplicate readings for each standard and sample. Concentrations from standard curve were then multiplied by dilution factor.

4.3 Plasma IL-18 Quantification

IL-18 was measured by ELISA, following manufacturer's instructions (MBL, Japan). Each sample was diluted 1:5 in Assay Diluent provided by the kit. 150µl of diluted samples and standards were transferred to a 96-plate pre-coated with anti-IL-18 antibody in duplicate. The plate was incubated for 1 hour at room temperature. The plate was then washed and 100 µl of conjugate solution (provided in the kit) were added to each well. The plate was incubated for 1 hour at room temperature. After 4 washed, 100 µl of Substrate Reagent were added to each well and the plate was incubated for 30 minutes at room temperature. The colorimetric reaction was stopped by pouring 100 µl of stop solution. Plate was read at 450 nm. The blank optical density was subtracted from the average of duplicate readings for each standard and sample. Standard Curve was created by generating a four parameter logistic curve-fit using iMark Bio-Rad software. Concentrations from standard curve were then multiplied by dilution factor.

4.4 Plasma IL-1β Quantification

IL-1β was measured by ELISA, following manufacturer's instructions (DuoSet R&D Systems). Briefly, A 96-well microplate was coated with 100 µl per of diluted capture antibody (provided in the kit) and incubated overnight at room temperature. The day after, plate was washed three times and subsequently each well was blocked by adding 300 µl of Reagent Diluent (provided in the kit) at room temperature for 1 hour. Plate was then washed twice. 100 µl of undiluted samples or of standards were added in duplicate to the plate and incubated for 2 hours at room temperature. Plate was washed three times and 100 µl of Detection

antibody were added to each well and incubated for 2 hours at room temperature. Plate was then washed three times and 100 µl of diluted Streptavidin-HRP Solution was added to each well and incubated for 20 minutes at room temperature in the dark. Plate was washed 3 times and 100 µl of Substrate Solution was added to each well and incubated for 20 minutes at room temperature in the dark. 50 µl of Stop Solution were added to each well and optical density was immediately determined at 450 nm with wavelength correction set at 595 nm. The blank optical density was subtracted from the average of duplicate readings for each standard and sample. Standard Curve was created by generating a four parameter logistic curve-fit using iMark Bio-Rad software. Concentrations were calculated from standard curve.

Plasma IL-1β was undetectable in some samples, therefore for samples that could not be detected by using a standard IL-1β ELISA, we used a IL-1β High Sensitivity ELISA kit following manufacturer's instructions (Quantikine HS ELISA R&D Systems). Briefly, 100 µl of Assay Diluent (provided in the kit) were added to each well of a 96-well pre-coated with anti-IL1β antibody and then 150 µl of standards or samples were added to each well and incubated for 3 hours at room temperature on an horizontal shaker. Plate was then washed six times and 200 µl of high sensitivity conjugate (provided in the kit) was added to each well and incubated for 2 hours at room temperature on an horizontal shaker. Plate was washed for six times and 50 µl of Substrate Solution was added to each well and incubated 1 hour at room temperature on an horizontal shaker. Then, 50 µl of Amplifier solution was added to each well and incubated for 30 minutes. 50 µl of stop solution were then added to each well and optical density was immediately assayed by reading the plate at 490 nm with wavelength correction set at 655 nm. The blank optical density was subtracted from the average of duplicate readings for each standard and sample. Standard Curve was created by generating a four parameter logistic curve-fit using

iMark Bio-Rad software. Concentrations were calculated from standard curve.

4.5 Plasma IL-6 Quantification

IL-6 was measured in plasma by Quantikine High Sensitivity ELISA Kit according to manufacturer's instructions(R&D). Briefly, 100 µl of Assay Diluent (provided in the kit) were poured into each well of a 96-well plate pre-coated with an antibody anti-IL-6. 100µl of standard or samples were added per well and the plate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker at 500 rpm. After 6 washes, 200 µl of IL-6 conjugate (provided in the kit) were added to each well and the plate was incubated for additional 2 hours, followed by 6 washes. 50µl of substrate solution was added to each well and the plate was incubated for 1 hour. Given that IL-6 levels in plasma are low, it is necessary to amplify the signal generated by the substrate solution. For this reason, 50 µl of amplifier solution (provided in the kit) were added to each well and the plate was incubated for additional 30 minutes. Finally, 50 µl of stop solution were added to each well. The plate was read at 490 nm with wavelength correction set to 655 nm. The blank optical density was subtracted from the average of duplicate readings for each standard and sample. Standard Curve was created by generating a four parameter logistic curve-fit using iMark Bio-Rad software. Concentrations were calculated from standard curve.

4.6 Plasma Caspase-1 Quantification

Caspase-1 is a IL-1β-converting enzyme (ICE), which is synthesized as an inactive precursor. Active caspase-1 is generated by dimerization and proteolysis and it produced an enzyme composed of two large (20 KDa) and two small (10 KDa) subunits and the active enzyme contains 2 active sites [306].In order to detect it in plasma we used a ELISA Kit (Quantikine, R&D), which allows the specific detection of the p20 subunit of caspase-1. Briefly, 50 µl of Assay Diluent (provided in the kit) were added to each well of a 96-plate previously coated with an antibody anti-caspase-1 p20 subunit. Samples were diluted 1:2 and then 100 µl of samples and standards were added into each well and incubated 1,5 hours at room temperature. Plate was then washed three times and 100 µl of caspase-1 antiserum was added to each well and incubated for 30 minutes at room temperature. Plate was then washed three times and 100 µl of caspase-1 conjugate was added to each well and incubated at room temperature. Plate was then washed and 200 µl of substrate solution was added to the plate and incubated for 20 minutes at room temperature. 50 µl of stop solution was added into each well and optical density was immediately read at 450 nm. Standard Curve was created by generating a four parameter logistic curve-fit using iMark Bio-Rad software. Concentrations were calculated from standard curve.

5. STATISTICAL ANALYSIS

Parametric (unpaired Student T test) and nonparametric (Mann Whiney) t-tests were performed to compare INRs and IRs. Samples distribution were assessed by KS normality test. Statistical analysis were performed using GraphPad Prism analysis software.

Results

1. STUDY POPULATION

In this study 39 HIV^+ ART-treated patients were enrolled at the Unit of Infectious Diseases, San Gerardo Hospital, Monza – Italy. Demographic and clinical characteristics are summarized in Table 4. All patients were virologically suppressed as plasma viral load was at undetectable levels (plasma HIV-RNA <50 cp/mL) for at least 12 months. Patients were divided into two groups according to their $CD4^+$ T cell counts: Immunological Responders (IR, $CD4^+$ T cell count >500 cells/μl) and Immunological Non Responders (INR, $CD4^+$ T cell count <350 cells/μl). INRs were older (median 60 *vs*. 43 years, p<0.001) and showed a higher rate of past AIDS-defining events, probably due to their low $CD4^+$ T cell counts (76.5% *vs*. 18.2%, p<0.001). Older age is associated with lower $CD4^+$ T cell counts and poorer immunological response to HAART [307].

Older individuals have a higher background of immune activation and this could contribute to inadequate immune recovery during HAART. Median CD4 count was 840 (IQR 718-1131) cells/μL in IR *vs*. 295 (IQR 256-343) cells/μL in INR.

	IR (n=22)	INR (n=17)	P
Age	43 (40-48)	60 (50-66)	<0.001
Male gender	16 (72.7)	14 (82.4)	0.704
Caucasian	19 (86.4)	14 (82.4)	1.000
Riskfactor:			0.769
Heterosexual	11 (50)	10 (58.8)	
Homosexual	5 (22.7)	4 (23.5)	
Unknown	6 (27.3)	3 (17.6)	
Years from HIV diagnosis	9.3 (6.2-20.2)	10.2 (3.8-15.6)	0.557
Past AIDS-definingevents	4 (18.2)	13 (76.5)	<0.001
CD4	840 (718-1131)	295 (256-343)	<0.001
CD4%	34 (28-40)	19 (16-21)	<0.001
CD8	1139 (781-1383)	685 (489-899)	0.001
CD8%	41 (36-47)	48 (37-53)	0.300
CD4/CD8	0.81 (0.60-1.03)	0.41 (0.29-0.58)	<0.001
CD4/CD8 >1	6 (27.3)	0	0.027
Months from last VL>50	53.7 (33.4-67.7)	59 (25.9-84.9)	0.617
Years from cART initiation	7.7 (4.8-11.2)	10.2 (3.3-13.4)	0.540
Months from last regimen initiation	48.5 (18.2-60.9)	16.8 (5.5-41.2)	0.209
NRTI	20 (90.9)	16 (94.1)	1.000
NNRTI	12 (54.5)	7 (41.2)	0.613
PI	6 (27.3)	6 (35.3)	0.851
InSTI	6 (27.3)	7 (41.2)	0.568
EI	0	0	nc

