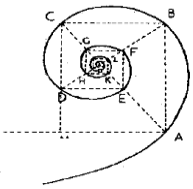

UNIVERSITÀ DEGLI STUDI DI MILANO

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TESI DI DOTTORATO DI RICERCA

BIO/13 Biologia Applicata

DISSECTING ERAP2 ROLE IN HIV-1 INFECTION

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ABSTRACT

Background: Immune response during HIV-1 infection and replication is quite complex because many viral and host factors, beyond individual variables, are involved at different levels. Many efforts have been made to evaluate the influence of these factors on HIV-1 infection and disease progression mainly focusing on the identification of genetic variants that can play a role in HIV-1 infection susceptibility. These studies have led to the concept of "immunological advantage". Moreover, the immunological advantage characterizing natural resistance to HIV-1 in HESN (HIV-exposed seronegative) has been associated with a number of polymorphisms (SNPs) in immune genes (e.g. ERAP2, APOBEC3H, MX2 and TLR3). However, the role of these SNPs in response to combination antiretroviral therapy (cART) has never been investigated. Among “protective” genes mentioned above, Endoplasmic Reticulum Aminopeptidase 2 (ERAP2) is involved in antigen presentation, trimming the amino-terminus of peptide precursors to the correct size for binding to HLA class I molecules. Haplotype-specific alternative splicing of the ERAP2 gene results in either a spliced (Hap B) or full-length (Hap A) mRNA. Interestingly, the frequency of the latter is higher in HESNs individuals and Hap A results correlated to *in vitro* HIV-1 infection natural resistance. Finally, the possibility of an alternative ERAP2 cellular localization has been recently investigated.
The project aims to: 1) deepen the correlation between ERAP2 haplotypes and *in vitro* HIV-1 susceptibility; 2) investigate the role played by ERAP2 genetic variants and progression of HIV-1 infection in cART treated patients; and 3) examine the molecular mechanism by which Hap A and Hap B control peptide production for antigen presentation and the resulting CD8 mediated immune response.

Methodology: To investigate the role of “protective” SNPs in response to cART, 300 HIV-1-infected patients from the Italian ICONA cohort undergoing a first HAART regimen were enrolled and genotyped for the “resistance” variants in ERAP2, TLR3, APOBEC3H and MX2 genes. Periodically (basal, 6 months and 1 year post cART initiation) HIV-infected patients’ clinical parameters (gender, CD4/CD8 cell count, viral load, therapies, year of infection, clinical evolution) were collected and analysed for possible correlation with the above mentioned genetic variants.
In parallel, to investigate the role of ERAP2 in *in vitro* HIV-1 susceptibility, PBMCs isolated from 30 healthy control (HC), grouped according to their ERAP2 genotype, were infected with HIV-$1_{Ba\text{-}L}$ in presence/absence of recombinant (rh) ERAP2 (100ng/mL) or with/without DG013A (100 nM/mL), an ERAP2 inhibitor, and analysed for: 1) susceptibility to HIV-1 infection (p24 ELISA assay); 2) expression mRNA levels of genes involved in the antigen presentation pathway; 3) MHC class I expression in cells and cytotoxic response by perforin/granzyme expression on CD8+ T cells. In addition, the trimming ability and modulation of CD8+ T cell response of Hap A and Hap B ERAP2 were assessed following transfection of DNA model constructs into ERAP2 deficient cells expressing a peptide precursor and evaluating by CPRG assay and B3Z response.

Findings: Results obtained from HIV-1-infected patients undergoing cART therapy did not show any significant correlations between the genotypes of TLR3, APOBEC3G and MX2 (single or combined) and the progression of HIV-1 infection.

The only exception was represented by ERAP2 gene, where HIV-infected Caucasian patients with a GG homozygosis condition for rs2549782 SNP (Hap A) showed a significant reduction ($p=0.042$) in viral load and a recovery in CD4+ T cells compared to heterozygous or homozygous TT patients (Hap B). Data obtained in Hap A HC showed a less susceptibility to *in vitro* HIV-1 infection compared to Hap B. Inhibition of ERAP2 activity by DG013A peptide increased HIV-1 infection in all PBMCs independently from their haplotypes. The role of ERAP2 in antigen processing in HIV-1 susceptibility has been confirmed by increased mRNA expression of other aminopeptidases and transporter, such as ERAP1 and TAP1 ($P<0.05$; $P<0.01$;$P<0.005$), in a condition where ERAP2 was inhibited. In line with these findings, transfection experiments showed that models for Hap A ERAP2 trimmed the peptide precursor efficiently, whereas Hap B ERAP2 constructs showed a reduction in trimming activity which resulted in a decreased CD8+ T cell response ($P<0.05$, $P<0.005$, $P<0.001$).
Addition of rhERAP2 to *in vitro* HIV-infected cells coupled with a reduction of viral replication in both ERAP2 haplotypes ($p<0.01$), without affecting cell viability. This protective effect was independent from an increase of HLA-ABC expression and/or of perforin and granzymes expression by CD8+ T lymphocytes.

Conclusions: Haplotype A of ERAP2 is associated with a reduced *in vitro* HIV-1 susceptibility as well as slower disease progression in cART treated HIV-1 infected patients. The effect may be explained by a greater control of antigen presentation pathway in Hap A subjects, in terms of trimming activity and consequently modulation of CD8+ T cell immune response. The role of ERAP2 in the extracellular milieu is still undisclosed and needs further investigation. However, data herein suggest a defensive feature mediated through an unconventional mechanism, distinct from immune system modulation.

SOMMARIO

Introduzione: La risposta immunitaria contro HIV è composita e complessa: entrano in gioco molti fattori appartenenti sia al virus che all'ospite e le variabili interindividuali non sono trascurabili e agiscono su vari livelli. Molti studi sono stati condotti per valutare l'influenza che questi diversi fattori possono esercitare sull'infezione da HIV-1 e sulla progressione della malattia e particolare interesse è stato rivolto all'identificazione di fattori genetici in grado di modulare la suscettibilità all'infezione da HIV. Questo orientamento ha portato alla elaborazione del concetto di "vantaggio immunologico" che sembra contraddistinguere la naturale resistenza ad HIV riscontrata in individui HIV-esposti sieronegativi (HESN). In particolare, in questi soggetti tale resistenza è stata correlata a diversi polimorfismi genetici (SNPs) in geni che esercitano un ruolo nell'organizzazione e funzionamento del sistema immunitario (es. ERAP2, APOBEC3H, MX2 e TLR3). Tuttavia, l'eventuale coinvolgimento di questi SNP in pazienti sieropositivi sottoposti ad una terapia antiretrovirale combinata (cART) non è mai stato investigato. Tra i geni "protettivi" menzionati sopra, Endoplasmic Reticulum Aminopeptidase 2 (ERAP2) è un enzima coinvolto nel processo di presentazione antigenica, infatti, taglia le estremità amino terminali di precursori peptidici adattandoli alla corretta lunghezza per il legame con la tasca delle molecole HLA di classe I. Splicing alternativi aplotipo-specifici sul gene di ERAP2 originano sia un trascritto alternativo (Hap B) sia un trascritto definito full-lenght (Hap A). E' Interessante osservare che la frequenza di quest'ultimo risulta maggiore in individui HESN e, inoltre, la presenza dell'aplotipo A è stata correlata alla naturale resistenza all' infezione da HIV *in vitro.* Infine, recentemente, è stata discussa la possibilità dell'esistenza di una localizzazione di ERAP2 alternativa da quella consueta a livello del reticolo endoplasmatico.
Alla luce di queste osservazioni il progetto di ricerca si propone di: 1) approfondire la correlazione tra aplotipi di ERAP2 e la suscettibilità *in vitro* al virus dell'HIV-1*;* 2) investigare il ruolo di varianti genetiche associate ad ERAP2 in rapporto alla progressione dell'infezione virale in pazienti sieropositivi riceventi terapia cART; ed 3) esaminare il meccanismo molecolare attraverso il quale Hap A e Hap B mediano la produzione di peptidi destinati alla presentazione antigenica e, di conseguenza, la risultante risposta immunitaria mediata da linfociti T CD8+.

Metodi: Per investigare il ruolo svolto da SNP definiti "protettivi" in risposta alla cART sono stati arruolati 300 pazienti provenienti dalla corte italiana ICONA, riceventi per la prima volta la terapia HAART, sui quali sono state condotte analisi per genotipizzare le varianti resistenti sui geni codificanti ERAP2, TLR3, APOBEC3H e MX2. Periodicamente (basale, 6 mesi ed 1 anno dall'inizio della terapia cART) sono stati raccolti i parametri clinici dei pazienti (genere, conta cellulare CD4/CD8, carica virale (VL), terapia, anno di infezione, evoluzione clinica) e, successivamente, analizzati per identificare una possibile correlazione con le varianti genetiche citate in precedenza.
In parallelo, per approfondire il ruolo rivestito da ERAP2 nella suscettibilità *in vitro* a HIV-1, PBMCs, isolate da 30 donatori sani (HC) suddivisi in funzione del loro

genotipo per ERAP2, sono state infettate in vitro con HIV-1_{Ba-L} in presenza/assenza di ERAP2 ricombinante (rh) (100ng/mL) o in presenza/assenza di DG013A (100 nM/mL), un inibitore di ERAP2. In seguito le culture cellulari sono state analizzate per valutare: 1) la suscettibilità all' infezione da HIV-1 (saggio ELISA); 2) l'espressione dei livelli dei trascritti (mRNA) di geni coinvolti nei meccanismi di presentazione antigenica; 3) l'espressione di molecole MHC di classe I e la valutazione della risposta citotossica attraverso misurazione dei livelli di perforina/granzimi su cellule CD8+. Inoltre, l'abilità di taglio e la modulazione della risposta cellulare CD8-mediata di Hap A e Hap B è stata valutata attraverso saggi di trasfezione con costrutti modello su cellule ERAP2 deficienti, esprimenti precursori peptidici e valutati attraverso saggio CPRG e risposta B3Z.

Risultati: I risultati ottenuti da pazienti HIV-1 infetti sottoposti a terapia cART non hanno mostrato nessuna correlazione significativa tra i genotipi di TLR3, APOBEC3G and MX2 (singoli o combinati) e la progressione dell'infezione da HIV-1. L'unica eccezione è rappresentata dal gene ERAP2, dove pazienti HIV-1 infetti di origine caucasica con condizione di omozigosi GG per rs2549782 SNP (Hap A) hanno mostrato una significativa riduzione ($p=0.042$) in carica virale e ripristino dei valori cellulari di linfociti CD4+, comparati ai pazienti eterozigoti o TT omozigoti (Hap B). I dati ottenuti da Hap A HC hanno mostrato una minor suscettibilità ad un'infezione *in vitr*o rispetto a Hap B. L'inibizione dell'attività di ERAP2, ad opera del peptide DG013A, ha portato ad un aumento dell'infezione da HIV-1 in tutte le PBMCs, indipendentemente dal relativo aplotipo. Il ruolo mediato da ERAP2 nel processamento antigenico correlato alla suscettibilità verso HIV-1 è stato confermato da un aumento dell'espressione di trascritti (mRNA) di altre aminopeptidasi e trasportatori specifici, come ad esempio ERAP1 e TAP1 ($P<0.05$; $P<0.01$;$P<0.005$), in condizioni nelle quali ERAP2 era inibito. In linea con questi dati, esperimenti di trasfezione hanno mostrato che i modelli per ERAP2 Hap A presentano un'attività di processamento peptidico più efficiente, mentre costrutti modello per ERAP2 Hap B hanno mostrato una ridotta attività di taglio risultante in una diminuzione della risposta cellulare CD8-mediata ($P<0.05$, $P<0.005$, $P<0.001$). L'aggiunta *in vitro* di rhERAP2 in cellule infettate con HIV ha portato ad una riduzione della replicazione virale in entrambi gli aplotipi di ERAP2 ($p<0.01$), senza influenzare però la vitalità cellulare. Questo effetto protettivo è stato raggiunto indipendente dall'aumento dell'espressione di molecole HLA-ABC e/o innalzamento dei livelli di perforina e granzimi sui linfociti CD8+.

Conclusioni: L'aplotipo A di ERAP2 è associato sia ad una riduzione della suscettibilità a HIV-1 *in vitro* sia ad una progressione più lenta della malattia in pazienti HIV-1 infetti sottoposti a trattamento cART. L'effetto può essere giustificato da un miglior controllo del meccanismo di presentazione antigenica in soggetti Hap A, definita in termini di attività di processamento peptidico e conseguente

modulazione della risposta immune T CD8 mediata. Il ruolo extracellulare di ERAP2 è ancora in discussione e richiede ulteriori indagini. Nonostante ciò, i dati ottenuti suggeriscono un ruolo protettivo mediato da ERAP2 nell'infezione da HIV-1, probabilmente esercitato attraverso meccanismi non convenzionali, indipendenti dalla modulazione del sistema immunitario.