Table 4: ***Clinical and demographic characteristics of HIV-infected patients enrolled in the study****. Median and interquartile ranges are shown. VL: viral load. cART: combinational antiretroviral therapy.*

2. T-CELL IMMUNE ACTIVATION

The main hallmarks of T cell immune activation during HIV infection are the expression of HLA-DR II molecules on $CD4^+$ T cells and of CD38 antigen on $CD8^+$ T cells [58], [308]. HLA-DRII is constitutively expressed on APCs and, during immune responses, it is expressed only on a subset of activated T cells and it is a marker of HIV disease progression [48]. CD38 is constitutively expressed by naïve T cells and it is down-regulated on memory cells and up-regulated on activated cells. It has been reported by several studies that the persistent immune activation of T cells during HIV infection leads to depletion of the naïve $CD4^+$ T cell and $CD8^+$ T cell pools [27], [309]. Therefore, we decided to conduct a immune phenotypic analysis in order to evaluate the basal immune activation status in the two groups. In particular, we analyzed HLA-DRII expression on $CD4^+$ T cells and $CD38^+$ expression on $CD8^+$T cells in IRs and INRs. Results are shown in Figure 17 and are expressed as percentage of $CD4^+$/HLA-$DRII^+$ T cells and of $CD8^+$/$CD38^+$ T cells.

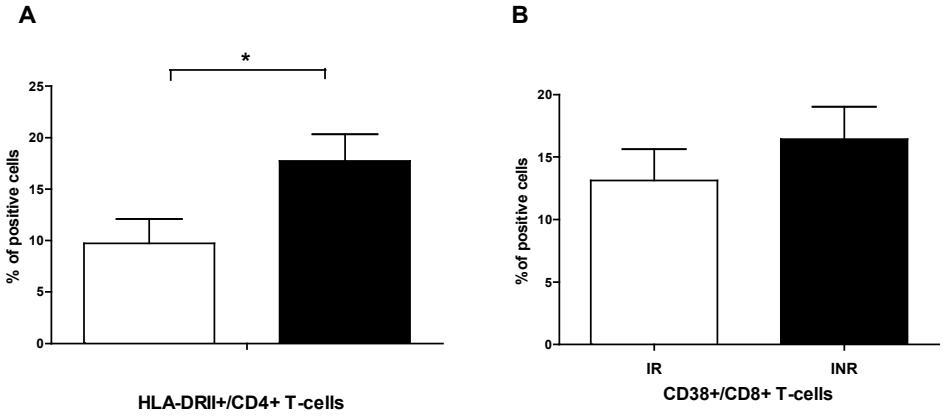


Figure 17: ***T-cell Immune Activation in IR and INR patients.*** *Panel A: percentage of HLA-DRII$^+$CD4$^+$T cells in INRs and IRs. Panel B: percentage of CD38$^+$CD8$^+$T cells in INRs and IRs. Mean values and SEM are shown. *= p< 0.05.*

We observed that INRs show a significant higher percentage of activated CD4$^+$ T cells and a trend of increased percentage of CD38$^+$/CD8$^+$ T-cells as well. These data indicate that INR individuals, even in presence of no detectable viremia, are more immune activated than IR individuals and this observation is in agreement with previous findings [129], [48], [142].

3. NLRP3 INFLAMMASOME ACTIVATION

It has been established that chronic immune activation in HIV infection has deleterious effects on the immune system by inducing maturation, differentiation and activation of many innate and adaptive immune cells [48]. In addition Bandera et al., recently suggested that innate immunity may play an important role in immune activation [74]. Indeed, recent reports indicate that inflammasome activation, especially NLRP3 inflammasome, may play a role in HIV infection and in $CD4^+$ T cell depletion [274], [290]. In addition a mutation in NLRP3 was associated with higher levels of susceptibility to HIV infection [271]. Despite these evidences, inflammasomes' role in HIV pathogenesis and during chronic infection has not yet been elucidated. For these reasons we decided to evaluate inflammasome activation in IR and INR patients.

3.1 NLRP3 expression in unstimulated PBMCs

NLRP3 expression was evaluated by real-time PCR in unstimulated PBMCs from IR and INR patients. Results (Figure 18) were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and they are shown as fold change expression.

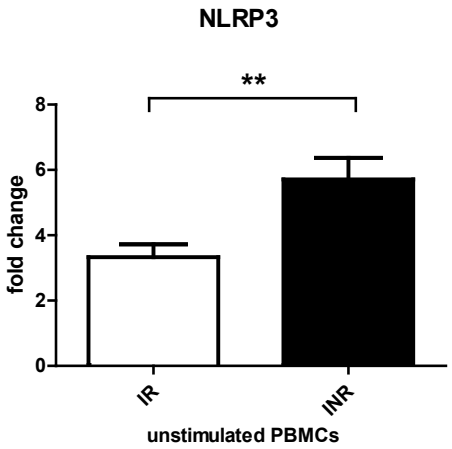


Figure 18: ***NLRP3 mRNA expression in unstimlatd PBMCs from IR and INR patients.*** *Mean values and SE are shown.**= p< 0.01*

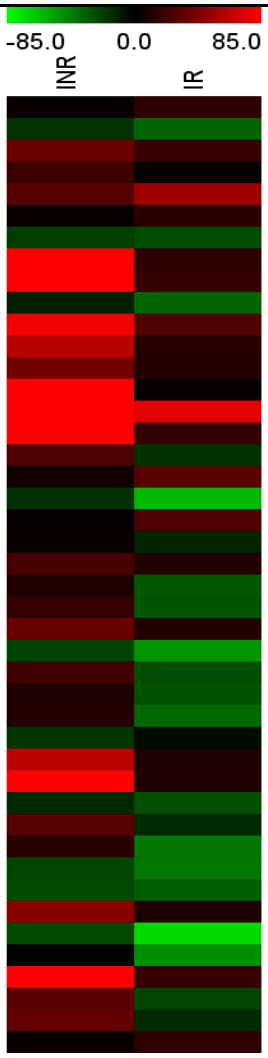
We observed a significant (p<0.01) increase in NLRP3 gene expression in INRs compared to IRs, suggesting a higher rate of inflammasome activation in these patients.

3.2. Inflammasome Pathway Gene Expression

Inflammasome pathway gene expression was further evaluated in PBMCs either unstimulated or stimulated with LPS or with AT2-treated HIV-1_{BaL}virions. LPS is known to induce inflammasome expression [220]. We also wanted to evaluated if HIV is able to induce inflammasome activation in the settings of chronic infection and for this reason AT2-treated HIV-1_{BaL}virions were used as well.

3.2.1 Inflammasome Pathway Gene Expression in LPS-stimulated PBMCs

To evaluate the inflammasome pathway gene expression in LPS-stimulated PBMCs, we performed a Real-Time PCR Array screening of 84 genes. Results are shown in Figure 19 and only targets showing a nfold>3 were considered significant. We could not find significant differences in the expression levels of unstimulated PBMCs, however INRs were more responsive to LPS stimulation than IRs: in particular, we observed an up-regulation of NLRP3, caspase-1 (CASP1), IL-1β and IL-18 in INRs compared to IR. These data suggest that inflammasome expression and possibly activation is higher in INRs. Interestingly, an up-regulation of many pro-inflammatory cytokines and chemokines (CCL3, CXCL2, IL12A, IL6) and of other genes associated with signal transduction and cell death (IFNB1, RIPK1, RIPK2, TRAF6, XIAP, ZBP1 and TNF) was detected in INRs compared to IRs.



Nfold>3	Gene	INR	IR
	CASP1	3.5	1.5
	CCL3	30.2	5.3
	CXCL1	9.06	1.54
	CXCL2	25.9	1.78
	IFNB1	8.1	2.7
	IL12A	6.1	1.3
	IL1B	3.8	1.18
	IL18	3.8	1.16
	IL6	8.04	7.5
	IL8	8	1.7
	NLRP3	3.5	1.19
	RIPK1	6.3	1
	RIPK2	8.6	1
	TRAF6	4.6	1
	XIAP	3.3	1.4
	ZBP1	4.2	1.6
	TNF	8.5	1.8

Figure 19: ***Real-Time PCR Array on LPS-stimulated PBMCs in INR and IR HIV-infected patients.*** *Gene expression (nfold) is shown as a color scale from green to red (-85 to +85) (MEV multiple experiment viewer software). Only targets showing >3-fold modulation are considered significant and are shown in table. Nfold LPS-stimulated PBMCs vs unstimulated PBMCs*

3.2.2 Gene Expression Profile in LPS-stimulated PBMCs

To confirm the results obtained in the array analysis and to further characterize the gene expression profile, we evaluated some targets by Real-Time PCR on each subject included in the study. In particular, we evaluated the expression of several pro-inflammatory cytokines and chemokines such as IL-1β, IL-18, TNF-α, IFN-γ, IFN-β and CCL3 (Figure 20).

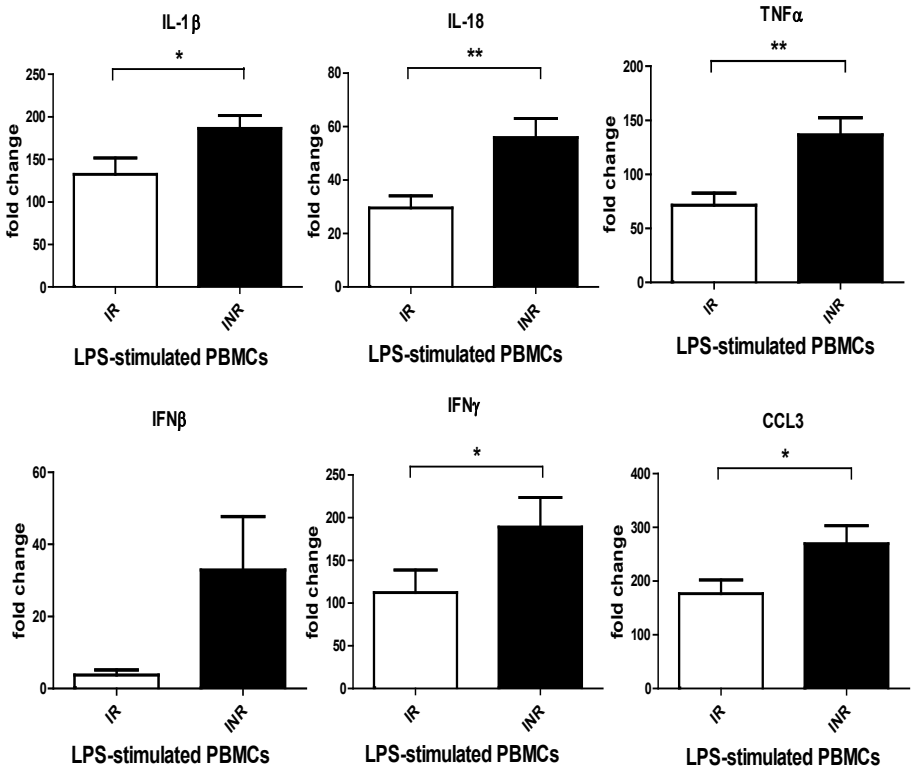


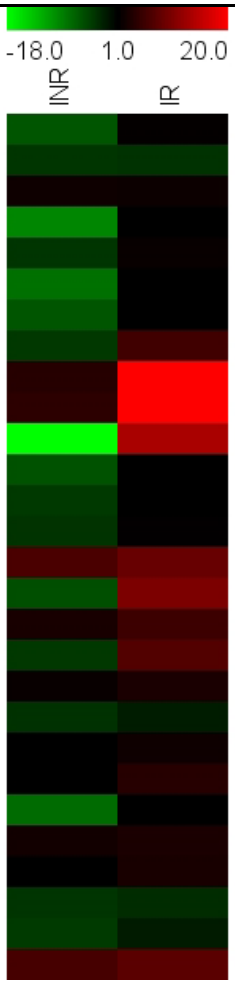
Figure 20: ***Gene expression profile of pro-inflammatory cytokines in LPS-stimulated PBMCs from IR and INR HIV-infected patients.*** *IL-1β, IL-18, TNFα, IFNβ, IFNγ and CCL3 expression was evaluated in IRs (white bar) and INRs (black bar). Mean values and SE are shown. *= p< 0.05; **= p< 0.01*

Results are shown as fold change expression and were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and relative to one calibrator untreated sample, according to Livak's method [299]. We observed a significant increase in inflammasome-related cytokines and other pro-inflammatory cytokines expression in INR patients, thus confirming the array results. In particular, we showed an increase in IL-1β ($p < 0.05$) and IL-18 ($p < 0.01$), suggesting a higher activation rate of inflammasome pathway in INR patients than in IRs, as well as higher activation rate of immune system and thus of immune activation in immunological non-responders.

3.2.3 Inflammasome Pathway Gene Expression in AT2-treated HIV-1_{BaL}-stimulated PBMCs

The same type of analysis was performed on AT2-treated HIV-1_{BaL}-stimulated PBMCs in order to evaluate inflammasome response to HIV in the two groups of patients.

As expected, HIV stimulation was less efficient in triggering inflammasome activation compared to LPS, probably due to the chronic infection settings, which may lead to exhaustion of immune system itself (Figure 21). However, we could still detect an up-regulation of NLRP3 and caspase-1 (CASP1) in both groups of patients, suggesting that, even in a scenario of chronic infection, HIV is able to stimulate NLRP3inflammasome activation. Interestingly, IFN-α and IFN-β were up-regulated in IRs, but not in INRs. Type I interferons are known to be important for immune responses against viruses, so the lack of Type I interferon up-regulation in response to HIV stimulation in INRs may indicate that, in these patients, HIV-specific responses against HIV is particularly skewed.



Nfold>2	Gene	INR	IR
	CASP1	2.26	2.09
	DMTB1	-3.18	5.95
	IFNA1	3.97	174.56
	IFNB1	4.47	185.53
	IL12B	-18.07	13.66
	IRF7	6.73	8.77
	MEFV	2.99	5.57
	MPO	-3.24	7.32
	MYD88	1.74	3.03
	NLRP3	1	2.13

Figure 21: ***Real-Time PCR Array on AT2-treated HIV-1_{BaL} virions-stimulated PBMCs in INR and IR HIV-infected patients.*** *Gene expression (nfold) is shown as a color scale from green to red (-18 to +20) (MEV multiple experiment viewer software). Only targets showing >2-fold modulation are considered significant and are shown in table. Nfold AT2-treated HIV-1_{BaL} virions-stimulated PBMCs vs. unstimulated PBMCs*

3.2.4 Gene Expression Profile in AT2-treated HIV-1BaL-stimulated PBMCs

To confirm the results obtained in the array analysis and to further characterize the gene expression profile, we evaluated some targets by Real-Time PCR on each subject included in the study. In particular, we evaluated the expression of several pro-inflammatory cytokines such as IL-1β, IL-18, TNF-α, IFN-γ and IL-6. NLRP3 expression was also evaluated in order to confirm if NLRP3 inflammasome could be induced directly by HIV. (Figure 22).

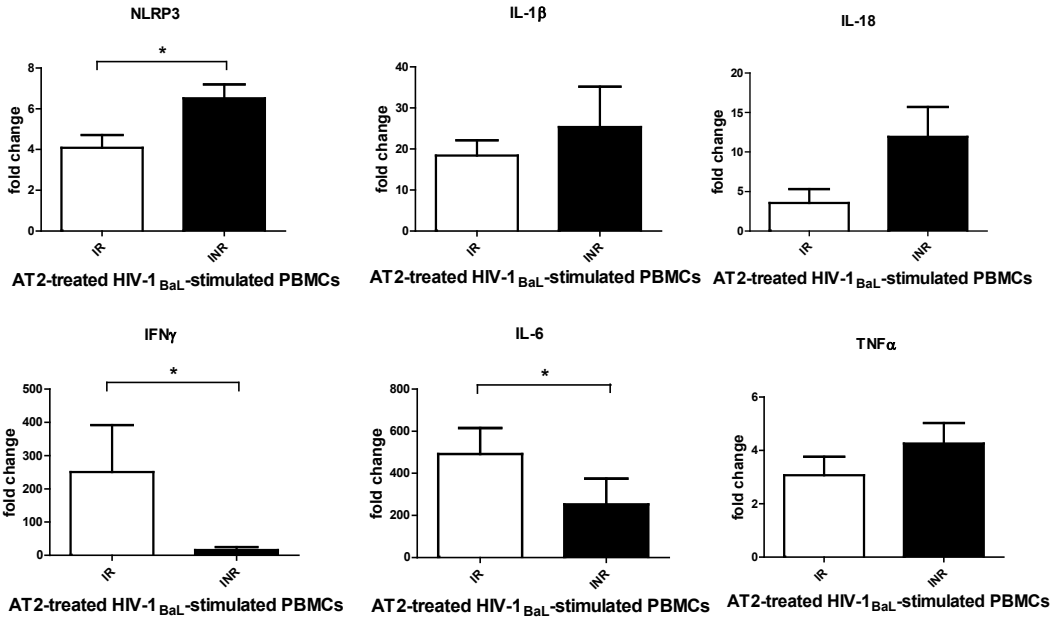


Figure 22: ***Gene expression profile of pro-inflammatory cytokines in AT2-treated-HIV-1$_{BaL}$-stimulated PBMCs from IR and INR HIV-infected patients.*** *NLRP3, IL-1β, IL-18, IFNγ, IL-6 and TNFα mRNA expression in IRs (white bar) and INRs (black bar). Mean values and SE are shown. *= p< 0.05; **= p< 0.01.*

Results are shown as fold change expression and were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and relative to one calibrator untreated sample, according to Livak's method [299]. We observed that NLRP3 and IL-18 were significantly more expressed in INRs compared to IRs ($p<0.05$ and $p<0.01$, respectively), suggesting that NLRP3 inflammasome pathway induction by HIV is increased in INRs. We could detect a higher expression of IL-1β in INRs too, even though it was not statistically significant. Interestingly, IL-6 was not increased in INRs, confirming the array data. IFN-γ expression was also lower in INRs compared to IRs. IFN-γ is important in the control of viral infections, as it prevents viral spreading by activating innate immune phagocytic cell activities. The fact that IFN-γ was lower in INRs may suggest, once again, that the immune response to HIV is particularly impaired in these patients.