LIST OF ABBREVIATION

ACE: angiotensin converting enzyme

AIDS: Acquired Immunodeficiency Syndrome

A-LAP: adipocyte-derived leucine aminopeptidase

APC: antigen presenting cells

APOBEC3H: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 H

ART: antiretroviral therapy

ARTS-1: aminopeptidase regulating type I TNF receptor

ART-START: Strategic Timing of Antiretroviral Therapy

AS: ankylosing spondylitis

ATP: Adenosine Tri-Phosphate

BH: bleomycin hydrolase

cART: combined antiretroviral therapy

CCR5: C-C chemokine receptor type 5

CDK: cyclin-dependent kinase

CHIC: UK Collaborative HIV Cohort

CPRG: chlorophenol red β-galactopyrannoside

CTLA: cytotoxic T-Lymphocyte Antigen

CTLs: CD8+ T lymphocytes

CXCR4: C-X-C chemokine receptor type 4

DC: Dendritic cells

dNTPs: deoxynucleoside triphosphates

ds: double strand

dsDNA: double stranded DNA

dsRNA: stranded RNA

EC: endothelial cells

ER: endoplasmic reticulum

ERAAP: ER aminopeptidase associate with antigen processing

ERAP1: Endoplasmic Reticulum Associated Aminopepetidase type 1

ERAP2: Endoplasmic Reticulum Associated Aminopepetidase type 2

ERAP2-AS: alternatively spliced ERAP2

ERAP2-FL: full-length ERAP2

ESN: exposed seronegatives

EU: exposed uninfected

FBS: fetal bovine serum

FIs: Fusion Inhibitors

GM-CSF: granulocyte-macrophage colony-stimulating factor

gp: glycoproteins

HAART: Highly Active Antiretroviral Therapy

Hap A: haplotype A ERAP2 (GG)

Hap B: haplotype B ERAP2 (TT)

Hap: Haplotype

HCMV: human cytomegalo virus

HIV: Human Immunodeficiency Virus

HLA: human leukocyte antigen

Homo: homozygosis

HPV: human papilloma virus

HTLV2: Human T-lymphotropic virus 2

IFN: interferon

IL: interleukin

IL-1RII: IL-1 decoy receptor

IN: Integrases

INSTI: Integrase strand transfer inhibitors

ISG: interferon-stimulated gene

LAG: lymphocyte activation gene

LAP: leucine aminopeptidase

LCMV: lymphocytic choriomeningitis virus

LNPEP: leucyl-cystinyl aminopeptidase

L-RAP: leukocyte-derived arginine aminopeptidase

LTNP: Long Term Non Progressors

LTRs: Long Terminal Repeats

MACS: Multicenter AIDS Cohort Study

MEU: multiple exposed uninfected

MHC: Major Histocompatibility Complex

MIP: macrophages inflammatory protein

MLV: murine leukaemia virus

MS: multiple sclerosis

MSM: men who have sex with men

MX2: Myxovirus resistance-2

NHP: Non-human primate

NIH: National Institute of Health

NK: natural killer

NMD: non-sense-mediated decay

NNRTIs: Non-nucleoside reverse transcriptase inhibitors

NRTIs: Nucleotide reverse transcriptase inhibitors

OPD: ortho- phenylenediamine-HCl

PAMPs: pathogen-associated molecular patterns

PBMCs: Peripheral Blood Mononuclear Cells

PBS: phosphate buffered saline

PD-1: programmed death 1 factor

PDI: Protein disulfide isomerase

PDK1: phosphatidylinositol-dependent kinase 1

PIC: pre-integration complex

PILS-AP: puromycininsensitive leucine-specific aminopeptidase

PIs: Protease inhibitors

P-LAP: placental leucine aminopeptidase

PLC: peptide loading complex

pMHC: peptide bound to MHC molecules complex

Pol: polymerase

PPR: pattern recognition receptors

PR: Protease

PrEP: Pre-Exposure Prophylaxis

PTC: papillary thyroid carcinoma

RT: Reverse Transcriptase

SDM: site directed mutagenesis

SHCS: Swiss HIV Cohort Study

SIVs: simian immunodeficiency viruses

SNP: single-nucleotide polymorphism

SNPs: Single Nucleotide Polymorphisms

ss: single stranded

ssDNA: single-stranded DNA

ssRNA: single-stranded RNA

SV5: simian virus 5

TAP: transporter associated with antigen processing

TCR: T cell receptor

Th: T helper CD4+ T

TIM: T cell immunoglobulin domain and mucim domain

TLR: Toll-Like Receptors

TNF: tumor necrosis factor

TNFR1: type I TNF receptor

TPPII: tripeptidyl peptidase II

Tr: Regulatory T cells

UPS: ubiquitin-proteasome system

vif: virion infectivity factor

VL: viral load

WHO: World Health Organization

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INTRODUCTION

1. HIV and Acquired Immunodeficiency Syndrome (AIDS): Origin and History

In 1980s for the first time, the human immunodeficiency virus (HIV) infection was described. In 1983 Barré-Sinoussi-Montagnier and, one year later Robert C. Gallo, demonstrated the existence of a new retroviral virus and its relation, as causative agent, with the acquired immunodeficiency syndrome (AIDS). Fourteen years later, in 2008, Barré-Sinoussi and her boss Luc Montagnier received the Nobel Price for this discover [1-3].

HIV was first found and described in Africa [4-5]. Kreiss JK et al., demonstrated an epidemic HIV status in East Africa explained by the presence of high percentage of seroposivity prostitutes in Nairobi [6].

Despite a slight reduction in new infection in recent years, the problem of prevention, drug treatment and adverse effects is still enormous. The impact of morbidity and mortality caused by HIV infection is high despite the availability of antiretroviral therapies. The World Health Organization (WHO) data report 1.6 millions of people dying for the disease every year and still 30 million living with HIV infection, most of them (70%) in Africa. It is estimated that HIV has infected 50 - 60 million people, causing death of more than 25 million individuals [7-8]. The presence of the virus causes a severe immunodeficiency condition, characterized by CD4+ T cell loss, leading to secondary effects as co-infection (by other pathogens like mycobacterium tuberculosis and hepatitis viruses), dementia and malignant tumours (mostly Kaposi's Sarcoma and B-cell lymphoma). Without a therapy, HIV-infection leads to Acquired Immunodeficiency Syndrome (AIDS) and ultimately to death.

2. HIV Virology

2.1 HIV classification and structure

HIV is a retrovirus belonging to viral retroviridae family and to the genus Lentivirus. Two different types of HIV have been identified: HIV type 1 and HIV type 2. They differ for genome structure but both cause AIDS syndrome. HIV-1 is more prevalent in America, Europe and Central Africa, while HIV-2 is present in West Africa and in Asia [8-10]. HIV-1 raised from cross-species transmission of a chimpanzee virus to humans and, based on genome differences, three groups of HIV-1 have been described: M, N and O [11]. Group M can be divided into 11 major clades (A1, A2, B-D, F1, F2, G-K) and most of HIV infections are due to them. Circulating recombinant forms (CRF) contribute to maintain and expand the diversity of HIV subtypes. Clades A1, D and CRFs are predominant in Kenya, whereas clade B is prevalent in North America and Europe [12]. Ten years ago, a new HIV-1 sequence was isolated from a single individual. Result show a stronger similarity between the sequence and a simian immunodeficiency virus (SIV) found in gorillas, compared with SIVs found in chimpanzee. The sequence has been placed in group P "pending the identification of further human cases" [13].

As for HIV-2 origin, it raised up from cross-species transmission of a sooty mangabey virus. The genomic structure and antigenicity is different and it has a lower infectivity, causing a slower progression of the disease compared with HIV-1 [14-16].

2.2 HIV Structure and Genome

HIV-1 is a single-stranded RNA (ssRNA) enveloped virus with a spherical shape and dimension about 100 nm; The genome contains two identical positive sense single-stranded RNA molecules (~ 9.2 kb long) and localizes into a capsid of p24 viral proteins surrounded by p17 matrix proteins. A cell

membrane host-derived phospholipid bilayer envelope packs the viral structure [17] (Figure 1.1).

Long terminal repeats (LTRs) in the viral genome regulates survival functions for the virus as viral replication, integration into host genome and gene expression. Structural genes encode the infection-envelope (Env) glycoprotein (gp) 120 and gp41, internal core (Gag) proteins and the enzymatic polymerase (Pol) necessary for viral replication. In detail, polymerase sequence encodes for reverse transcriptase (RT), integrases (IN) and protease (PR). There are also regulatory genes, such as Tat and Rev, and accessory genes like Vif, Vpr, Vpu and Nef [18].

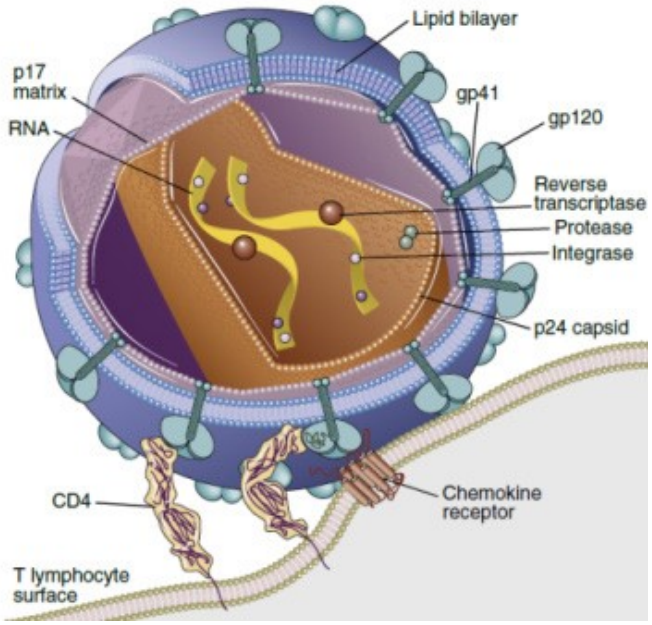


Figure 1.1. HIV-1 Structure. HIV-1 virion binding to a T cell through the CD4 and other chemokine receptors on the target cell surface. Image from *"Cellular and Molecular Immunology,* 8th Edition" [17].

Vif is the acronym of “virion infectivity factor” and it relates with viral infectivity; Nef is a virion–associate protein, the deletion origins an asymptomatic infection in macaques [19-21]. Vpu is present only in HIV-1 and shares similar functions with nef and gp120-env. It down regulates CD4 molecules from the plasma membrane of infected cells. Further Vpu counteracts BST-2 (or Tetherin), a cellular protein which hampers the release of new progeny virions in the final step of the virus life cycle [22-23] (Figure 1.2).

Only in HIV-2, the presence of the virion-associated protein Vpx facilitates virus replication in T cells and macrophages by counteracting the host factor SAMHD1, which hydrolyses intracellular deoxynucleoside triphosphates (dNTPs) required for the synthesis of viral DNA [24].

Tat and Vpr (virion–associated protein) are transcriptional activators of HIV-1, in particular in non-dividing cells such as mature macrophages [25]. Besides Vpr’s control at cellular level (apoptosis and G2/M cell cycle arrest), it makes part of pre-integration complex (PIC). PIC chaperons the viral genome into the cell where conversion from RNA to DNA by virion–associated reverse transcriptase enzyme takes place (nucleus).

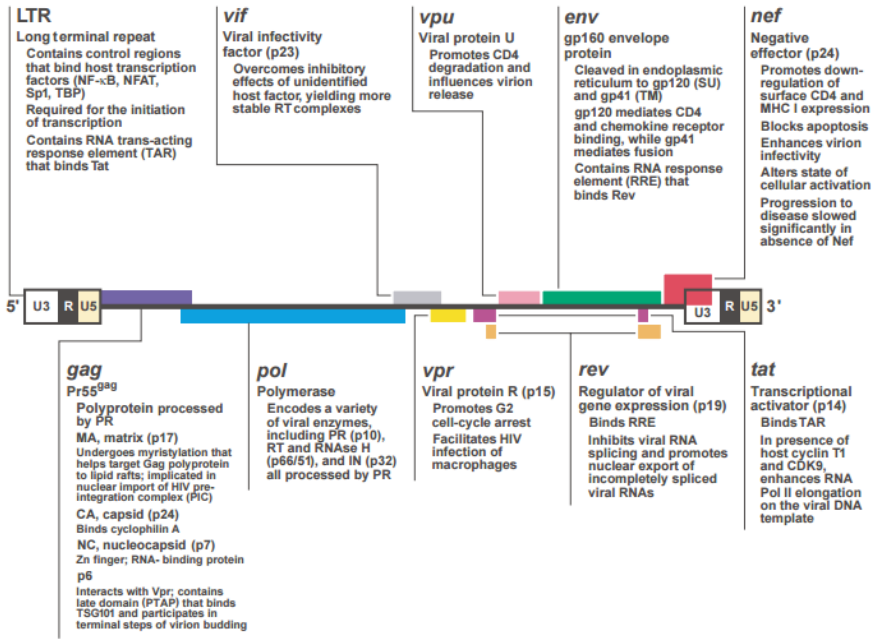


Figure 1.2. HIV-1 genome. For each gene, a different colour is associated. The common sequence used by different genes is shown by overlapping blocks. Genes, whose code separate sequence and require RNA splicing to produce mRNA, are related to coloured block separated by lines. Image from "*AIDS and the immune system"* [26].

2.3 HIV life cycle

In HIV infection, the first step for the virus' entrance is the bond between the viral protein gp120 and CD4 molecules present on the surface of host target cells. The bond induces a conformational change followed by a second bond between another gp120 protein and the co-receptors CCR5 or CXCR4, depending on viral tropism. R5X4 HIV variants are able to bind both described chemokine receptors, X4 variants use CXCR4 coreceptor, while R5 variants bind CCR5 chemokine receptor [27]. CCR5 is expressed on macrophages and activated T-cells, while CXCR4 expression is ubiquitous. This bound induces the presentation of a hydrophobic region that starts the

fusion process between the virus envelope and target cell membrane. Once the cell is infected, different gp120 and gp41 proteins are shown on the cellular surface, thus favouring cell-cell fusion, by receptor bindings and new infections (Figure 2.3) [28]. Once inside, the reverse transcription process starts via enzyme activation: the viral reverse transcriptase converts RNA genome into a single stranded (ss) DNA copy immediately replicated to produce a double stranded (ds) molecule [29]. The reverse transcription process is error-prone and it allows to increase the diversity of progeny genomes. Subsequently, the viral dsDNA enters into cell nucleus with the viral integrase which catalyses the insertion of the viral DNA into the host genome (provirus) [17; 30-31]. The provirus could stay transcriptionally inactive long time (months or years) and this explains the latency of HIV infection for long periods in infected individuals. 5' LTR sequence (containing polyadenylation signal sequences -TATA box promoter sequence) regulates the transcription process, induced by cytokine-mediated T cell and macrophage activation . Cytokines like interleukin (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, while IL-2 and tumor necrosis factor (TNF) stimulate HIV gene expression in infected T cells [32-37]. Inter-individual variants may play a key role in conditioning HIV replication and the pathogenesis of AIDS because the physiological response of latently infected cells to exogenous stimuli may increase viral replication and spread, favouring infections in new cells.

We can divide HIV gene expression into two stages: an early one, where regulatory genes are expressed and a late stage, in which structural gene expression takes place.

After transcription, viral polyproteins are translated in the host cell cytoplasm and cleaved, by a mix of viral and cellular proteases, into individual viral

proteins. Soon after, the assembly of infectious viral particles starts and is followed by budding from the plasma membrane of progeny virions [27] (Figure 1.3). Viral full-length RNA transcripts are packaged within a nucleoprotein complex (gag core proteins and pol encoded enzymes) and during bud-process env and host glycoproteins are included in the viral envelope.

Notably, some host cells may express prevent-virion release factors, such as APOBEC3 (Apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3) which interferes with viral replication [17]. Another example is represented by TRIM5α a host factor identified in 2014 by Edward Campbell, which brings in non-human primates to viral transcriptase's proteosomal degradation and premature viral uncoating [38].

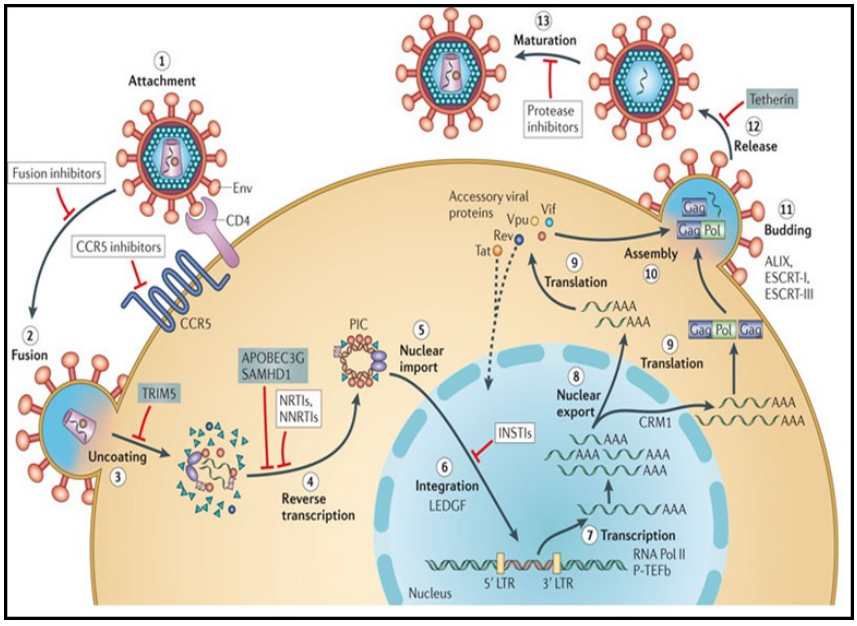


Figure 1.3. Schematic representation of HIV life cycle. Image from "*The structural biology of HIV-1: mechanistic and therapeutic insights"* [39].