We could not detect any differences in the gene expression levels of unstimulated PBCMs, except for NLRP3, as shown in paragraph 3.1.

4. CASPASES, IFI16 AND PRO-INFLAMMATORY CYTOKINES EXPRESSION PROFILE

Inflammasome activation leads to the activation of caspase-1, which is able to mediate activation of both IL-1β and IL-18 and pyroptosis. We decided to evaluate caspase-1 expression in samples from INRs and IRs. In particular, we evaluated caspase-1 expression in unstimulated, LPS- and AT2-treated-HIV-1_{BaL}-stimulated PBMCs. Results are shown (Figure 23) as fold change ($2^{\Delta\Delta Ct}$) expression and were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and relative to one calibrator untreated sample, according to Livak's method [299].

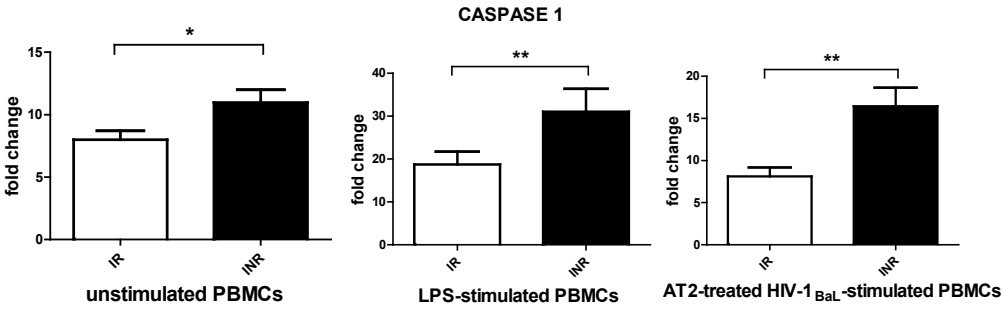


Figure 23: ***Caspase-1 expression in unstimulated, LPS- or AT2-treated HIV-1BaL stimulated PBMCs in IRs and INRs.*** *Caspase-1 expression in IRs (white bar) and INRs (black bar). Mean values and SE are shown. *= p< 0.05; **= p< 0.01.*

Casapse-1 expression was increased in INRs, in each condition analyzed. These data suggest that, indeed, inflammasome activation is higher in INRs compared to IRs. In addition, these data is in agreement with the array data previously reported. Caspase-1 up-regulation in INRs in each culture

condition is of particular interest because it strongly suggests that these patients could be exposed to cellular death by pyroptosis more than IRs.

Given the gene expression results, we decided to further investigate caspase-1 expression in INRs and IRs (Figure 24) and for this purpose plasma levels of caspase-1 were quantified in IRs and INRs. Although, statistical significance was not reached, INRs showed higher levels of caspase-1 (median IRs 375.3 pg/ml *vs*. median INRs 526.7 pg/ml), suggesting an increased susceptibility to cell death by pyroptosis.

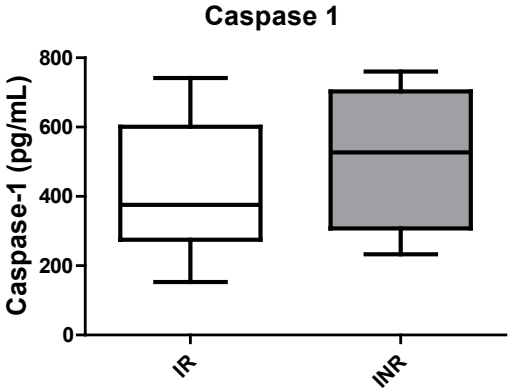


Figure 24: ***Plasma quantification of caspase-1 in IR and INR patients.****Caspase-1 expression in IRs (left) and INRs (right). Median values and max and min values of distributions are shown.*

To further characterize if inflammasome pathway is upregulated in INRs, we decided to evaluate IL-1β and IL-18 plasma levels in each patient included in the study. As shown in figure 25, we could not detect any differences in IL-18 expression, however we could see an increased IL-1β expression in INRs, even if it is not statistically significant. Of note, IL-1β detection in plasma is rather tricky as it is not released in high quantities. Neverthless, this observation leads us to speculate once more that inflammasome NLRP3 is indeed more activated in INRs compared to IRs.

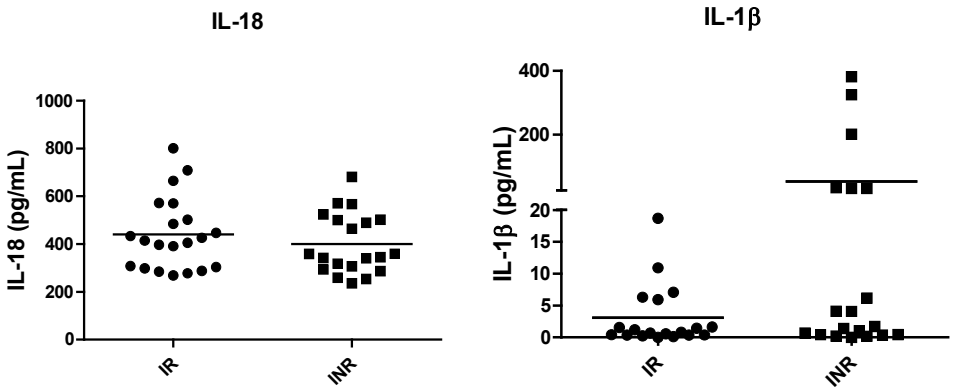


Figure 25: ***IL-18 and IL-1β plasma quantification in IR and INR patients.****IL-18 and IL-1β expression in IRs (dots) and INRs (squares). Horizontal lines indicate mean values.*

To further characterize the inflammatory profile of INRs, we also evaluated IL-6 expression in plasma. IL-6 is a pro-inflammatory cytokine and it has been considered a predictor of non-AIDS-morbidity in HIV-infected patients [312]. Results are shown in Figure 26. We could not detect any differences between INRs and IRs in agreement with recently reported data on IL-6 expression in INRs by Tincati C *et al*. [313].

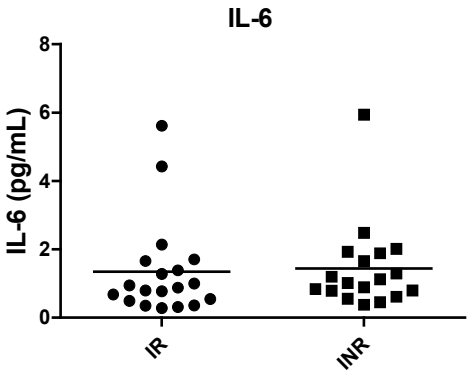


Figure 26: ***IL-6 plasma quantification in IRs and INRs.*** *IL-6 expression in IRs (dots) and INRs (squares). Horizontal lines indicate mean values.*

NLRP3 activation may be induced in a "non classical" pathway by caspase-11 in mice and by caspase-4 and caspase-5 in humans. Therefore we decided to evaluate caspase-4 and caspase-5 expression in unstimulated or stimulated with LPS or AT2-treated HIV-1_{BaL} PBMCs. Results were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and to a calibrator untreated sample and they are shown (Figure 27) as fold change expression, according to Livak's method [299].