3. HIV pathogenesis: transmission and progression

HIV-transmission from one individual to another may occur by different routes. Among horizontal transmission, the most common is sexual contact: homosexual or heterosexual. The latter accounts for the majority of HIV cases worldwide [40].

Follow parenteral transmission by needle sharing and blood contaminated transfusions, although nowadays the index of infections due to contaminated blood products is significantly decreased compared with the past because of routine laboratory screenings.

Nowadays it is also possible to prevent vertical transmission, which occurs when the virus passes from mother to child during pregnancy (in utero) or postnatal life (trough breast-feeding). Treating the mother with antiretroviral drugs during the prenatal period prevent the transmission.

Three different phases characterise HIV-infection and progression in absence of antiretroviral therapy (ART): acute phase, chronic phase and AIDS (Figure 1.4). Three months after the transmission, infected individuals may undergo to non-specific symptoms of infection, such as acute mononucleosis-like syndrome, which occurs in 50-70% [17]. At this primary stage, the immune system is not able to control and contrast the infection, and therefore viremia peaks are commonly observed. HIV usually enters into the host via mucosal epithelia where it causes a rapid lymphocyte depletion infecting memory CD4+ T cells in mucosal lymphoid tissues. Moreover, epithelial dendritic cells, which are able to capture the virus, migrate into lymphnodes where, by direct cell contact, can infect CD4+ T cells increasing viremia levels. At this condition, HIV can spread across the body and infect helper T cells, dendritic cells and macrophages.

In the meantime the immune system progressively develops an HIV-specific humoral and cell-mediated immune response which partially control the viral spread and infection causing a sharp drop in viral load more or less 12 weeks after primary infection. This low point in viral load (VL set point) has been positively associated with the progression of HIV-disease: the higher the VL of the set point, the faster the virus will progress to AIDS; the minor the VL of the set point, the longer the patient will persist in clinical latency [41-42].

A longer chronic phase follows acute infection. Even if HIV-1 is still present in organs like lymphnodes and spleen, the immune system seems to become competent at managing various infections. Actually a consistent CD4+ T cell turnover and a progressive decline of these cells in the periphery take place. The more the disease progresses the more the host becomes susceptible to new infections. The presence of opportunistic infections, that the immune system attempts to cope with, facilitates HIV-1 replication.

Two-hundreds cells/mm3 CD4+ T cell count is the threshold that marks the onset of AIDS and it is considered the final and lethal phase of the disease. During this stage, the host immune system is unable to contrast new opportunistic infections and infectious challenges [43]. The immune system is severely depleted and usually secondary effects (as neoplasms, central nervous system degeneration and kidney failure) lead to death [44-45].

However nowadays, the natural course of HIV-infection has been deeply modified by the availability of an efficient antiretroviral therapy that has improved the course of disease as well as time and quality of survival of the patients.

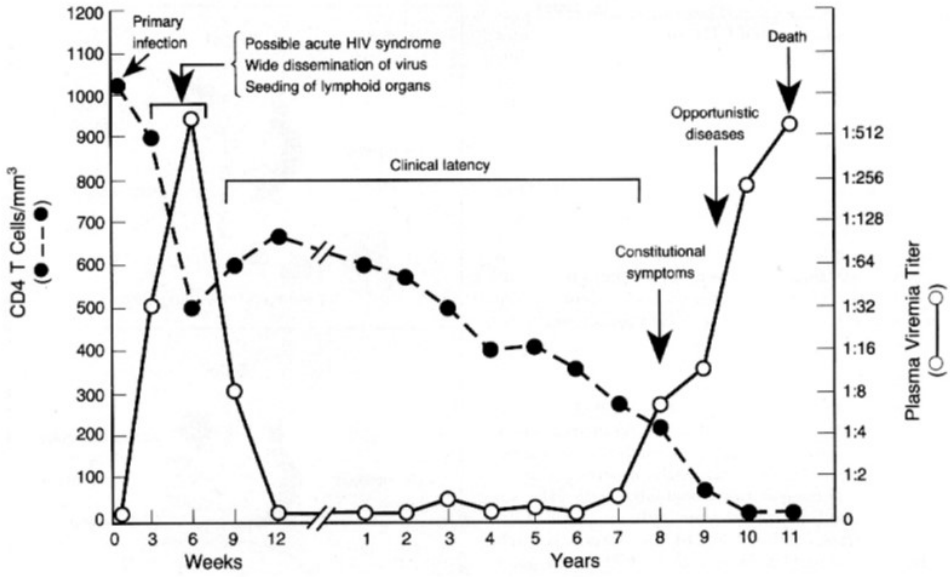


Figure 1.4. Natural course of HIV-infection. Image from "*The Immunopathogenesis of Human Immunodeficiency Virus Infection*" [44].

4. Highly Active Antiretroviral Therapy (HAART)

Antiretroviral drugs, able to inhibit HIV replication cycle at different levels, are used to control and manage HIV-infection. In 1987 zidovudine (AZT) was approved as first antiretroviral drug by the US Federal and Drug Administration. In the following years, different antiviral drugs were discovered and approved for the treatment of HIV-infection. In the 90s a "combinational antiretroviral therapy", consisting of 3-drug therapy into clinical practice, leaded to an AIDS rate's decline (80%), reducing hospitalization and death in HIV-infected patients. Basically, introduction of ART transformed HIV-infection from being a fatal condition to a chronic and non-fatal one [46].

We can classify antiretroviral drugs in 6 different groups according to their action mechanism in viral life-cycle:

- ✓ Nucleotide reverse transcriptase inhibitors (NRTIs): as the name suggests, these drugs are able to prevent HIV-replication, inhibiting viral reverse transcriptase. NRTIs act as competitive inhibitors by blocking nucleosides addition to the DNA chain during the HIV transcriptional process. Commercial drugs belonging to this group are Abacavir (ABC), Emtricitabine (FTC), Lamivudine (3TC), Stavudina (d4T), Tenofovir (TDF) and Zidovudine (ZDV).

- ✓ Non-nucleoside reverse transcriptase inhibitors (NNRTIs): also this category of drugs is able to prevent HIV-replication, however their mechanism of action is different from NRTIs. Indeed they are able to interfere with reverse transcriptase viral activity, binding the hydrophobic pocket p66 subunit of the enzyme and leading to a conformational change that blocks the catalytic site of the reverse transcription enzyme. First generation commercial NNRTIs are, for example, Nevirapine (NVP) and Efavirenz (EFV), instead Rilpivirine (TMC278) and Etravirine (TMC125) are considered second-generation drugs. HIV-2 presents a natural resistance to this type of drugs.

- ✓ CCR5 antagonists: CCR5 chemokine receptor is a co-receptor used by R5 HIV to enter into host cells. These antagonists act by binding the chemokine receptor and resulting in a virus internalization inhibition. The use of this drug could shift HIV tropism, allowing HIV to target CXCR4 (the other main co-receptor) so some cautions are requested when using these drugs. Maraviroc (MVC) is a CCR5 antagonist.

- ✓ Fusion Inhibitors (FIs): drugs belonging to this category block HIV-fusion with the cellular membrane of target cells and virus entry process by competing with gp41. Enfuvirtide (ENF) belongs to this class.

- ✓ Integrase strand transfer inhibitors (INSTI): These drugs block the integration of HIV-genome into host cell's genome. Raltegravir (RAL) and Elvitegravir are INSTI drugs.

- ✓ Protease inhibitors (PIs): Viral proteases are responsible of the packing (and make infective) of new virions. This class of drugs is able to prevent and to inhibit this process. Atazanavir (AZT), Darunavir (DRV), Fosamprenavir (FPV), Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV) and Tipranavir (TPV) are all PI.

Mono-therapy showed to be unsuccessful because a single inhibition mechanism favours the onset of new mutant resistance virus. This could be explained by an intense HIV genetic variability together with a selective pressure induced by the drug.

The use of two NRTIs plus one NNRTIs or a combination of two NRTIs plus one PI, defined as Highly Active Antiretroviral Therapy (HAART), is the current therapeutic approach used as standard antiretroviral regimen. It is based on the administration of a combined therapy, which hinders different stages of viral replication to achieve a synergistic effect in infection control. As a matter of fact, the approach based on a combined therapy suddenly increases CD4+ T cell counts and, at the same time, brings to a stable and drastic viral load suppression. In general, HAART efficacy is achieved in 4-6

months after initiation with an increase of 100-150 cells/mm3/year of CD4+ T cell count and a suppression of viral replication (< 50 copies/ml) [47-49].

That being said, at the time of HIV-diagnosis current medical guidelines suggest to perform a genotype drug resistance test in order to opt to the right and more effective combination of antiretroviral drugs. As expected, the success of therapy 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.

As all the therapies, ART has side adverse events. Anaemia, diarrhoea, dizziness, headaches, fatigue, nausea, vomiting, pain and nerve problems as well as coetaneous rashes are considered short-term effects. Instead among the long term effects we can list: diabetes caused by insulin resistance, lipodystrophy, increase in cholesterol and triglycerides levels (lipid abnormalities), lactic acidosis and reduction of bone density. The scientific as well as the medical community suggested to suspend the HAART regimen for shorts period in the attempt to reduce side and toxic events due to long-term therapy and to improve patient's compliance. However, a large trial funded by National Institute of Health (NIH), called SMART, demonstrated more benefits following a continuous antiretroviral regimen compared with the risks of long-term collateral effects. Changing the drug regimen is one of the possibilities to avoid long-term complications of HAART.

Another aspect to be considered is when starting HAART therapy. Currently medical guidelines suggest, the antiretroviral drug regimen should be started at the time of HIV-diagnosis in particular if CD4+ T cell count is less than 350 cells/mm^3. However two large randomized controlled clinical trials, named TEMPRANO and ART-START (Strategic Timing of Antiretroviral Therapy), demonstrated a reduction of morbidity and mortality among HIV-infected

patients with CD4 T cell count >500 cells/mm^3. The studies were randomized in order to receive ART immediately after diagnosis versus delaying initiation of therapy [50-55]. Notably, some recent evidences correlate HAART with the prevention of HIV-infection and transmission (PrEP: Pre-Exposure Prophylaxis). In particular, studies on HIV-serodiscordant heterosexual couples show lower HIV plasma levels correlated with lower transmission rate as well as a decrease in the virus concentration in genital secretions [56-58].

The fact that antiretroviral therapy has been universally approved, has allowed the development of new perspectives of study on HIV-infection. New cohorts of HIV-positive patients, following an ART/HAART regimen or not, raise up in order to analyse different aspects of the disease. The goals of these projects are to have interdisciplinary observations for human immunodeficiency virus, such as clinical trial, translational studies, basic, epidemiological and social sciences and, also, for addressing public health questions. These studies allow monitoring challenges of modern antiretroviral treatment (like effectiveness, drug resistance and toxicity), treatment-duration, virus–host interactions, cell biological and genetic mechanisms of the disease.

For example, in 1988 the Swiss HIV Cohort study (SHCS) was established dissecting different aspects of the pathology. Other groups to be mentioned are the UK Collaborative HIV Cohort (CHIC) and the HIV-Brazil cohort. The Multicenter AIDS Cohort Study (MACS) is a US 30-year study of the HIV-1 infection in gay and bisexual men conducted since 1984 (Retrained from: http://www.shcs.ch/; http://www.ukchic.org.uk/; http://aidscohortstudy.org/).

In the Italian panorama, ICONA is a unique cohort that joins other prestigious European and American cohorts. It started as a Foundation in 2007 but it has been operating since 1997. It consists in a national Italian network designed

to collect clinical data and select biological materials from 50 medical centres operating throughout Italy and coordinated by 6 University Centres. In the last 20 years, more than 14.500 patients have been enrolled. Notably, the only eligibility criterion for participants is that they must never have taken antiretroviral drugs, indeed all the patients were “naïve” to antiretroviral therapy at the enrolment time. It, therefore, stands out as the only cohort in the world capable of supplying reliable data on the impact of initial treatment on epidemiological, clinical, biological, and behavioural parameters.

5. HIV immunology

The first immune system response to HIV infection is similar to the one mounted for other viruses, however it has a limited protection against the infection.

5.1 Innate response to HIV infection

Innate immune system provides the first line of defence mediated by the host system. Competent cells are able to recognize by pattern recognition receptors (PPR) evolutionary conserved structures known as pathogen-associated molecular patterns (PAMPs). Among these, toll-like receptors (TLRs) identify conserved motifs unique to microorganisms. In terms of viral infection, the best characterized are TLR-9 which is specific for DNA molecules, TLR-7, TLR-8 and TLR-3, which recognize both single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA) and viral proteins [59]. Interestingly HIV-1 ssRNA encodes for different TLR-7 and TLR-8 ligands, which can directly activate the immune system *in vitro.* The bond between receptor and specific-substrate triggers a cascade of cytokine production. In particular, the expression of interferon α (IFNα) is enhanced, followed by a transient increase in interleukin (IL)-10, IFNγ, tumor necrosis factor (TNF) α, IL-15, IL-18, IL-22, and CXCL10. IFNα is able to activate the expression of different genes known as interferon stimulated genes (ISG) and this process

is able to inhibit viral life cycle at different stages [60-61]. *In vitro*, the interferon cascade-inhibition has been demonstrated in macrophages, monocytes and humanized mouse models of HIV-1 infection [62]. The production of IL-10 and IFNγ casually correlates with the beginning of virus-specific adaptive immune response [63]. Due to high levels of pro-inflammatory cytokines secreted by specific cells (in particular by monocytes and dendritic cells), the activation and expansion of natural killer (NK) cells occur. This step correlates with the acute HIV-1 infection phase, which precedes any antibody response [64]. After an initial peak, NK levels start to decrease and a persisting viral replication leads to disease progression.

5.2 Adaptive response to HIV infection

The response of adaptive immune system is another important aspect of host response against HIV. It can be divided into cellular and humoral response.