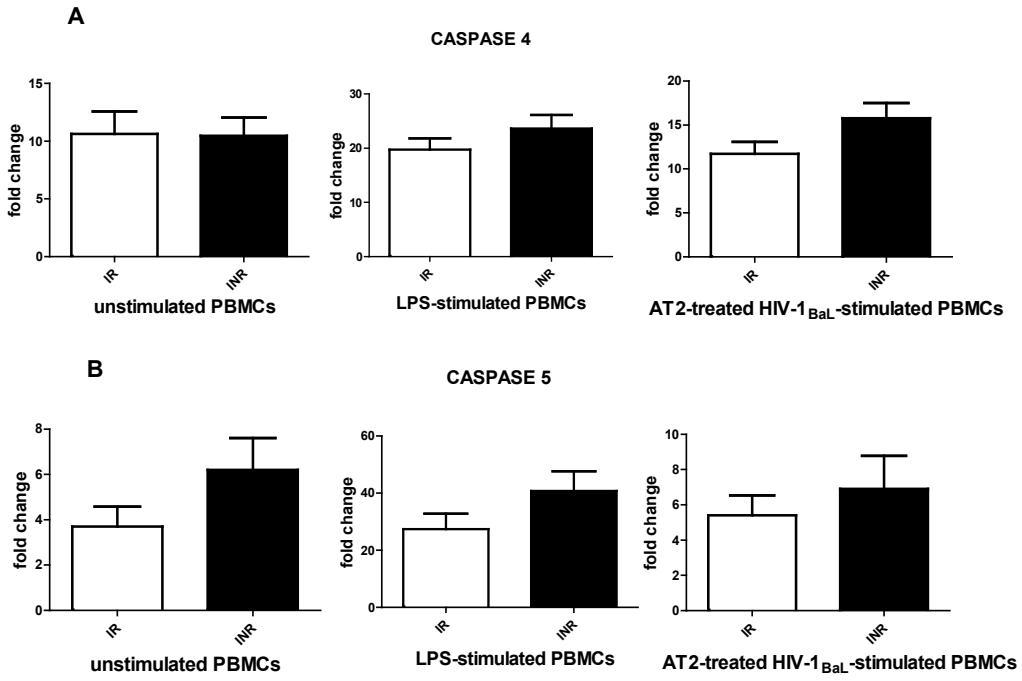


Figure 26: ***Caspase-4 and Caspase-5 gene expression in unstimulated, LPS- and AT2-treated-HIV-1_{BaL}-stimulated PBMCs.*** *Caspase 4 (Panel A) and 5 (Panel B) expression in IRs (white bars) and INRs (black bars). Mean values and SE are shown*

As shown in Figure 26, we could not detect any differences in caspase-4 and caspase-5 expression between IRs and INRs in none of the conditions analyzed. These data suggest that in these cohorts of patients NLRP3 inflammasome activation may not depend on the "non classical pathway".
Next, we decided to evaluate also caspase-3 expression in IRs and INRs. Caspase-3 is an apoptotic caspase known to induce cell death by apoptosis. We evaluated caspase-3 expression in unstimulated, LPS- and AT2-treated HIV-1_{BaL}-stimulated-PBMCs.

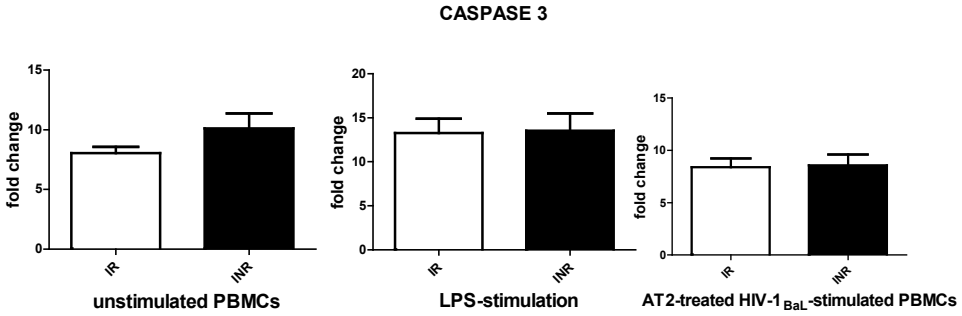


Figure 28: ***Caspase-3 gene expression in unstimulated, LPS- and AT2-treated-HIV-1$_{BaL}$-stimulated PBMCs.*** *Caspase-3 expression in IRs (white bar) and INRs (black bar). Mean values and SE are shown*

We could not detect any differences in caspase-3 gene expression between IRs and INRs, suggesting that apoptosis is probably not the main cell death pathway in INRs.

IFI16 is a PPR, which recently has been associated with HIV pathogenesis [314]. Therefore we decided to evaluate the expression levels of IFI16 in IRs and INRs. We evaluated IFI16 expression in unstimulated or LPS- or AT2-treated HIV-1$_{BaL}$-stimulated-PBMCs as shown in Figure 28. Results were calculated relative to the mean expression of housekeeping genes GAPDH and β-actin and to a calibrator untreated sample and they are shown (Figure 29) as fold change expression, according to Livak's method [299].

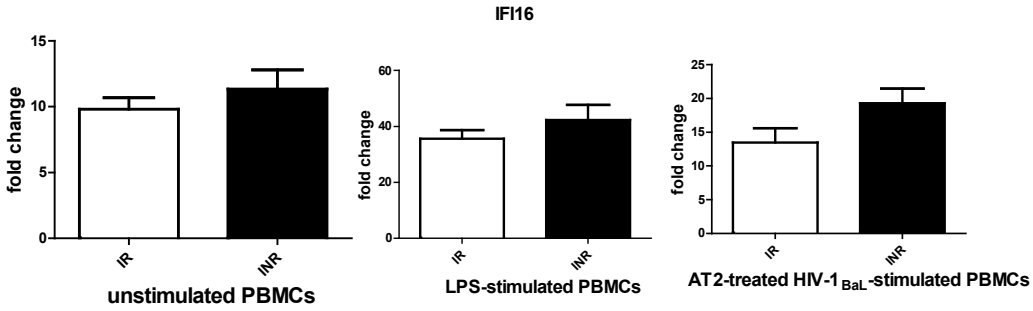


Figure 29: ***IFI16gene expression in unstimulated, LPS- and AT2-treated-HIV-1$_{BaL}$-stimulated PBMC.*** *IFI16 expression in IRs (white bars) and INRs (black bars). Mean values and SE are shown.*

We could not detect any differences in IFI16 gene expression between IRs and INRs. It should be mentioned that IFI16 expression has been evaluated on HLAC model system and it has been suggested that peripheral blood cells do not express high levels of this receptor [292], [314].

5. PERIPHERAL TH17 T-CELLS EVALUATION IN IRS AND INRS

Inflammasome activation can induce Th17 differentiation as it has been demonstrated that IL-1β is necessary for Th17 differentiation [261], [315]. In addition, little is known about Th17 role in INRs. Therefore, we decided to evaluate Th17 T cells subset in our patients. In order to identify Th17 T cells we used a combination of three markers: CD4, RORγT and IL17A. RORγT is a transcriptional factor that promotes differentiation of T cells into Th17. We evaluated the percentage of $IL17A^{+}/ROR\gamma T^{+}/\ CD4^{+}$ T cells in PBMCs either unstimulated or stimulated for 18 hours with LPS or AT2-treated HIV-1_{BaL}. Results are shown in Figure 30.

IL17A-producing, RORγT-expressing CD4$^+$ T cells

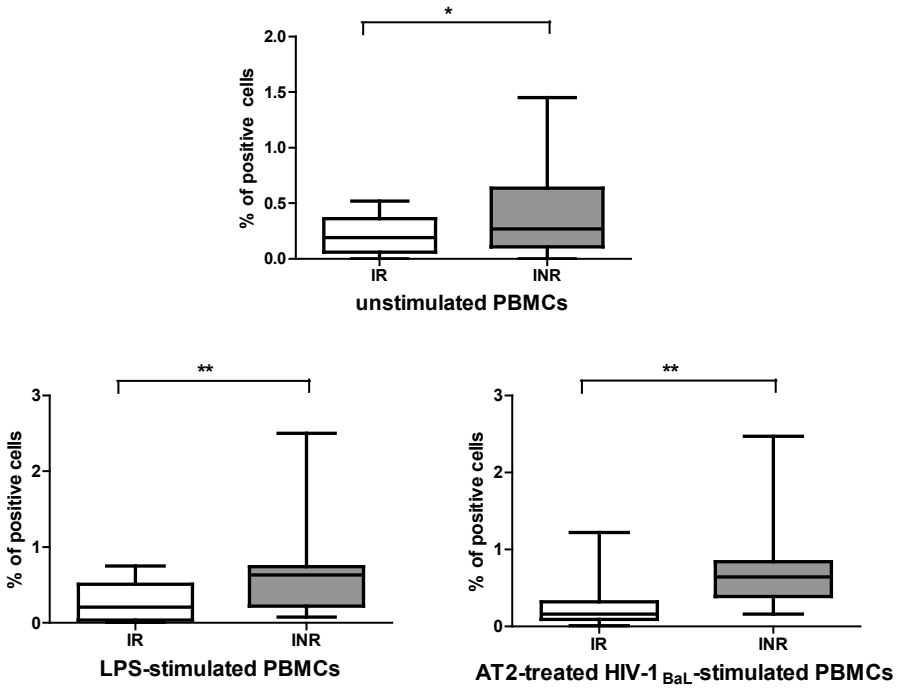


Figure 30: ***Th17 cells in IRs and INRs. Percentage of Th17 cells (CD4$^+$, IL17A$^+$ and RORγT$^+$ cells) in IRs (white plot) and INRs (grey plot) in unstimulated,*** *LPS- or AT2-treatedHIV-1$_{BaL}$-stimulated PBMCs. Median values and max and min values of distributions are shown. *= p < 0.05; ** = p< 0.01.*

INRs showed increased percentages of Th17 T cells in all analyzed conditions (basal: IRs *vs.* INRs p=0.04; LPS: IRs *vs.* INRs p=0.02; AT2: IRs *vs.* INRs p=0.02). This is somewhat surprising considering that Th17 depletion in the gut has been reported in HIV infection and has been associated with disease progression. On the other hand, our data were obtained from peripheral blood cells and evidences of the role of peripheral

Th17 T cells during HIV infection are still debated. Yet, Th17 T-cells are known for their pro-inflammatory action therefore these data may suggest the involvement of this T-cell subset in the mechanisms of persistent immune activation that characterize INR patients.

6. MICROBIAL TRANSLOCATION

Recently microbial translocation has been considered one of the main drivers of immune activation in HIV-infected patients [91]. Therefore, our hypothesis was that microbial translocation could be the main driver of inflammasome activation observed in our cohort. To verify this hypothesis we quantified sCD14 and LPS levels in plasma from IRs and INRs. sCD14 and LPS are considered the main hallmarks of microbial translocation and increased plasma levels of sCD14 and/or LPS have been associated with higher immune activation rates during HIV infection [78].

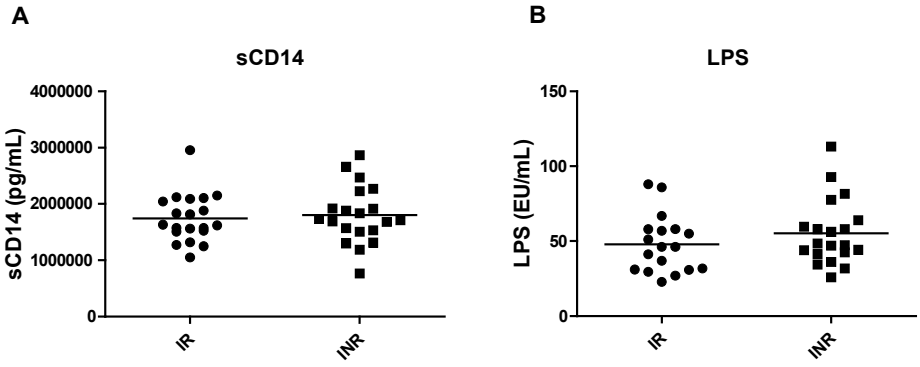


***Figure 31:* sCD14 and LPS quantification in plasma from INRs and IRs.** *sCD14 (Panel A) and LPS (Panel B) plasma expression in IRs (dots) and INRs (squares). Horizontal lines indicate mean values.*

As shown in Figure 31, surprisingly, we could not detect any significant differences between IRs and INRs, suggesting that – at least in our cohort – microbial translocation does not seem to be the main driver of immune and inflammasome activation and that probably other mechanisms come into play. Nevertheless, these data are in agreement with a very recent report by Tincati C. *et al* [313], in which the authors reported no significant differences in LPS and sCD14 plasma concentrations in INRs ($CD4^+$ T cells <350 cells/μl and or $\Delta CD4^+$ change from baseline <30%) and IR ($CD4^+$ T cells >350 cells/μl and/or $\Delta CD4^+$ T cells change from baseline >30%).

Discussion

In recent years, HIV infection has been regarded not only as immune deficiency syndrome but also as a chronic inflammatory disease and, nowadays, systemic chronic immune activation in HIV infection is considered one of the main drivers of $CD4^+$ T cell loss. HAART is extremely successful in inhibiting viral replication, however it has been extensively demonstrated that in HAART-treated HIV-infected patients, immune activation and inflammation levels still remain higher than in uninfected individuals, making HIV-infected individuals more susceptible to cardiovascular diseases and other morbidities [48]. In addition, approximately 20% of all HIV-infected individuals fail to restore their $CD4^+$ T cells counts despite fully suppressed viral replication: these patients are commonly referred to as "immunological non-responders". INRs display higher immune activation and inflammation rates and, for these reasons, they are even more susceptible to non-AIDS related morbidity [307]. The reasons behind the lack of immune reconstitutions have not been yet fully elucidated, even though many factors – such as residual HIV replication, impaired $CD4^+$ T-cell production and excessive loss – have been associated to this condition. Yet, none of these factors provide a full explanation for the lack of immune recovery observed in INRs. Recently, Wilson et al. reported in a large study including 670 HIV-infected participants, that plasma inflammatory biomarkers were strongly associated with monocyte activation and migration and not with $CD8^+$ T-cell activation phenotypes [316]. A further study by Bandera et al. suggested that innate immune activation contributes to the pathogenesis of inflammation and poor immune reconstitution [317]. In addition, many reports have indicated a possible role for innate immunity receptors, such as inflammasomes in the pathogenesis of HIV and in $CD4^+$ T cell loss [272], [273], [282], [291]. Given these premises, we decided to evaluate the possible associations

between inflammasomes and T cell activation and the degree of immune reconstitution in HIV-infected ART-treated patients.

To this purpose, we first investigated T cell immune activation by evaluating the percentage of HLA-DRII$^+$ and CD38$^+$ cells respectively on CD4$^+$ and CD8$^+$ T cells. We observed that INRs had a higher proportion of activated T cells, which is in agreement with other scientific reports (reviewed in [48]) and confirms the higher immune activation status of these patients.

Next, we decided to evaluate the basal expression of NLRP3 inflammasome in PBMCs. We observed a significant increase of NLRP3 gene expression in INRs. This result is of particular interest because, to this date, there are no reports of NLRP3 expression and/or activity in INRs. It has been reported that HIV is able to trigger NLRP3 expression upon infection, but clear analysis on inflammasome's role during chronic infection are still missing [272]–[274]. Interestingly, only very recently inflammasome activation – especially of IFI16 and in part of NLRP3 – has been implicated in HIV pathogenesis, suggesting that their activation, especially in the lymph nodes, leads to pro-inflammatory cytokine release, such as IL-1β and IL-18, and to pyroptosis of CD4$^+$ T cells, which is a form a programmed cell-death that, unlike apoptosis, is able to trigger inflammation. Therefore, the significant higher expression of NLRP3 in INRs could indicate a higher rate of inflammasome and immune activation.

To better characterize inflammasome activation in IRs and INRs, we stimulated PBMCs either with LPS or with AT2-treated HIV-1_{BaL}. LPS is known to be a potent activator of inflammasomes' pathway and we wanted to evaluate if HIV-1 could induce NLRP3 expression even in the settings of chronic infection. In LPS-stimulated PBMCs we were able to observe that INRs were much more responsive to LPS stimulation compared to IRs, suggesting a higher immune activation status, with an up-regulation of NLRP3 and caspase-1 gene expression. In addition, Real-Time PCR on each subject included in the study, showed a significant increased

expression of inflammasome-related cytokines, such as IL-1β and IL-18, which are directly linked to inflammasome activation. We were also able to observe an increase of other pro-inflammatory cytokines and chemokines such as IL-6and CCL3. Further, we performed the same type of analysis on PBMCs stimulated with AT2-treated-HIV-1_{BaL}. As expected, HIV was less efficient in stimulating the inflammasome pathway compared to LPS, which is in agreement with other reports. Pontillo A. *et al.*, reported that HIV was able to induce NLRP3 gene expression only in dendritic cells from healthy individuals but not from HIV-infected individuals, suggesting that HIV was able to induce NLRP3 expression upon infection, but that failed to do so in chronically activated cells from infected individuals [272]. Interestingly, when we evaluated NLRP3 gene expression by individual Real-Time PCR, we observed a significant up-regulation of NLRP3 in INRs compared to IRs, suggesting that in INRs HIV is able to significantly elicit NLRP3 expression. Further to that, we also evaluated by individual Real-Time PCR if IL-1β and IL-18 were increased in INRs after HIV stimulation. In agreement with the increased expression of NLRP3, IL-1β and IL18 were increased too in INRs suggesting that inflammasome pathway was more induced in these patients compared to IRs. Of interest, IFNγ expression, as well as IL-6 expression, upon HIV stimulation in INRs was significantly lower compared to IRs which may due to a deeper impairment of specific antiviral immune response in INRs compared to IRs. Next, we decided to evaluate caspase-1 expression either in unstimulated or LPS- or AT2-treated-HIV-1_{BaL} treated cells. In all conditions, a significant increase in caspase-1 expression in INRs was observed. Given that caspase-1 is induced by inflammasome activation [216], these data further confirm that in INRs inflammasome activation is, indeed, higher compared to IRs. In addition, caspase-1 mediates a form of high inflammatory programmed cell death called “pyroptosis” [266]. The higher expression levels of caspase-1 in INRs suggest that these patients may be more susceptible to pyropotos is