5.2.1 Cellular Immune Response

The main cell types that play a central role in this process are T lymphocytes. We can identify two main clusters of T cells: T helper (Th) CD4+T cells and cytotoxic CD8+ T lymphocytes (CTLs). Th cells can separate into different T cell subsets: Th1, Th2, Regulatory T cells (Tregs) and Th17. Type 1 cytokines like IFNγ, TNFα and IL-2 are requested for events such as proliferation, activation and differentiation of Th and CTLs into memory or differentiated cells and are produced by Th1. Humoral immune response is activated by Th2, mediated by IL-4, IL-5 and IL-13 secretion. A robust HIV-specific CD4+ T cell response is associated with better control of HIV viral replication [65]. The detection and elimination of infected cells is made by CTL. The mechanism of action of these cells is associated with the recognition and bond of viral antigens to MHC I molecules that present them on the cell surface. Once activated, the production and release of perforins and granzymes by CD8+ T cells can directly eliminate infected cells [66]. The

elimination of infected cells can occur also by apoptosis following the interaction between Fas ligand, on CTL surface, and Fas receptor, expressed by infected cells [67]. As explained above few weeks after primary infection a decline in peak viremia occurs and it correlates with HIV-specific CD8+ T cell onset. These HIV-specific T cells play a key role in viral replication control and usually are specific for Env and Nef peptides, while later, during infection, CD8+ T cells specific for other viral proteins take place [68]. In line with this concept, CTLs isolated from HIV-infected individuals are able to inhibit viral replication in *in vitro* assay [69]. In addition, the depletion of CD8+ T cells from SIV-infected macaques results in a loss of viral control and rapid disease progression [70].

The immune responses, in particular the cellular one, play a critical pressure in viral evolution explained by viral isolates able to escape from CTLs and CD4+ T cell control. This occurs for the loss of their original CTLs and CD4+ T cell epitopes [71]. However, a functional exhaustion of HIV-specific T cells undergoes and T cells die by apoptosis after losing proliferative and cytotoxic abilities. Different efforts have been made to study T-cell exhaustion phenomenon and factors 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 have been identified as markers of the process [72]. In support of this, PD-1 has been identified as a critical negative regulator in HIV-infection [73] while Tim-3 has been linked to elevated sensitivity to Treg-mediated suppression [74].

The function of Treg cells is to maintain peripheral tolerance and prevent autoimmune diseases and chronic inflammation. This is reached by suppression of antigen-specific T-cell responses and by control of immune activation [75]. Inhibition of antiviral immune responses contributes to pathogen persistence and disease progression. Conversely, Tregs help to

suppress immune activation, thereby slowing disease progression. Notably HAART increases Treg level, normally decreased with disease progression [76-78].

Th17 cells produce the pro inflammation cytokine IL-17, especially at mucosal level [79]. In HIV population an imbalanced Tregs-Th17 ratio and has been described and a lot of efforts have been made to clarify the Th17 controversial protective-harmful double meaning, however, its role in HIV-infection is not yet fully understood [80].

5.2.2 Humoral Immune Response

Another force of adaptive response is represented by humoral immune response. Dendritic cells (DC) internalise viral proteins and process these into smaller viral peptides, which are shown on the membrane in order to activate specific humoral response. The presentation occurs mainly through the antigen presentation machinery Major Histocompatibility Complex (MHC) II that binds the viral peptide and to CD4+Th cells, which then stimulate naïve B cells by cytokines production. After the recognition of specific epitopes through surface antibodies, B cells differentiate in part into plasma cells, which subsequently produce specific antibodies such as IgG, IgA, IgE, the other part into memory B cells. The seroconversion is related to the detection of an antibody response to HIV that takes place within 6-9 weeks after infection [81]. The efficacy and neutralization power of early antibodies are not high, despite of it 3 months from infection proper neutralizing antibodies appear. However, they are not able to cope with infection progression and to control viremia. The most immunogenic proteins of HIV are the envelope glycoproteins, indeed high levels of anti-gp41 and anti-gp120 can be detected in HIV-infected patients for a couple of years.

The panel of B cells in HIV-infected patients shows to have an abnormal distribution compared with an uninfected control. For example, a lower

frequency of memory B subset (CD27+B220−) and higher frequency of plasma blasts (CD20lo/−CD27hiCD38hi) have been reported. Additionally, tissue-like memory B cells (usually characterized to be exhausted cells with a reduced proliferation index) are shown to be increased in HIV-infected patients compared to uninfected controls. Nearby B cell chemotactic capability in HIV-patients is altered [82].

Non-human primate (NHP) studies demonstrated a passive protection of neutralizing monoclonal antibodies against virus challenge [83]. Thereby, in the last years a lot of efforts have been made to discover and characterize several highly potent antibodies to use in a not far future vaccine scenario. New findings from an early-stage clinical trial (based on 2600 women under risk for contracting HIV) of a potential HIV vaccine candidate have proven to be encouraging [84].

6. Models of Natural Protection

As explained above, immune responses against HIV are quite complex as many viral and host factors, as well as individual variables, interplay [85]. Indeed in the world population, subjects with an unconventional infection/progression relationship with the virus have been identified. Among these, Long Term Non Progressors (LTNP) are patients who present peculiar virus-specific immune responses that allow them to control infection progression for many years, even in the absence of therapy [86-88]. HIV-patients, called Elite Controllers, are able to maintain normal CD4+ T cell values and very low plasma HIV RNA levels for many years after infection without any therapy [89-90].

The observation that parenteral exposure to HIV-1 does not inevitably results in infection became evident a few years later the explosion of the AIDS epidemics. In the late 80's, epidemiologic studies showed that frequent

unprotected homosexual contacts did not mandatory result in a sereoconversion. The study has been based on a small cohort of men from UCLA Multicenter AIDS Cohort Study (MACS) [91].

In addition, in 1992 Clerici described the presence of an HIV-specific T helper cell response in these exposed seronegative individuals, suggesting a relation between this resistance condition and an immunologic and/or genetic protection [92].

Later, also in a Kenyan heterosexual female sex worker cohort the existence of differential susceptibility to HIV-infection was reported [93]. Shortly thereafter, protection from HIV-infection was described in health care workers accidentally exposed to HIV, intravenous drug users, subjects who have unprotected sex with their seropositive partners (in both heterosexual and homosexual scenario) and haemophiliacs who had received HIV-contaminated factors during blood transfusions. The resistance has been shown as well in vertical transmission scenario. A particularly interesting cohort is represented by uninfected newborns from HIV-infected mothers. At the birth, most of them result uninfected (65-75%) despite being exposed in a constant way to an infected environment during pregnancy. The positive serostatus is due to a passive of maternal anti-HIV antibodies.

Since the first decade of 2000s, many efforts were done trying to explain this complex phenomenon and trying to identify immunological and/or genetic correlates of protection. Comparative data obtained in different laboratories from various cohorts was problematic even because of the lack of a codified and clear classification of what is an exposed uninfected condition. Until that time, HIV-exposed uninfected individuals were referred to as exposed seronegatives (ESN), exposed uninfected (EU) or multiple exposed uninfected (MEU). In 2009 in Canada, the first International Symposium on Natural Immunity to HIV was organised with the intent to define the inclusion

criteria of the ESN individuals and consequently compare the results obtained from different ESN cohorts. A year later, a second meeting agreed upon a new classification of these individuals as HIV-Exposed Sero-Negatives (HESN). A summary of the topics discussed within these meetings can be found in Journal of Infectious Diseases Volume 202 Supplement 3 November 10, 2010 (Figure 1.5).

Although many studies in the last years have tried to evaluate the influence of immunological factors on HIV-1 infection and disease progression and many efforts were dedicated to the identification of genetic variants that can confer either resistance to infection or slow disease progression, key information are still lacking. Indubitably, the resistant phenotype is not dependent on a single variable and its characterization requires multidisciplinary approaches as it results from a combination of genetic, immunological and environmental factors.

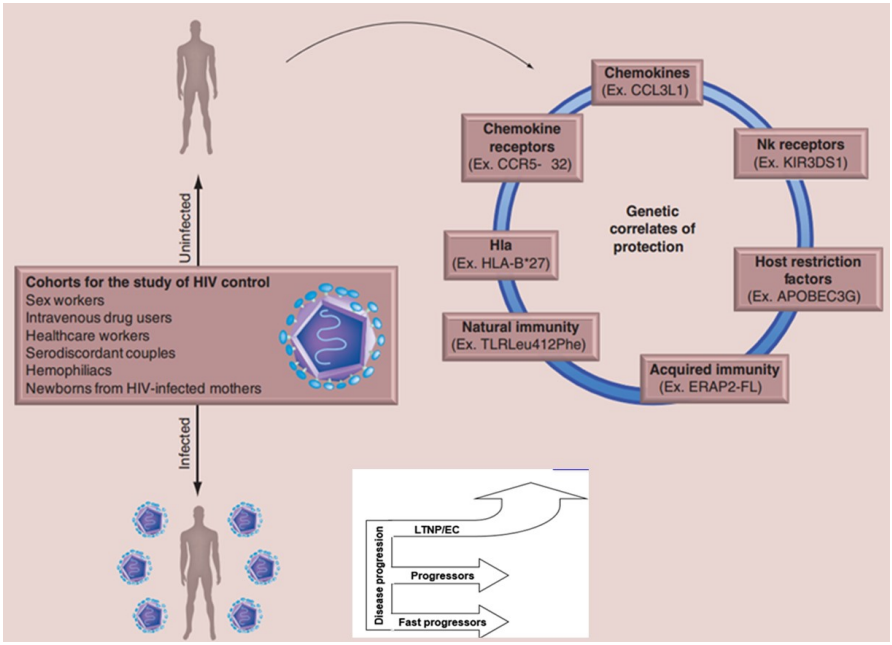


Figure 1.5. Different cohorts that control HIV infection are used to assess the impact of hereditably factors on HIV-1 infection and/or progression. The main genetic correlates of protection to HIV infection so far identified by candidate gene approach are summarized in this panel.

6.1 Genetic correlates of protection

6.1.1 Candidate-gene studies

The first data supporting a genetic contribution in protection's phenomenon dates back to 1996, when it was discovered that the homozygous variant allele of the CCR5 receptor (the main co-cellular receptor for R5 HIV-1 virus), called Δ32, gives an almost absolute protection to HIV-1 infection *in vitro* [94-95]. These data have led researchers to investigate host genetic restriction factors that can influence host susceptibility to HIV, thus determining the genetic characteristics of individuals that have a peculiar response to the virus.

Candidate-gene studies are focused on genes which functions are directly related with HIV-1 life cycle or those genes responsible for the immune response against the viral infection. Consequently, the knowledge about gene function in HIV pathogenesis is a priori fundamental [96]. Remarkably, the most important restriction genes so far identified (and confirmed by genome wide studies) belong to the **human leukocyte antigen** (HLA) class I locus [97]. For example HLAB*27 expression seems to have an important role in maintaining a stable bound between gag peptide, in particular with arginine amino acid in position 2, and HLAB pocket. Instable B27-gag complex is correlated with a faster AIDS progression [98]. Another protective role is displayed by HLAB57. Indeed, HLAB57-restricted cytotoxic T lymphocytes are able to target a high variety of HIV-peptides as proven by different studies. The B5701 allele in Caucasian and the B5703 allele in Africans showed a broad cross-reactivity against both common and rare variants of dominant gag epitopes, associated with delayed progression of HIV disease [99-100]. Quite an opposite effect is exhibited by B*35 allele, as it has been associated with a faster progression of the disease [101]. Notably, B*35-Cw*04 haplotype is associated with a disease development only in Caucasian population, suggesting a negative influence on HIV pathogenesis [102-103].

The evidence that **NK** activation plays a defensive effect in HIV-infection has been established by different genetic studies. African HESN sex workers present a higher frequency of inhibitory KIR genes (in the absence of their associated HLA genes) and a higher rate of the AB KIR genotype, resulting in a stronger KIR activation. Further studies on the activating receptor allele KIR3DS1 bring on the defence mechanism displayed following NK activation. Homozygosis expression of this receptor is higher in HESN compared with HIV-infected controls [104], in addition Carrington reported that the presence of its presumed HLAB ligands brings to a stronger protective function of this

allele [102]. While Pelak showed that copy number variations of activating and inhibitory KIR influence the expansion of protective KIR3DS1+ NK cells in peripheral area, which in turn may determine differences in HIV-1 control post infection [105].

Contradictory is the role exercised by **chemokines and chemokine receptors** on HESN phenotype. For example, ratio between CCL3L1 copy number and susceptibility to HIV-1 infection showed an inverse correlation of CCL3L1 levels with CCR5 expression on CD4+ T lymphocytes [106]. Additional results performed in a cohort of Human T-lymphotropic virus (HTLV)-2-infected individuals indicated a higher ratio of median copy number of CCL3L1 and the CCL3L1/CCL3 mRNA in HESN and in LTNP compared with healthy controls.

APOBEC3H is part of the APOBEC3 family that includes seven subtypes (A, B, C, D, E, F, G, H) that are able to contribute at different levels to HIV-1 resistance [107]. Among APOBEC3 family, an important role is played by APOBEC3G (A3G) which causes a replacement of G/A base in the viral genome and whose action is opposed by the viral protein VIF [108]. Recently, Cagliani et al., in a case-control association analysis, reported that an APOBEC3H haplotype, called HAPI, is associated with protection from HIV-1 when it is sexually transmitted. HAPI produces a destabilized variant protein, which confers resistance to Vif-mediated degradation. This observation suggests that a protein with a lower stability may have been replaced by a more stable one, in order to obtain a greater ability to escape vif-mediated degradation [109].

Viral replication can be inhibited by type-I interferon (IFN) and the expression of a number of gene products with specific anti HIV-1 activity is induced by type-I IFN [110-111]. **Myxovirus resistance-2 (MX2)** is known as an interferon-induced inhibitor of viral infection. Goujon showed a modest MX2-

mediated suppressor effect on divergent simian immunodeficiency viruses (SIVs) and different HIV strains but not an inhibition of other retroviruses (as, murine leukaemia virus (MLV) [112]. While depletion of MX2, using RNA interference method, reduced the anti-HIV-1 potency of IFNα [113]. Nowadays it has been demonstrated that MX2 interferes with viral replication, thus decreasing nuclear accumulation and integration of viral DNA. Mutations in the HIV-1 capsid protein, which is involved in nuclear import, can overcome the inhibition of MX2. Therefore, it has been hypothesized that MX2 targets the viral capsid and that it interacts with other cellular proteins, such as Cyclofilin A, which blocks the nuclear import of HIV-1 [113-114]. In population studies, it has been observed a haplotype associated to SNP rs2074560 (G / A) that is more represented in HESN and the protective haplotype is determined by the homozygous GG. The results obtained suggest that the G allele of rs2074560 protects from HIV-1 infection regardless of the transmission route [115].

Toll-like receptors is a family of receptors capable of recognizing stereotypical characteristics common to different pathogens. Previous studies reported that Peripheral Blood Mononuclear Cells (PBMCs) isolated from HESNs were more responsive to stimulation with TLR3 agonists (Poly I:C), TLR4 agonists (LPS), TLR7 agonists (imiquimod) and TLR7/8 agonists (ssRNA40) compared to those isolated from HIV-infected patients and healthy controls [116]. More recently, a study performed in two cohorts of HESNs with different exposure routes reported a higher frequency of TLR3 SNP rs3775291 (Leu412Phe), which confers protection from HIV-infection. *In vitro* studies demonstrated that TLR3 SNP rs3775291 was associated with a control of viral replication and with an increase of the responsiveness induced by specific agonists [117]. The mechanism behind this protection needs to be better characterized, nevertheless in HESN it seems to be

associate with an increase in full-length form of the adaptor protein MyD88 expression, which acts as an intracellular negative regulator of this pathway.

In the immune response, antigen presentation plays a crucial role. Following this concept, many candidate genes studies put their efforts on this host defence pathway. **ERAP2 (Endoplasmic Reticulum Associated Aminopepetidase type 2)** is a gene involved in antigen presentation [118]. This enzyme cuts the amino-terminal end of peptide precursors that are processed in proteasomes in order to adjust their size to the pocket of HLA class I molecules. Therefore this process modulates the antigenic repertoire presented to CD8+ T lymphocytes and the subsequent immunological response [119]. ERAP2 has been target of long-standing balancing selection [120]. This process has maintained two highly differentiated ERAP2 haplotypes at intermediate frequency in most human populations [121]. The two haplotypes, hereafter referred to Hap A and Hap B, differ at multiple variants in tight linkage disequilibrium. Hap B harbours the T allele for rs2549782 (Asn392Lys) and the G allele for rs2248374; this latter determines the activation of a cryptic splice site in intron 10 and the production of an alternatively spliced ERAP2 mRNA (ERAP2-AS) with an in-frame stop codon. Thus, the predicted protein product of Hap B-derived transcripts is a truncated protein of 534 amino acids, whereas the canonical ERAP2 protein, consisting of 960 amino acids, is encoded by a full-length mRNA (ERAP2-FL) transcribed from Hap A. Since the frequency of haplotype A is higher in HESNs, it was hypothesized that this variant is able to generate a peptide repertoire qualitatively and/or quantitatively different, which in turn is able to confer natural resistance to infection. The data collected so far seem to validate this hypothesis [120-122]

During HIV-1 infection and replication, each of these genes is able to act at different levels and the genetic variants previously mentioned modulate such

interactions by altering gene functionality. There are no studies and it is not yet known whether these protective gene variants may correlate with different responses to therapy in HIV-positive subjects undergoing ART.

6.1.2 Immune correlates of protection

There are evidences that innate immunity gives a contribution in the protection upon primary exposure to HIV before adaptive immunity is altered. NK activation, for example, has a main importance in resistance to HIV-infection [123]. As well, there are many data demonstrating the role of TLR pathway in natural resistance to infection. PBMCs, isolated from HESN cohort, showed to be more responsive to TLR triggering by production of pro-inflammatory cytokines and chemokines [116].

Further, it has been observed that secreted factors could play a role in HIV-transmission at mucosal level. β-chemokines macrophages inflammatory protein (MIP)-1α, MIP-1β and RANTES compete with virus for co-receptor CCR5 which, results in a decrease of HIV infection in target cells [124].

Elevated IL-22 levels are associated with resistance in HESN phenotype and it occurs by induction of acute–phase serum amyloid A, which downregulates CCR5 expression on target cells [125]. In conclusion, elevated salivary of β-chemokines have been shown to be associated with oral sexual behaviour in men who have sex with men (MSM) HESN [126].

Different evidences have supported the association between T cell response and resistance against HIV infection in the last years in different HESN cohorts. The fundamental role of HIV-specific CTLs in resistance mechanism is recurring in data obtained from HESN cohorts. In the majority (>70%) of different HESN cohorts data shown the presence of HIV-specific CTLs cells at both systemic and mucosal level. This evidence is interesting because represents a key step in the disclosure of the resistance mechanism

characterizing HESN subjects. Briefly, an HIV-specific CTL response is possible only following a complete cycle of viral replication leading to an effective viral-antigen presentation mediated by human leukocyte antigen (HLA) class I molecule to CD8+ T cells. This means that in exposed individuals the infection takes place but their immune system is able to control and contain the infection progression still in an unknown way. Otherwise, the discovery of a cross-priming process, an alternative processing-presentation mechanism mediated by dendritic cells, might explain the presence of HIV-specific CTLs in absence of viral replication. HIV-specific CTLs isolated from HESN individuals are able to recognize various HIV epitopes that are different from those recognized by cells of HIV-infected patients, which could suggest an increased efficacy of the alternative presentation mechanism. Additionally, HESN CD8+ T lymphocytes seem to be more responsive to gag-stimulation, in terms of perforin and granzyme production levels [127-128].

As for humoral response, Devito et al., described HIV-specific IgA molecules into female genital tract (FGT) of HESN CSW and female uninfected partners of discordant couples [129]. As well, specific-IgA were reported in Pumwani sex worker cohort, unfortunately they seemed to not have virus neutralization activity [130].

7. Antigen processing and presentation

Antigen processing and presentation mechanisms are fundamental for immune surveillance mediated by CTLs. The generation of peptides to present on MHC molecules at the cell surface is mediated by three pathways. The endogenous pathway plays a role in processing proteins and peptides derived from intracellular pathogens and it is reached through the major histocompatibility complex class I (MHC I) that present peptides to CD8+

CTL. A second pathway, called the exogenous pathway, processes antigens derived from endocytosis of circulating peptides and is subsequently directed to lysosomes. MHC class II (MHC II) molecules present these peptides at the cell surface to CD4+ T cells. The last pathway to describe is the cross presentation pathway; interestingly, this process involves exogenous antigens being presented by MHC I. Briefly, in all these pathways, MHC molecules bind to peptides forming a complex (pMHC) that is recognised by antigen receptors present on the T cell surface (T cell receptor; TCR). Once bound this interaction allows the activation of the T cell, which eventually will eliminate target cells presenting the antigen.

The MHC gene region, located on chromosome 6 in humans, encodes for highly polymorphic MHC molecules and is divided into three different regions: MHC I, II, III. Class I encodes HLA A, HLA-B and HLA-C, while HLA-DP, HLA-DQ, HLA-DR and HLA–DO are encoded by MHC class II region. Finally, MHC III encodes other immune system components involved in the complement system.

7.1 Endogenous antigen presentation pathway

As explained above, the endogenous presentation pathway is involved in presentation of antigen derived from endogenous proteins, such as viral proteins in the case of virus infection. In the cytosol, the proteasome and several peptidases trim peptides that are then transported by the transporter associated with antigen processing (TAP) protein into the endoplasmic reticulum (ER). For presentation, peptides need to be the correct length of 8-10 amino acids [132-133]. Longer peptides require additional trimming in order to bind with a high enough affinity to the peptide binding pocket of HLA [133-135]. In humans, the N-terminal trimming of longer peptides is catalysed by two aminopeptidases, ERAP1 and ERAP2, while in mouse the process is mediated by ERAAP (endoplasmic reticulum aminopeptidase associated

with antigen processing) [136-139]. Once the peptides are the correct length to bind, they are loaded to MHC I molecules and the resulting pMHCI complex translocates to the cell surface in order to be recognised by specific CD8+ T cells [140-141]. The final goal is the CTL-mediated monitoring of the presence of 'foreign' peptides from invading pathogens [142]. As described, these events follow a multistep process and is shown below in Figure 1.6.

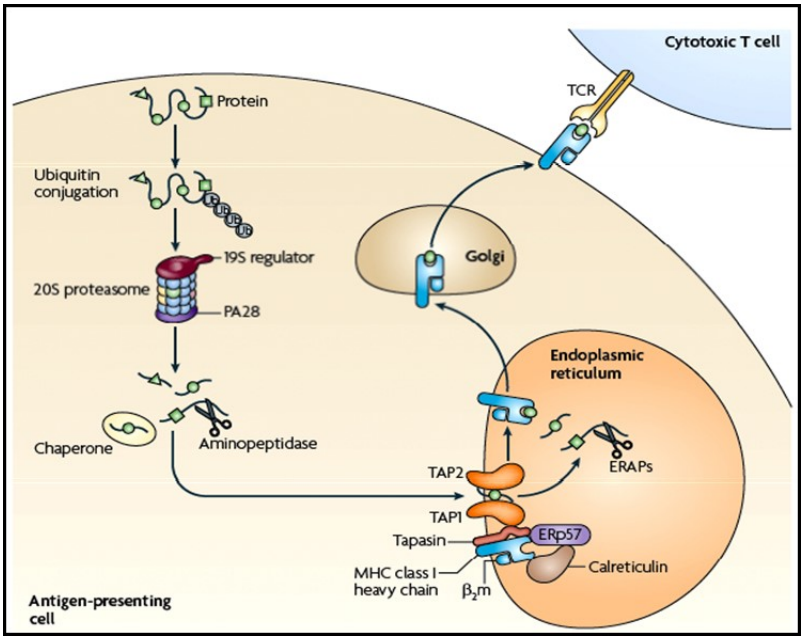


Figure 1.6. MHC I antigen processing and presentation. Degradation of cytosolic and nuclear protein is proteasome-mediated and often followed by cytosolic aminopeptidase trimming. TAP proteins transport peptide into the lumen of the ER. Alongside this, MHC I heavy chain folding occurs with the assistance of the chaperones calnexin and calreticulin, the peptide editor tapasin and the oxidoreductase ERp57 (the latter three, alongside TAP, form the peptide loading complex; PLC). Protein disulfide isomerase (PDI) helps in the formation of the disulfide bond in the α2 domain of the MHC I molecule. ERAPs trim N-terminal extensions from antigenic precursor, thus resulting in the generation of final pMHC I complex that exit the ER and translocate via the golgi to the cell surface [250].

7.2 Exogenous antigen presentation pathway

Competent antigen presenting cells (APC) are characterised by dendritic cells (DCs), monocytes-macrophages, B cells and thymic epithelium cells, all of which express MHC II molecules. The processing pathway starts with the internalisation mediated endocytosis through lysosomal and endosomal vesicles of protein antigen. In contrast to MHC class I molecules, the MHC II complex mediates a CD4+ T cell response. Briefly, the MHC II are synthesised and folded within the ER and consist in a tethered heterodimeric complex (α1, α2 and β1, β2). α2 and β2 bind the MHC to the membrane, while α1 and β1 domain define the peptide binding groove. Antigenic peptide lengths are longer compared with the MHC I, approximately 12-15 amino acids [143]. Intriguing, the two ends of the peptide binding groove are in an open conformation and have residues that allow longer length peptides to bind (Figure 1.7).

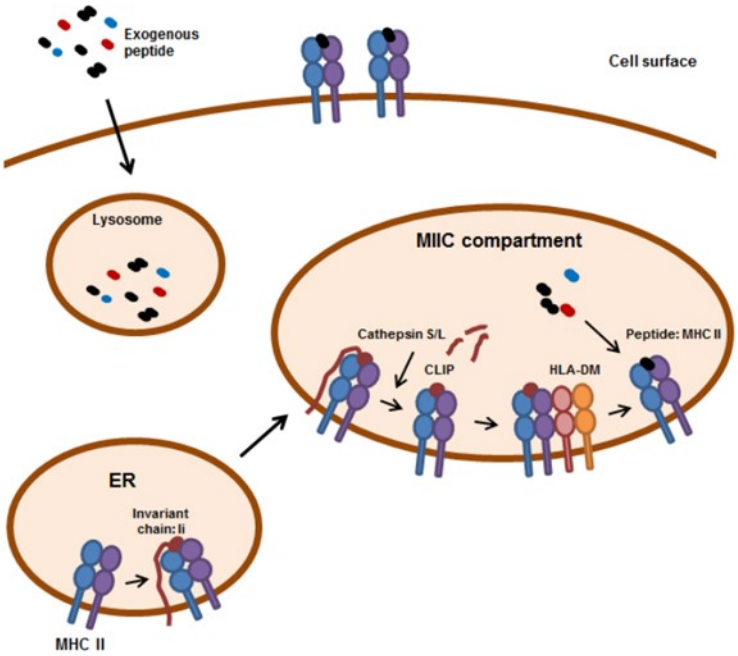


Figure 1.7. Endogenous peptides are internalised into endosomes. Immature α and β chains associate with Invariant chain (Ii) and then degrade, leaving a small fragment bound in the peptide binding grove (CLIP). HLA-DM allows CLIP's dissociation and let the association of the αβ chain with peptide antigen for the final expression at the cell surface. Image from "*Functional consequences of single nucleotide polymorphisms in ERAAP"* [144].

Folded MHC II α and β chains are transported from the ER to lysosomal vesicles via the invariant chain, which binds through occupation of the peptide binding groove. Once in the lysosomal vesicles, the invariant chain is cleaved by a number of peptidases (e.g. cathepsins), resulting in a final CLIP peptide being bound. In MHC II rich vesicles called MIIC, the CLIP peptide is displaced by antigenic peptides, a process facilitated by HLA-DM and HLA-DO molecules [145]. Following the binding of stable exogenously-derived peptide, the peptide bound MHC II molecules translocate to the cell surface for presentation to circulating CD4 + T cells [146].

7.3 Cross-presentation of exogenous antigens

Cross presentation is a combination between the exogenous and endogenous antigen presentation pathways (Figure 1.8). Exogenous peptide antigen can be internalised, processed and then loaded (and consequently expressed) on MHC I. DCs and macrophages, expressing both MHC molecules, have the ability to internalise exogenous antigens and use this particular process of presentation. In the TAP-dependent pathway, exogenous products are internalised, by endocytosis or phagocytosis, transported into the cytosol and degraded by the proteasome [147]. The TAP protein transports them into the ER and they are loaded on to MHC I [148]. In the TAP-independent pathway the internalisation process is as above, but in this case antigens are degraded by endosomal proteases. Rock and Shen reported the presence of MHC I in the endosomes suggesting MHC I trafficking into these compartments allows loading and expression of MHC I, though the mechanism of trafficking is unknown [149]. An alternative pathway has been suggested where phagosomes containing exogenous antigen fuses with the ER allowing loading onto MHC I molecules [150].

In conclusion, understanding cross-presentation mechanisms could be crucial to identify vaccination strategies in infectious diseases and cancer. Interestingly, an enhanced immune protection, mediated by both effector CD4+ and CD8+ T cells, was only obtained when long synthetic peptides were used (compared to short peptides). Cross-presentation by DCs was found to be fundamental to inducing this long lasting T cell stimulation lead to eradication of tumours or infective pathogens [151-152].