compared to IRs, which could – at least in part – explain the lack of immunological recovery during ART. Indeed, according to Doitsh *et al*., caspase-1 activation in HIV-infected individuals may be present even in absence of actual productive ongoing viral replication and thus it may lead to pyroptosis and cell death even if the settings of ART treatment [314]. According to this model, if HIV infects resting and non-permessive $CD4^+$ T-cells (which account for 95% of all $CD4^+$ T-cells), abortive infection occurs, leading to an accumulation of incomplete viral DNA transcripts that are detected by IFI16. IFI16 is, in turn, able to assemble into an inflammasome and thus to activate caspase-1, which is then able to trigger pyroptosis and inflammation. This high inflammatory status and the release of ATP by pryototic $CD4^+$ T cells would then be responsible for the activation of other inflamamsomes, such as NLRP3, and for new rounds of both pyroptosis and inflammation even in absence of ongoing productive HIV replication [314]. The authors also reported that this process would take primary place in the lymph node, rather than in the blood. Indeed, the authors noticed that even though quiescent blood $CD4^+$ T-cells supported HIV entry and fusion, they were highly resistant to pyroptotic cell death [292] and this is probably due to their deeper state of cellular rest. Deeper resting state would lead to fewer incomplete reverse transcripts following abortive infections and thus of lower expressions of PRRs, such as IFI16. Nevertheless, we were able to observe increased caspase-1, NLRP3, IL-1β, IL-18 and IFI16 expression in PBMCs from INRs and plasma concentration of caspase-1 and IL-1β were higher in INRs as well. This could be due to the fact that we analyzed PBMCs, which are a heterogeneous cell population compromising not only $CD4^+$ T cells but other lymphocytes and monocytes too. Monocytes are known for constitutive caspase-1 expression [318] and release of IL-1β upon TLR4 stimulation; therefore the higher expression levels of caspase-1, NLRP3 and IL-1β could depend on increased activation of monocytes in INRs compared to IRs. Of note, the array data of LPS-stimulated PBMCs,

showed a significant up-regulation of TLR4 in INRs compared to IRs. In addition, expression levels of caspase-4 and caspase-5, involved in the "non-canonical" activation of NLRP3 inflammasome in human monocytes, were higher, even though not statistically significant, in INRs compared to IRs [319]. These observations could be of high interest because they suggest that monocytes in INRs are much more activated than IRs and thus probably contributing to a higher inflammation and immune activation status observed in INRs. All together these data suggest that inflammasome pathway and possibly pyroptosis are upregulated in INRs and could likely contribute to the lack of immune recovery observed in these patients. Interestingly, very preliminary data from Doitsh *et al.*, suggest that in HIV-infected patients, caspase-1 can be detected in the lymph nodes at higher levels compared to uninfected individuals even under HAART [320], further supporting our evidences. Even more interestingly, a recent report investigating caspase-1 and caspase-3 plasma levels in a $CD4_{high}$ group ($CD4^+$ T cells > 450 cells/µl) and in a $CD4_{low}$ group ($CD4^+$ T cells < 250 cells/ul), showed that while in the $CD4_{high}$ group caspase-1 and caspase-3 levels increased rapidly and decreased within a short time during early HIV-1 infection, levels in the $CD4_{low}$ group were increased after 1 year of HIV-1 infection [296]. These data appear to be in agreement with our observations of higher levels of caspase-1 expression in INRs and strongly suggest an involvement of caspase-1 in the regulation of cell death and inflammation in these patients. We have also observed increased plasma levels of IL-1β in INRs even though it was not significant. Nonetheless, these evidences further support the idea that inflammasome activation is higher in INR patients.

We could not detect any differences in IL-6 plasma levels between INRs and IRs. This is somewhat surprising because IL-6 is one of the most important pro-inflammatory cytokines and it has been associated with AIDS progression and non-AIDS related morbidity [48]. It was therefore

reasonable to expect higher IL-6 levels in INRs. Despite this, a recent report from Tincati C. *et al*. on IL-6 plasma levels in IRs and INRs are in agreement with our data, indicating that probably this cytokine does not play such a central role in INRs' higher immune activation status [313]. In agreement with our data, there is also a very recent report by Stiksrud B. *et al.* The authors performed an extensive analysis on plasma inflammatory markers in INRs and IRs and could not find increased IL-6 levels in INRs [202]. It has been demonstrated that inflammasomes are also able to regulate cellular immune response by stimulating the differentiation of $CD4^+$ T cells into Th1 or Th17 T cells [251]. Given the lack of consistent evidences about the role of peripheral Th17 T cells in HIV pathogenesis and immune activation, we decided to evaluate this cellular subset in PBMCs from IRs and INRs. Interestingly, we observed significant increased levels of Th17 T cells in INRs, even in the settings of HIV-1 or LPS stimulation. Th17 T cells are known for their pro-inflammatory role, therefore the increased levels in INRs could underlie some inflammatory processes that could elicit immune activation. These data are interesting because it has been extensively demonstrated that intestinal Th17 T cells are particularly sensitive to HIV infection and are rapidly depleted upon primary infection, contributing to the disruption of the intestinal mucosal barrier and to microbial translocation. It is therefore of interest that we found Th17 T cells to be increased in INRs. Our data are somewhat in agreement with Girard A. et al. The authors reported increased frequencies of β7+ Th17 T cells in INRs compared to IRs and healthy controls [204]. β7+ is a gut homing marker and the authors suggested that mucosal Th17 T cells are recruited to the Gut-Associated Lymphoid Tissue (GALT) due to previous depletion; once in the GALT, these cells would become more sensitive to infection and this condition would lead to an altered Th17/Treg ratio and a disturbed intestinal homeostasis, which could lead to microbial translocation. Next, we therefore decided to focus on microbial

translocation. In recent years, microbial translocation has been considered one of the main drivers of immune activation and chronic inflammation in HIV-infected individuals [321]. For this reason, our hypothesis was that higher levels of microbial translocation in INRs could be responsible for the higher inflammasome activation that we observed in these individuals. To test this hypothesis we quantified plasma levels of LPS and sCD14, which are two of the main markers of microbial translocation. Surprisingly, we could not detect any differences between the two groups of patients, suggesting that microbial translocation is not the main driver of inflammasome activation in these patients. Interestingly, Tincati C. *et al* data is in agreement with ours: they reported comparable levels of LPS and sCD14 between HIV-infected individuals which recover or not $CD4^+$ T cell count [313]. Stiksrud *et al*., recently measured LPS and sCD14 in a cohort of INRs and IRs and they could not detect any differences between the two groups either, in agreement with our results [202]. Moreover, it has been suggested that probably while microbial translocation markers have been shown to predict clinical outcome in untreated HIV infection, their use in HAART-treated patients may not be as accurate [189]. Given these premises, it is therefore tempting to speculate that higher inflammasome activation rates observed in INRs may probably be due to residual HIV defective replication, which would, in turn, lead to inflammation, immune activation and cell death. Indeed, despite full suppressed viremia during HAART, there is an important ongoing debate on its real efficacy at completely inhibiting viral replication. As it has been reviewed so elegantly by J. Martinez-Picado and S. G. Deeks, while there is ample evidence that modern HAART is effective at inhibiting viral replication, there is also ample evidences of the contrary: even during HAART, HIV may still replicate at low levels and perhaps only in a subset of individuals [322]. Indeed, there have been evidences that cell-to-cell transmission of HIV is not inhibited by HAART and, even more interestingly, cell-to-cell transmission effectively

triggers pryoptotic cell death of lymphoid-tissue-derived $CD4^+$ T cells[293], [314], [323]. Therefore if antiretroviral drugs only sub-optimally inhibit cell-to-cell transmission, inflammation and depletion of $CD4^+$ T cells would still take place in spite of suppressed viremia. In addition, antiretroviral drug distribution is not homogenous with higher concentration reported in PBMCs, and lower concentration in certain tissues, such as lymph nodes and ileal and rectal mononuclear cells. These anatomical sites represent "immune sanctuaries", in which the virus could still be able to replicate. It should also be taken into consideration that antiretroviral drugs are also mostly ineffective at clearing cells harboring latent HIV. Nonetheless, further evidences are necessary to fully prove that in INRs, HIV replication – being it defective or productive – still happens and it should be of primary interest to evaluate it as it could explain many of the immunological features observed in these patients.