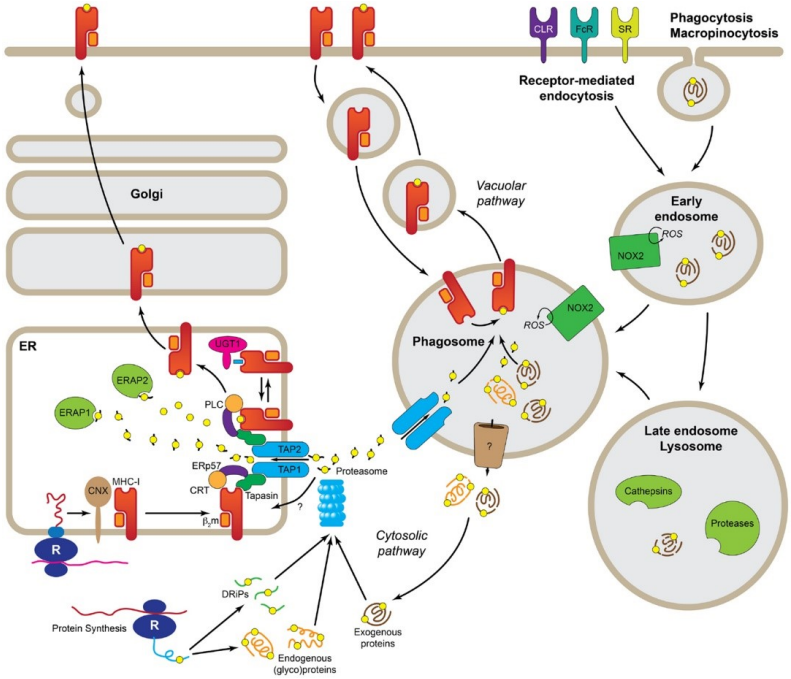


Figure 1.8. Molecular cross-presentation pathways in DCs. Once internalised antigens, they undergo several degradation mechanisms (pH, cathepsins or proteases). At this point, optimal peptides can be directly loaded onto MHC molecules, while peptides that need to be further processed are transported to the cytosol where proteasomal degradation occurs. TAP or different transporters translocate peptides into the ER where they are loaded into MHC-I. Image from "*Understanding the biology of antigen cross-presentation for the design of vaccines against cancer*" [151].

7.4 The MHC class I

The MHC I genes encode polymorphic membrane glycoproteins, which present self or non-self peptides to CD8+T cells. As explained above, HLA proteins are encoded by HLA-A, HLA-B and HLA-C genes and by three less polymorphic genes HLA-E, HLA-F and HLA-G (expressed only in a tissue-restricted area). MHC I has three components: two polypeptide chains, a heavy chain (α-chain) and a smaller invariant light chain β2-microglobulin

(β2m) subunit, and peptide antigen to present (Figure 1.9). The heavy polypeptide chain consists of three domains: α1, α2 and α3. α1-α2 domain folding creates the peptide-binding cleft, which is important in the modulation of the diversity of peptide presented. Consequently, these two domains have shown a highly polymorphic state in order to provide optimal T cell-mediated responses [153-154]. The transmembrane-domain α3 is essential for anchoring at the cell surface and it is non-covalently associated ß2m domain [155]. The invariant β2m is a member of the immunoglobulin family of proteins, but it is not encoded within the MHC gene region. The final component of MHC I is the peptide antigen. A small peptide of 8-10 amino acids bind to the MHC I groove following a non-covalent interaction, which induces conformational changes in this region [156] and defines the antigenic epitope that can be recognised by T cells. Once the TCR of CD8+ T cells recognises and binds a specific MHC I complex, a T cell signalling cascade is initiated and promotes the elimination of abnormal or virus-infected cells. MHC I folding, from an immature to a mature state, occurs within the lumen of the ER and, as other proteins with the same origin, are made for secretory pathways. This folding happens prior to engagement with the peptide loading complex (PLC) that consists of four main components (ERp57, calreticulin, tapasin, and TAP). Through interactions with the PLC, the MHC I molecule is loaded with peptide antigen completing the correct folding leading to the dissociation of the PLC and translocation to the cell surface through the trans-golgi [157-158].

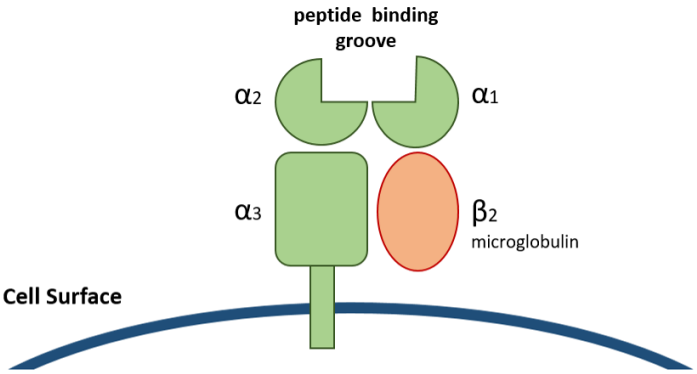


Figure 1.9. MHC class I complex consists of β2m, heavy α chains and peptide. α domains fold to form the peptide binding pocket (between α1 and α2).

7.5 The ubiquitin-proteasome system

The generation of the final component of MHC class I complex, antigenic peptide, starts within the cytosol of the cell, and the proteasome, a multicatalytic protease complex found in the nucleus and cytosol of eukaryotic cells and involved in non-lysosomal protein degradation, plays a central role in this process [159]. Poly-ubiquitinated proteins are targetted for degradation through the ubiquitin-proteasome system (UPS), which is responsible for protein turnover and prevent aggregation of misfolded and unfolded proteins [160]. The 26S proteasome is the main proteolytic enzyme in the UPS system, and consists of a 20S multiple subunit core and 19S caps. The 20S subunit is responsible for the catalytic activity and forms a barrel shape made up of four rings, each containing seven subunits [161]. The two identical outer rings contain seven distinct α subunits (α1-α7) while the two inner rings contain seven ß subunits (β1-β7) that make a central chamber where proteolysis events occur. Among these ß subunits, β1, β2

and β5 have proteolytic activity. The two 19S caps act as regulatory ATPases and their role is to recognise and bind peptide targets with a polyubiquitin chain, with the S6 subunit of 19S cap primarily responsible for the recognition. Upon recognition and binding of the peptides to the 19S-20S complex, ubiquitinated targets are unfolded and then translocated into the core groove where they are cleaved into smaller peptide fragments [162-163].Interestingly, inhibiting proteasomal activity with specific drugs reduces the antigenic peptide supply and almost completely reduces MHC I presentation at the cell surface [164-165]. This further highlights the importance of the proteasome in antigen processing and presentation

IFN-γ stimulation up regulates the expression of the proteasome and induces a change in three β catalytic subunits of the 20S proteasome. Homolog subunits LMP2, LMP7 and MECL1 are incorporated into the proteasome core replacing β1, β2 and β5 respectively [166-167]. This 20S proteasome with alternative active subunits is termed immunoproteasome for its role in the generation of antigenic peptides [168] and *in vitro* studies have shown a more efficiency of immunoproteasome in processing a number of immunogenic peptides for MHC I presentation. Recent *in vivo* studies have shown that the lack of all three subunits, LMP2, LMP7 and MECL1, resulted in an impaired presentation of antigenic peptide epitopes and an overall reduction (~50%) in surface MHC I expression when compared to wild type mice [169]. Furthermore, when assessed *in vitro* using synthetic fluorogenic substrates, the immunoproteasome showed a reduction in cleavage of acidic amino acids and an increase in cleavage after hydrophobic and basic residues, which bind more optimally to MHC I molecules [170]. IFN-γ stimulation can also induce an alternative regulatory subunit: PA28 (or 11S REG). PA28 is a multimeric ring-shaped proteasome activator unit and can bind both ends of the 20S such as 19S cap [171], and appears to influence

the uptake and cleavage of shorter peptides and consequently play an important role in the generation of antigenic peptides [172-173]

Peptides with an optimal length of 8-11 amino acids are a small minority of the proteasome products; usually antigenic peptides generated have a correct hydrophobic C-terminus although they often contain an extended N-terminus and therefore a further processing step is required before stable binding on to an MHC I molecule [174]. This final peptide processing event occurs in the ER by specific proteases in order to generate an optimal N terminusand allow a stable MHC I complex formation.

7.6 Peptide transport across membranes

Transporter associated with Antigen Processing (TAP) protein belongs to the large family of ABC transporters, which utilise Adenosine Tri-Phosphate (ATP) to translocate substrates across membranes [175]. TAP consists of a heterodimer TAP1-TAP2 complex [176]. Both the ATP-hydrolysing subunits, TAP1 and TAP2, contain an ATP-binding cassette and transmembrane domains and are required for antigen translocation and incorporation into the PLC for the loading of peptide [177-178]. The peptide binding process is ATP independent but translocation into the ER requires ATP hydrolysis. TAP mutants that are unable to bind ATP, are also unable to bind the peptide [179]. Interesting is that peptide binding and ATP hydrolysis of TAP are each targeted by several different viral immune-evasion proteins [180]. The translocation of peptides by of TAP can be inhibited by these viral proteins, such as ICP47,a protein encoded from the Herpes simplex virus.. The effect of ICP47 on TAP transport subsequently reduces peptide supply to nascent MHC I, and this inhibition of function correlates with a loss of MHC I expression in malignant cervical carcinoma [181]. It is well characterised that peptide transporters are selective for both length and the N- and C-terminal structure [182]. In humans, a hydrophobic C-terminal is favoured by TAP,

although basic residues can also be accommodated, and the optimal peptides length for translocation into the ER is between 8 and 16 residues. However, despite these length preferences, longer peptide substrates can be transported through TAP, albeit with lower efficiency [183].

TAP associated glycoprotein (tapasin) is a 48kDa glycoprotein that plays an important role in the generation of stable peptide-MHC I complex through its role as a peptide editor. [184]. Tapasin serves as a bridge between MHC I, TAP and calreticulin, however also act as quality controller of peptides [185]. In tapasin deficient cells, a reduction of MHC I expression is observed and correlates with subsequent decrease in stability and ability to present peptide antigens, highlighting the crucial role of tapasin in the optimisation of peptide cargo for stable pMHC I expression [190].

7.7 The peptide-loading complex

As previously discussed, the folding and peptide loading of MHC I molecules occurs in conjunction with the peptide loading complex (PLC). PLC is a multisubunit structure in the ER containing TAP assembled with MHC I, ß2m, tapasin, calrecticulin, ERp57, and possibly protein disulfide isomerase (PDI) [186-187]. Tapasin, ERp57 and PDI are fundamental for TAP stabilisation and control the disulfide bod in the peptide-binding groove of the MHC I heavy chain [188]. PLC dissociation occurs immediately after stable peptide loading and the pMHC I complex is released and transported (via trans-Golgi) to the cell surface. As expected, deficiency or alterations in expression of any of the components of the PLC can result in severe consequences on 1) expression levels, 2) overall stability and 3) quality of the pMHC I repertoire expressed at the cell surface [189].

7.8 N-terminal trimming of peptide precursors

Due to the preferences of TAP in transporting peptides between 8-16 amino acids in length into the ER, and the optimal peptides for stable loading onto

MHC I being 8-11 amino acids in length, further trimming of peptides are often required after proteasome cleavage but before peptides can be loaded on to MHC I molecules [188]. Interestingly, in the cytosol, peptides of >15 residues can be further processed by cytosolic peptidases such as tripeptidyl peptidase II (TPPII), leucine aminopeptidase (LAP) and bleomycin hydrolase (BH). LAP was firstly identified as an IFN-γ inducible cytosolic protease [191] and cells deficient in LAP expression demonstrate that this aminopeptidase can trim peptide precursors, however its activity may not be responsible for the generation of MHC I peptide ligands [192]. BH is a cytosolic cysteine protease, which can influence some antigenic peptide presentation, even if with a largely redundant effect in presence of other aminopeptidases [193]. However, despite the presence of cytosolic proteases, a subset of peptides can directly translocate into the ER lumen by TAP. Within the ER, the N-terminus of these proteins may be further processed by resident aminopeptidases. ERAP1 and ERAP2 in humans, and ERAAP in mice, provide this final ER trimming process in order to create optimal length peptides (8-11 amino acids) to bind and load on MHC I molecules, with potential to result in pMHC I specific NK cell CD8+ T cell responses [137]. The human ER aminopeptidases, in particular ERAP2, is a major focus of this study, and a better characterisation of these will be discussed in more detail below.

8. Endoplasmic Reticulum Aminopeptidases

Proteasome/immunoproteasome-derived peptides are often N-terminally extended upon entering the ER, and consequently ER proteolysis and processing events occur to generate the optimal N-terminal for MHC I binding. In 1995, Elliot et al., identified a proteolysis phenomena within the ER compartment, which suggested that the peptide trimming process is not restricted solely within the cytoplasm [194]. In 2002, two independent groups

identified aminopeptidases responsible for N-terminal peptide trimming: ER aminopeptidase associate with antigen processing (ERAAP) in mice and in ER aminopeptidase (ERAP1) in human. Approximately one year after these initial ERAP1 studies, a closely related ER aminopeptidase (ERAP2) in humans was discovered. [136; 139; 195]. ERAP proteolytic activity has been shown to depend on the length of the peptide substrate, with studies revealing that upon incubation with shorter 8mer peptides, the trimming activity of ERAP1 ceased, when compared to incubation with peptides longer in length. This substrate length dependency of ERAP is related to the fact that peptides of 8-9 amino acids confer a higher affinity of MHC I binding [138]. Importantly, ERAP aminopeptidase activity cannot be compensated by any other aminopeptidases within the ER, revealing the exclusive role of ERAP in production of MHC I ligands [196].

Mouse ERAAP and human ERAP1 and ERAP2 are members of zinc metalloprotease family and belong to oxytocinase M1 subfamily that share consensus zinc-binding motif within the active site that is fundamental for enzymatic activity [215]. These aminopeptidases are normally expressed in a variety of tissues, preferentially where there is a high expression of MHC I molecules and are strongly induced after stimulation with IFNγ and TNFα [195]. After IFNγ treatment, an increase in N terminal aminopeptidase activity was seen in comparison to limited antigen processing in the absence of stimulus [197].

Mouse ERAAP and human ERAP1 share 86% homology, most conservation is seen in the active site regions [198]. Interestingly, ERAP2 has no equivalents in rodents, and evolution studies suggest a relatively recent duplication origin from ERAP1 [119]. ERAP primarily resides within the lumen of the ER, where they carry out their main function of peptide trimmers, and have been shown to localise with proteins containing the KDEL ER retention

motif [199]. In addition, the involvement of ERAP1 in shedding of cytokine receptors, and also in the regulation of innate immune and inflammatory responses has been observed [200]; conversely, there are no reports of ERAP2 participation in cytokine receptor shedding. Other cellular and regulatory functions of ERAP1 and ERAP2 include promotion of angiogenesis and blood pressure regulation through their role in cleavage of bioactive peptide hormones in the renin-angiotensin system [201]. The nomenclature ERAP1 and ERAP2 have been approved by the Human Genome Organization Nomenclature Committee, however ERAP1 is also known as adipocyte-derived leucine aminopeptidase (A-LAP), puromycininsensitive leucine-specific aminopeptidase (PILS-AP), or aminopeptidase regulating type I TNF receptor (TNFR1) shedding (ARTS-1), whereas ERAP2 is known as leukocyte-derived arginine aminopeptidase (L-RAP). These alternative names relate to the different proposed biological functions of ERAP1/2.

8.1 ERAP1 structure and its variants

The ERAP1 gene is located on the long arm of chromosome 5q15 and the derived protein consists of 941 amino acids, with a molecular weight of 106kDa. Among the 20 exons spanning 47 kilo-bases, exon 6 contains the active site region of the protein which includes the zinc metalloprotease catalytic motif HEXXH(X)18E, and a 5 amino acid GAMEN motif, which is responsible of enzymatic function [199; 202]. Moreover, differences in the exon 20 sequence can generate two isoforms of ERAP1: a longer isoform a (ERAP1a) and the shorter isoform b (ERAP1-b); this latter has found to be more abundant in several cells [203].

The crystal structure of ERAP1 shows an open conformation in absence of peptide, revealing a large internal cavity where GAMEN and HEXXH(X)18E motifs are present. A closed conformation of ERAP1 has also been described

when bound with the protease inhibitor bestatin, however currently there are no crystal structures that include full length peptide binding to ERAP1. Based on these observations, a plausible mechanism of activity has been hypothesized, in which ERAP1 is peptide receptive in the 'open' conformation and when bound with the substrate, leads to a conformational alteration and results in the 'closed' conformation that results in catalytic enzyme activation and trimming [204-205].

Genetic variation in the coding region of ERAP1 can affect the biological functions. A number of SNPs have been identified and that are associated with disease and these resultant amino acid changes are located throughout the protein; rs17482078 (R725Q) and rs27044 (Q730E) located on inner surface of the C-terminal cavity and rs2287987 (M349V) can influence substrate sequence or length specificity [144]. Polymorphisms rs27044 (R725Q), rs30187 (K528R), rs26653 (R127P) and rs10050860 (D575N) conduct to a conformational change that could indirectly affect both specificity and enzymatic activity. In particular, using synthetic peptide substrate and antigen precursor peptides, rs30187 and rs27044 have shown a significant reduction in aminopeptidase activity [206].

8.2 ERAP2 structure and its variants

The ERAP2 gene is located on chromosome 5q15 between ERAP1 and leucyl-cystinyl aminopeptidase (LNPEP). It contains 19 exons and the two highly conserved motifs, the zinc binding site HEXXH and GAMEN motif, are encoded by exon 6, while essential E residue is encoded by exon 7. ERAP2 shows a 49% of homology with ERAAP and ERAP1, and a 40% of homology with placental leucine aminopeptidase (P-LAP). The resulting 960 amino acid ERAP2 protein has a significantly hydrophobic N terminal region, similar to ERAP1, and preferentially cleave basic N terminal residues (arginine and lysine), however it shows no preference for peptides with hydrophobic C-

termini. Crystal structure analyses display the same domain organisation of ERAP2 in comparison with ERAP1, with a central internal cavity where trimming processes occur [207]. Interesting ERAP2 has been shown to form a homodimer or heterodimer complex with ERAP1. Recently studies have demonstrated that this dimerization of ERAP1 and ERAP2 creates complex with a higher and more efficient peptide-trimming activity, resulting in an increase in the number of peptides trimmed [208].

ERAP2 gene has been target of long-standing balancing selection [120]. This process has maintained two highly differentiated ERAP2 haplotypes at intermediate frequency in most human populations [121]. The two haplotypes, Hap A and Hap B, differ at multiple alleles in tight linkage disequilibrium. As explained above, predicted protein product of Hap B-derived transcripts is a truncated protein of 534 amino acids (ERAP2-AS), whereas the canonical ERAP2 protein, consisting of 960 amino acids, is encoded by a full-length mRNA (ERAP2-FL). The truncated version, also termed L-RAP, deems to be non-functional and it has been showed a lack of aminopeptidase activity when tested using various aminoacyl-MCAs [195].

The biological significance of the ERAP2-AS mRNA is currently unknown. Indeed, it is possible that ERAP2-AS mRNA is be translated into a ERAP2-alternative spliced truncated protein of 60kDa, or alternatively degraded by non-sense mediated decay (NMD). For rs2248374 allele, A/G heterozygous and A/A homozygous haplotypes results in the expression of a 120KDa ERAP2-full length protein, detectable by western blot. Conversely a G/G homozygous haplotype should result in the production of ERAP2-AS protein, which resulted in a reduction in surface MHC class I expression when observed in human lymphoblastoid cell lines [120] (Figure 1.10).

Since the frequency of the HapA was found to be higher in HESNs compared to the word population, it was hypothesized that this ERAP2 variant confer

natural resistance to HIV infection by qualitatively and/or quantitatively altering the peptide repertoire presented at the cell surface.

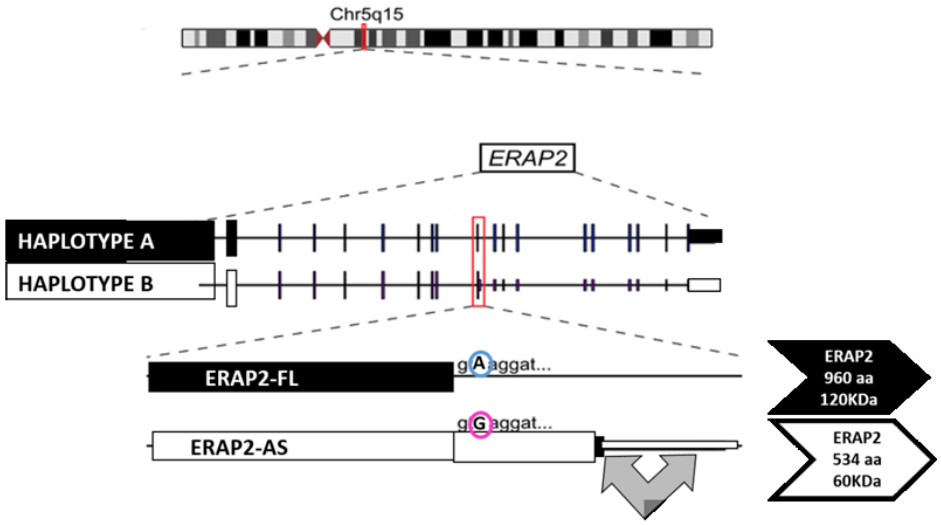


Figure 1.10. The genomic organization of ERAP2 gene. The two spliced forms of ERAP2 are shown for Haplotype A (black) and Haplotype B (white). The different alleles of rs2248374 are indicated with a blue or purple circle in base position, respectively. Arrows indicate the premature stop- codons in the Haplotype B mRNA ERAP2-AS.

8.3 Trimming activity of ERAP1 and ERAP2

ERAP1 is crucial in trimming 9–16 amino acids peptides, which are transported into ER by TAP, to their optimal length of 8-9 amino acids [209]. Studies using ERAAP deficient mice have highlighted the contribution of aminopeptidase in generation of peptides presented on MHC class I molecules. In *in vivo* experiments using ERAAP deficient mice, the levels of cell surface MHC I, H-2Kb and H-2Db, are reduced and correlate with a decrease in specific CD8+ CTL responses [210]. Notably and as expected, MHC class II levels remained unaffected. Moreover, when cell surface

expression of all 5 MHC I molecules, H-2Kk, -Kb, -Db, -Dd, -Ld were assessed, they showed a significant reduction in surface expression in ERAAP deficient mice compared to wild type. Furthermore, immunization of ERAAP deficient mice with wild-type cells resulted in a potent increase of CD8+ T cell responses, highlighting the significant contributions of ERAAP in determining the peptide-MHC (pMHC) class I repertoire in both a quantitative and qualitative manner [211]. Of note, ERAP1 activity is reduced towards peptides with either the presence of proline in position 2 (X-P-Xn) [152], and/or with less than 8- amino acids length which is the optimal length for binding to MHC I molecules [209]. However, a preference in catalytic activity towards lysine, leucine, asparagine and tyrosine residues has showed [136; 139; 199; 211]. ERAP1 presents a marked preference in trimming peptides with large hydrophobic C-terminal amino acids [209]. Despite this, the enzyme activity was also shown to be affected by the internal residue structure/sequence of peptides; Amino acid positions 2, 5 and 7 were shown to be important for the peptide sensitivity of ERAP1 processing [206]. Based on these observations, Chang et al., proposed a "molecular ruler mechanism" for the hypothesis of trimming activity in which ERAP1 binds substrates with a higher affinity hydrophobic C-terminus within the hydrophobic pocket, and the active site accommodates the N-terminal extended region, trimming the precursor in order to obtain optimal length. In support of this model, studies using recombinant ERAP1 and a selection of synthetic peptides, show a substrate length preference of ERAP1.

The closely related ERAP2 shows a strong preference for the basic arginine and lysine residues located at the N terminal region of the peptide. ERAP2 is present an overall domain I-IV organization (figure 1.11), in which domain IV closes into the proximity of domain II, the catalytic site, and the S1 pocket. This S1 pocket is the specific region where N-terminal peptide will be accommodated and cleaved. This domain organisation creates an internal

cavity where trim-function takes place within the active site. Residues 54−271 (N-terminal domain I) exhibit a β-sandwich structure, residues 272−546 (Domain II) have a thermolysin α/β-fold and contain the high conservative motifs, HExxHx18E and GAMEN. Residues 547−647, referred to domain III, present also as a β-sandwich conformation that acts as bridge between domains II- IV. In the end, domain IV (residues 648−960), an all α-helical domain interacting closely with domain II, caps the Zn (II) catalytic site (Fig. 1.11). Interactions between II and IV domains create large internal cavity, close to the catalytic site, which has almost no access to the external solvent but exhibits numerous water molecules trapped inside [206]. Crystal structures show a different distribution of electrostatic potential in the cavity compared to ERAP1, suggesting distinct selective pressures in the antigenic peptide repertoire mediated by ERAP2. Asparagine at position 198 is important in the selectivity for the N-terminal specificity.

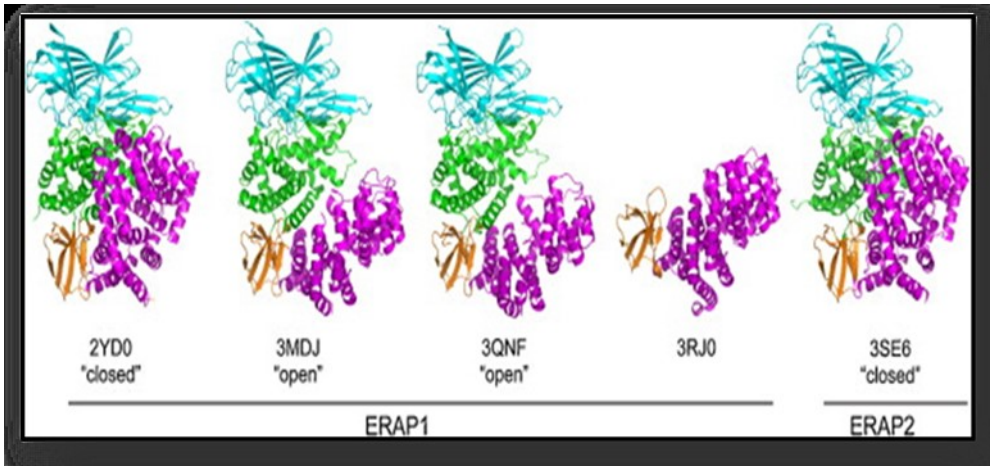


Figure 1.11. Representations of ERAP1 (left) and ERAP2 (right) crystal structures. Cyan shows domain I, green shows domain II, orange refers to domain III in and domain IV is in magenta. Note the conformation of domain IV to domains I and II into a “open” versus the “closed” conformations. Image from “Antigenic peptide trimming by ER aminopeptidases Insights from structural studies” [119].

8.4 Localisation/secretion of ERAP1 and ERAP2

There is a strong body of evidence highlighting that ERAP1 and ERAP2 play a fundamental role in ER proteolysis and their subsequent ER localisation. However, the role of these aminopeptidases in other biological processes means that the overall subcellular localization of the two aminopeptidases remains debatable [136]. A cytosolic localisation of ERAP1 has been previously described by several studies [205]. Moreover, its role in cytokine shedding at the cell surface and binding the specific receptors (IL-1RII, IL-6R and TNFR1), suggests a plausible ERAP1 translocation from its canonical (and described) subcellular localization in the ER. However, this may depend on the cell type or changes in the cellular environment (stress response). In support of this idea, Goto et al., reported the secretion of ERAP1 after LPS/IFN-γ stimulation in a dose-dependent manner [212]. Interestingly, in a macrophage model (RAW264.7 cells) the secretion seems to be mediated through the TLR signalling pathway by induction of pro-inflammatory cytokines IFN-β and TNF-α [213]. The authors have suggested a Ca^{2+} mediated mechanism for the secretion, in which cytokines increase cytoplasmic Ca^{2+} levels, which ultimately led to the induction of ERAP1 secretion through calmodulin activation. All these data were obtained using specific inhibitor molecules such as BAPTA-AM and the calmodulin inhibitor W7. It is plausible that this localisation change of ERAP1 upon stimulation of cells with cytokines may result in its multifunctional properties within the cell [213]

In early 2018, information regarding the extracellular localisation of ERAP2 was highlighted. A multidisciplinary integrated analysis revealed an overexpression of ERAP2, along with others candidate genes, in papillary

thyroid carcinoma (PTC) and the cystic fluids counterpart, suggesting a possible property of change localisation also for ERAP2 [214].

9. Alteration of ERAP function in human diseases

ERAP1 and ERAP2 are identified to be highly polymorphic aminopeptidases with numerous polymorphisms associated with the pathogenesis of diseases such as systemic arterial hypertension, diabetes mellitus,cervical carcinoma, preeclampsia, psoriasis and ankylosing spondylitis [215-217]. SNPs within these genes may result in an altered ERAP1 / ERAP2 function which can lead to an altered presentation process of pathogen-derived peptides, leading to an inadequate immune responses and disease development.

9.1 Hypertension

The Lys528Arg (rs30187) polymorphism in ERAP1 has shown a strong association with a reduction in bone mass density in a premenopausal Japanese women cohort. Moreover, a screening-study for 33 polymorphisms within ERAP1 gene, has related K528R SNP to an essential hypertension condition, causing by a hypothesised reduction in bradykinin formation and/or a lower angiotensin II inactivation [215;220]. In support of the latter, functional studies assessed K528R mutant activity using L-AMC substrate, and revealed a decrease in enzymatic activity. Collectively, a 60% reduction in the cleavage of both angiotensin II and kallidin hormones into angiotensin III and bradykinin respectively, supports the idea of the essential hypertension is due to a reduced ERAP1 enzymatic activity due to the presence of K528R [221].

Hypertension is associated with a number of morphological changes. Among these, left ventricular hypertrophy, during anti-hypertensive treatment in hypertension patients, has been associated with the K528R variant of ERAP1 [222]. Furthermore, a genetic link has been observed between

altered ERAP2 expression in first trimester placentas of pregnant women and development of pre-clampsia, which is a heritable pregnancy disorder characterised by new-onset hypertension and proteinuria. This association with ERAP1 expression and pre-eclampsia has been confirmed a Norwegian and Australian cohort study [218; 223], and more recently, a genetic variant (rs2549782) of ERAP2 has been found to be associated with an increased risk of preeclampsia [224].