Conclusions

In conclusion, we observed that increased immune activation levels and percentage of Th17 T cells and higher levels of inflammasome and caspase-1 expression could be detected in INR patients. The up-regulation of these pro-inflammatory mechanisms may plausibly contribute to the persistent immune activation that characterize INRs. Notably, higher levels of caspase-1 expression are likely to induce $CD4^+$ T cell loss via pyroptosis, contributing to the unsatisfactory $CD4^+$ T cell recovery observed in INRs. We could not detect any differences in microbial translocation markers between INRs and IRs and therefore microbial translocation is unlikely to be one of the main drivers of inflammasome activation. It is reasonable to speculate that other mechanisms are involved in triggering inflammasome upregulation in these patients. It is tempting to speculate that residual HIV replication may be at fault, however more experimental evidences are needed to prove this hypothesis. In the future, it would be of high interest to further characterize the inflammasome pathway upon HIV stimulation in macrophages and in lymphoid $CD4^+$ T cells from IR and INR HIV^-infected-ART treated patients and to evaluate the eventual residual HIV replication in these patients.

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Scientific Products

List of scientific products produced during the PhD Course (January 2014-December 2016)

PUBLICATIONS

The manuscript describing the results presented in this thesis is in preparation.

- Piconi S., Pocaterra D., Rainone V., Cossu M., **Masetti M.**, Rizzardini G., Clerici M., Trabattoni D. "*Maraviroc Reduces Arterial Stiffness in PI-Treated HIV-infected Patients*." Sci Rep. 2016 Jun 29;6:28853. doi: 10.1038/srep28853. PMID:27352838

- Trabattoni D., Gnudi F., Ibba S.V., Saulle I., Agostini S., **Masetti M.**, Biasin M., Rossignol J.F., Clerici M. "*Thiazolides Elicit Anti-Viral Innate Immunity and Reduce HIV Replication.*" Sci Rep. 2016 Jun 2;6:27148. doi: 10.1038/srep27148. PMID:27250526

- D'Adamo P., **Masetti M.**, Bianchi V., Morè L., Mignogna M.L., Giannandrea M., Gatti S. "*RAB GTPases and RAB-interacting proteins and their role in the control of cognitive functions*." NeurosciBiobehav Rev. 2014 Oct;46 Pt 2:302-14. doi: 10.1016/j.neubiorev.2013.12.009. Epub 2014 Jan 9. PMID: 24412241

ABSTRACTS

Abstracts about the results presented in the thesis:

- **M. Masetti**, M. Fabbiani, M. Biasin, A. Muscatello, N. Squillace, E. Colella, M. Clerici, A. Gori, D. Trabattoni, A. Bandera. "*Inflammasome and pyroptosis are involved in the lack of immune response during cART*". Presenting Author. To be presented as poster presentation at Conference on Retroviruses and Opportunistic Infections (CROI) 2017, Seattle, Washington, USA, 13-17 February 2017.
  Oral presentation at CROI-ICAR Awards 2017 (CROI Affiliated Event For Italian Young Investigators), Seattle, Massachussets, USA, 24 February 2016. This work has been awarded a "CROI-ICAR Award" for Young Italian Investigators.

- **M. Masetti**, M. Fabbiani, D. Trabattoni, A. Muscatello, M. Biasin, N. Squillace, I. Saulle, M. Clerici, A. Gori, A. Bandera "*Inflammasome and Th17 Activation in HIV+ ImmunologicalNonresponders*"
  Presenting Author. Poster Presentation at Conference on Retroviruses and Opportunistic Infections (CROI) 2016, Boston, Massachusetts, USA, 22-25 February 2016.
  Oral presentation at CROI-ICAR Awards 2016 (CROI Affiliated Event For Italian Young Investigators), Boston, Massachussets, USA, 24 February 2016. This work has been awarded a "CROI-ICAR Award" for Young Italian Investigators.

Other abstracts:

- V. Giacomet, D. Trabattoni, P. Nannini, **M. Masetti**, F. Forlanini, M. Clerici. G.V. Zuccotti "*Immunological Response After a Booster Dose of HBV Vaccine in HIV-Infected Youth*" To be presented as poster presentation at Conference on Retroviruses and Opportunistic Infections (CROI) 2017, Seattle, Washington, USA, 13-17 February 2017.

- I. Saulle, M. Biasin, **M. Masetti**, M. Sironi, S. Lo Caputo, F. Vichi, W. Aguilar-Jimènez, D. Trabattoni, C. Brander, M. Clerici "*6-Amino-Acid Insertion/Deletion Polymorphism in TIM1 Confers Protections Against HIV1*" Conference on Retroviruses and Opportunistic Infections (CROI) 2016 tenutosi a Boston, Massachusetts, USA, 22-25 February 2016. CROI-ICAR Awards 2016 Winner

- A. Berzi, S. Ordanini, **M. Masetti,** M. Biasin, D. Trabattoni, A. Bernardi, M. Clerici. "*A pseudo-glycodendrimer inhibits DC-sign-mediated HIV trans-infection and interferes with DC-sign signal"*. Presenting Author. Poster Presentation. 8° IAS Conference on HIV Pathogenesis, Treatment and Prevention, Vancouver, British Columbia, Canada, 19-22 July 2015.

- Saulle, S. Yahyaei, M. Biasin, M. Garziano, **M. Masetti**, A. Berzi, D. Trabattoni, S. Lo Caputo, F. Mazzotta, M. Clerici. "*Plasma and PBMC miRNA Profile in sexually HIV-1 exposed seronegative individuals*" Presenting Author. Poster Presentation. 8° IAS Conference on HIV Pathogenesis, Treatment and Prevention, Vancouver, British Columbia, Canada, 19-22 July 2015.

- M. Garziano, I. Saulle, F. Gnudi, **M. Masetti**, A. Berzi, F. Mazzotta, S.L. Caputo, D. Trabattoni, M. Biasin, M. Clerici."*Immune activation is present in HIV-1 Exposed Seronegative Individuals (HESN) and is independent from microbial translocation*" Poster Presentation. 8° IAS Conference on HIV Pathogenesis, Treatment and Prevention, Vancouver, British Columbia, Canada, 19-22 July 2015.

- **M. Masetti**, D. Trabattoni, F. Gnudi, S.V. Ibba, I. Saulle, M. Garziano, A. Berzi, M. Biasin, J. Rossignol, M. Clerici."*Thiazolides elicit anti-viral innate immunity and drastically reduce HIV replication in vitro*" Presenting Author. Oral Presentation 7° Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015.

- A. Berzi, S. Ordanini, **M. Masetti**, I. Saulle, M. Garziano, M. Biasin, D. Trabattoni, A. Bernardi, M. Clerici "*A pseudo-glycodendrimer Inhibits DC-SIGN-Mediated HIV Trans-Infection and Interferes with DC-SIGN Signal*" Oral Poster Discussion 7° Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015.

- I. Saulle, S. Yahyaei, M. Biasin, M. De Luca, F. Gnudi, A. Berzi, M. Garziano, **M. Masetti**, D. Trabattoni, S. Lo Caputo, F. Mazzotta, M. Clerici. "*Plasma and PBMC miRNA profile in sexually HIV- exposed seronegative individuals*" Oral Poster Discussion 7° Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015. Sivit Award.

- M. Garziano, I. Saulle, F. Gnudi, **M. Masetti**, A. Berzi, V. Rainone, S. Lo Caputo, F. Mazzotta, D. Trabattoni, M. Biasin, M. Clerici. "*Immune activation is present in HIV-1 Exposed Seronegative Individuals (HESN) and is independent from microbial translocation*" Oral Presentation 7°

Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015.

AWARDS

- **CROI-ICAR Awards 2017 for Young Italian Investigators**. Seattle, Washington,, U.S.A., February 15, 2017 and Siena, Italy, June 12, 2017.

- **Young Investigator Scholarship** to attend the 2017 Conference on Retroviruses and Opportunistic Infections (CROI 2017), Seattle, Washington, U.S.A., 13-17 February 2017. Awarded by the International Antiviral Society (IAS)-USA.

- **CROI-ICAR Awards 2016 for Young Italian Investigators**. Boston, Massachusetts, U.S.A., February 24, 2016 and Milan, Italy, June 6, 2016.

- **Young Investigator Scholarship** to attend the 2016 Conference on Retroviruses and Opportunistic Infections (CROI 2016), Boston, Massachusetts, U.S.A., 22-25 February 2016. Awarded by the International Antiviral Society (IAS)-USA.

- **Towards an HIV Cure Symposium Scholarship** to attend the 2015 Towards and HIV Cure Symposium, Vancouver (Canada), 18 & 19 July 2015 and the 8th IAS Conference on HIV Pathogenesis, Treatment & Prevention (IAS 2015), Vancouver, 19-22 July 2015. Awarded by the International AIDS Society (IAS).

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“The mere formulation of a problem is far more essential than its solution, which may be merely a matter of mathematical or experimental skills. To raise new questions, new possibilities, to regard old problems from a new angle requires creative imagination and marks real advances in science”

- *Albert Einstein*