9.2 Bacterial and Viral infections

ERAP1 plays an important role in immune response to viruses. Studies in ERAAP deficient mice have shown marked differences in specific lymphocytic choriomeningitis virus (LCMV) CD8+ T cell responses when compared to wild type mice. In addition, the normal hierarchy of CD8+ Tcell responses observed in wild type mice appears to be markedly impaired in ERAAP$^{-/-}$ mice [225]. The ability of virus to escape from immune detection has been observed, in 2004 Draenert et al., first shown that in HLA-B57+ HIV infected individuals, a mutation from alanine to proline at position 146 of HIV Gag protein alters antigen processing mediated by ERAP1 [226]. The variant, positioned immediately before the NH_2 terminus of a dominant HLA-B57 restricted CTL epitope, prevents the N-terminal end cleavage by ERAP1. Interestingly, variants in ERAP2 have been shown to confer resistance to HIV-1 infection, possibly through an indirect modulation of peptide repertoire to CD8+ T cells [121]. Intriguing, ERAP1 is a host target of human cytomegalo virus (HCMV) microRNA miR-US4–1 as result of miRNA-based immunoevasion strategy. Interfering with ERAP1, miR-US4–1 influences the HCMV-derived supply and result in a immunoevasion process [203].

The most significant risk factor in the pathogenesis of cervical carcinoma is the infection with the human papilloma virus (HPV). HPV infects the

epidermis and mucous membrane, which consequently causes alterations within the cells that leads to cervical neoplasms. SNPs in the ERAP1 gene have been described in different cervical carcinoma cases. SNP at 56 and 127 position give rise to a significant increase in risk of cancer and a decrease in overall survival [217]. Loss function studies show ERAP1 as a predictor of survival in cervical carcinoma patients [227]. It is plausible that polymorphisms within ERAP1, which alternate enzyme activity or specificity and consequently peptide generation, facilitate or not the disease progression modulating immune response.

9.3 Autoimmune diseases

Fierbracci et al., have demonstrated though GWAS the involvement of ERAP1 and ERAP2 with the susceptibity to different autoimmune diseases and their linkage with particular MHC I alleles [228]. An example is the link, demonstrated by different independently replicated studies, between ankylosing spondylitis (AS), an autoimmune inflammatory disorder, and ERAP1 genetic variants [229-230].

The variant K528 has been found in a cohort of HLA-B27-positive AS patients and it seemed to have faster trim-activity rate of peptide precursors then wild type variant. It suggests an aberrant peptide trimming mediated by ERAP1 that leads to an impaired peptide presentation mediated by HLA-B27 as causes in the AS pathogenesis [231]. Moreover, the mutant variant seems to increase susceptibility to multiple sclerosis (MS) based on an Italian population study [232]. In a recent genome wide meta-analysis study, ERAP2 has been identified to be linked with a risk of developing Crohn's disease [233].

9.4 Cancer

As well as screening for SNPs associated with essential hypertension, Mehta et al., has verified 13 SNPs within TAP transporters, LMP2, LMP7 and ERAP

genes and has described polymorphisms in ERAP1 associated with an increased risk in cervical carcinoma development [216; 234].

The ERAP1 and ERAP2 expression in several tumour cell lines (such as melanomas, leukemia-lymphomas and carcinomas of breast, colon, lung, chorion, skin, prostate, cervix, kidney and bladde) reveals a variation in protein expression (down and up regulated) independently from each other [235-236]. Following this, Fruci et al., analysed 150 neoplastic lesions and found an altered expression of both aminopeptidases (lost, acquired or retained) compared to the normal sample [237]. As the expression of ERAP1 and MHC I molecules are related, as expected, the altered expression of ERAP1 results in abnormal cell surface expression of MHC I molecules in tumour cell lines [236]. An increase in expression in ERAP1 and/or ERAP2 proteins has been described in thyroid and colon carcinomas, while a downregulation of the same genes has been detected in lung, ovary and breast carcinomas [237]. Losses and gains of ERAP1 and ERAP2 protein expression were identified in RCC lesions with an altered frequency in different renal tumour types [263]. Altered ERAP1 expression has been detected in 64% of endometrial carcinomas and, interestingly, this correlated with CA-125 levels, suggesting an involvement of the enzyme in tumour progression [238]. In addition, analysis of ERAP1 and ERAP2 expression in malignant cells has demonstrated that the levels of these enzymes undergo significant changes and may correlate with the ability of cancer cells to evade immune responses modulating antigen processing. Thereby ERAP modulation might be a novel tool that can guide the development of innovative approaches to cancer immunotherapy. Interfering with ERAP1 expression leads to tumour rejection in syngeneic animals by activation NK cells, and subsequently T cell-mediated cytolysis. This rejection was mainly due to NK cell response and is dependant upon the MHC I peptides presented [239-240].

Recently, a correlation between tumor suppressor factor p53 and ERAP1 has been highlighted, suggesting that p53 may be an activator of ERAP1 and it could be able to modulate MHC I molecule expression [241], thereby playing a crucial role in the regulation of immune responses, specifically in several aspects of the cytotoxic T-lymphocyte (CTL)-tumor cell interaction [242].

9.5 Cytokine Receptor Shedding

In addition to the role in peptide processing, an involvement of ERAP1 has been suggested in the facilitation of interleukin receptor shedding from the cell surface, resulting in the soluble form of these receptors. Firstly, Cui et al., demonstrated a potential interaction between the extracellular domain of the TNFR1 and ERAP, giving rise to the formation of a TNFR1/ERAP1 complex. In this context, ERAP1 is referred as aminopeptidase regulator TNFR shedding 1 (ARTS1) [198]. Overexpressed ERAP1 correlates with an increase in soluble TNFR1, which competes with cell surface TNF receptors, obtaining an attenuation of TNFα bioactivity when the levels are elevated and a rescue of TNFα when the levels have decreased. The data obtained suggest an indirect mechanism mediated by ERAP1 in cytokine receptor cleavage; this is more consistent due to supporting evidences that suggests an involvement of ERAP1 in the recruitment of other enzymes that act as endopeptidases or sheddases [200; 243]. In both human and mouse cell systems the role of ERAP in IL-6R shedding has been described. Subsequently, the same group demonstrated the role of the enzyme in type II IL-1 decoy receptor (IL-1RII) cleavage [200;244].

There are data on ERAP1 variant that can modify the ability of ERAP in mechanism of facilitating cytokine receptor cleavage. E320A mutant showed an abrogation in facilitate IL-6R shedding, as well R725Q and D575N

variants [157]. However, the ability for the SNP variant ERAP1 molecules to alter IL-6R ectodomain cleavage is still unclear.

10. Non-immunological function of ERAP

ERAP1 controls left ventricular mass through the renin-angiotensin system, essential for regulation of blood pressure [246]. This physiological system starts with the generation of angiotensin I, derived from cleavage of angiotensinogen. Successively angiotensin I is converted to angiotensin II by angiotensin converting enzyme (ACE). This last product can control blood pressure by modulation of hormone release [222]. Hattori et al., has demonstrated an efficient ERAP1 cleavage activity of angiotensin II to angiotensin III and IV [245], while Tanioka et al., highlight the ability of ERAP2 to cleave angiotensin III to angiotensin IV [195]. Notably, in the same study it was demonstrated that both enzymes can convert kallidin to bradykinin.

ERAP1 has been reported to control post-natal physiological process involving in growth of new blood vessels from pre-existing microvessels, referred to neo-angiogenesis. It occurs because ERAP1 seems to be involved in regulation of proliferation and migration events in endothelial cells (EC) [246]. Suppression of ERAP1 expression in this EC model inhibited VEGF-stimulated proliferation, migration, vessel network formation in vitro, and angiogenesis in vivo. It has been showed that ERAP1 can modulate VEGF stimulated G1-S transition during EC cell proliferation and it occurs binding phosphatidylinositol-dependent kinase 1 (PDK1). The resulting complex ERAP1-PDK1-S6 kinase activates cyclin-dependent kinase (CDK) 4/6 leading to G1/S-phase transition in the cell cycle and EC proliferation [247].

AIMS

Immune response during HIV-1 infection and replication is quite complex because many viral and host factors, beyond individual variables, are involved at different levels [85]. Many efforts have been made to evaluate the influence of these factors on HIV-1 infection and disease progression, mainly focusing on the identification of genetic variants that can play a role in HIV-1 infection susceptibility.

ERAP1 and ERAP2 enzymes are involved in antigen presentation, trimming the amino-terminus of peptide precursors to the correct size for binding to MHC class I molecules. Haplotype-specific alternative splicing of the ERAP2 gene results in either a spliced (Hap B) or full-length (Hap A) mRNA being expressed. Interestingly, the frequency of the latter is higher in HIV-Exposed Sero-Negative (HESNs) individuals [121] suggesting that Hap A is involved in HIV-infection resistance [122].

Based on these premises, the present study strive for deepening the correlation between ERAP2 haplotypes and HIV-susceptibility and investigating the molecular mechanism by which Hap A and Hap B control peptide production for antigen presentation mechanisms and the subsequent CD8 mediated immune response.

In particular, primary aims of this study are:

- To evaluate the frequency of single and combined SNPs within four immune genes (APOBEC3H, TLR3, ERAP2 and MX2), known to be “resistance genes” in viral context, in HIV-positive patients receiving ART.
- To identify a possible correlation between the frequency of single and/or combined previously mentioned polymorphisms (“supergenotype”) and the course of infection in HIV-positive patients

receiving ART, using the viral load slope and CD4/CD8 cell count as indexes of response to therapy.

- To confirm and better characterize the correlation between ERAP2 and HIV-1 susceptibility, by adopting specific experimental conditions (recombinant ERAP2 protein and ERAP2 inhibitor addition to cell culture).
- To investigate the trimming phenotype of the two ERAP2 haplotypes and to assess their impact on the peptide repertoire and related CD8+ T cell immune response.

MATERIALS AND METHODS

1. Role of ERAP2 variants in HIV1-infected ART-treated patients (ICONA Cohort)

1.1 Sample collection

300 HIV-infected patients from the ICONA cohort undergoing a first ART regimen were enrolled in the study. Blood samples were collected from each patient in EDTA Vacutainer® tubes every 6 and 12 months. Immediately after collection, peripheral blood mononuclear cells (PBMCs) were isolated and stored at -80°C. Successively cells were transferred to the laboratories of the University of Milan for genotyping analyses. Periodically (6 months and 1 year) HIV-infected patients' clinical parameters (genotype, gender, CD4 cell count, viral load, therapies, year of infection, clinical evolution) were analyzed and inserted into a predisposed database (NetCare, Healthware Technology s.r.l., Salerno). The study was designed and conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the hospitals involved in the study. After thoroughly explaining the project and clarifying any doubt concerning the research, all subjects signed an informed consent and the biological material collected was anonymized to ensure the privacy of each individual.

1.2 Genotyping analyses

DNA was extracted by the Maxwell® RSC Instrument automated following a nucleic acid purification method (Promega, Madison, Wisconsin, USA) associated with Maxwell® RSC Blood DNA Kit Technical Manual (Promega, Madison, Wisconsin, USA). Genomic DNA was used as template for PCR amplification using TaqMan probes specifically designed to perform a SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). The amplification and fluorescence reading were carried out using a Real-time PCR instrument (CFX Real Time, Biorad). The determination of the allele presence in each sample was done by a specific software (CFX

Manager Software, Biorad), which assesses the fluorescent signals measured during the plate reading. The list of the gene variants analysed by this strategy and the code of the TaqMan assay used are listed in the table below:

Gene	Code Variant	Assay ID
ERAP2	rs2549782	C__3282749_20
TLR3	rs3775291	C__1731425_10
APOBEC3H	rs1389297	C__7541955_10
	rs1389299	C__2243031_10
MX2	rs2074560	AN9HJGJ

Table 3.1 List of genetic variants analysed and correspondent code. *MX2 probe is a custom TaqMan SNP genotyping assay.

Moreover, genomic DNA was used to detect polymorphic 32 bases pair deletion at the CCR5 locus using PCR specific custom primers.

1.3 Statistical analyses

The allelic discrimination method was used to determine the frequency of each genetic variant (SNP). The main characteristics of the study population at the time of ART initiation were described and tabulated. Separate models were employed to evaluate the association between the detection of the genetic variants and HIV-RNA, CD4 count and CD8 count (as well as their ratio) responses to ART. For the HIV-RNA response, the time to achieving a value ≤50 copies/mL after ART initiation was evaluated by means of standard survival analysis and Kaplan-Meier curves. The date of the first HIV-RNA value ≤50 copies/mL after ART initiation (a single value) was used to define the time of achievement of viral suppression. Participants' follow-up was censored at the date of their last available viral load. For each gene and all four endpoints (HIV-RNA, CD4, CD8 and CD4/CD8 ratio) a linear mixed

model was used to compare intercept and slopes according to variants detection. Variability between patients was controlled using random intercepts and slopes. Viral load was fitted in the log10 scale to achieve distribution symmetry. Besides testing the associations with each gene variant, also a genotypic score was constructed by allocating 1 point every time a variant was detected and summing all points per participants (i.e. a patient in which ERAP2 rs2549782 G/G was detected as well as rs3775291 C/T or T/T for TL3 and none of the others would have a value of 2 for the score, etc.). In detail, participants were grouped and compared according to whether they had a score >3 vs. 0-1. The score is referred as 'supergenotype' throughout the results of this chapter. All models were fitted on the whole dataset of 300 participants and after restricting to 255 participants of Caucasian origins.

2. Role of ERAP2 *in vitro* HIV-1 infection

2.1 Sample collection

Blood samples were collected from 30 HIV-1 seronegative Italian healthy controls of Caucasian origin by venepuncture in Vacutainer tubes containing EDTA (Ethylene diamine tetra acetic acid) (BD Vacutainer, San Diego, CA). All subjects provided written informed consent to participate in this study.

2.1.1 Isolation of PBMCs

The separation of PBMCs by whole blood was made on lymphocyte separation medium (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA). Briefly, after centrifugation on a Ficoll discontinuous density gradient the sample was separated in the following layers from top to bottom: plasma and platelets, PBMCs, Ficoll and red blood cells, covered by a granulocyte layer. The PBMCs layer was carefully removed from the tube and transferred to a new one. After washing with phosphate buffered saline (PBS) buffer, cell

number and cellular vitality were determined by ADAM MC Automated Mammalian Cell Counter (NanoEnTek Inc).

2.2 Genotyping

Automated DNA Purification was performed by Maxwell® RSC Whole Blood DNA Kit according to the manufacturer instruction (Promega, Madison, Wisconsin, USA). Genomic DNA was used as template for PCR amplification using TaqMan specific probes for rs2549782 ERAP2 SNP (G/T) (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, California, USA). Allele analyse was performed by allelic discrimination PCR method on a CFX BIORAD Real Time instrument (Biorad)

2.3 Cell cultures and in vitro HIV-infection

After isolation, PBMCs were cultured at 37°C and 5% CO_2 in RPMI 1640 (Euroclone) supplemented with 20% Fetal Bovine Serum (FBS). $2x10^6$ PBMCs isolated from 15 GG (Hap A) and 15 TT (Hap B) healthy donors were cultured in RPMI 1640 containing FBS (20%) with or without 100ng/mL of recombinant (rh) ERAP2 (R&D) or with or without 100 nM/mL of ERAP2 inhibitor (DG013A) [248]. After 3 hours, $0.5ng/1x10^6$ cells HIV-1_{Ba-L} virus was added to each well and incubated for 24 hours at 37°C and 5% CO_2. Cells were then washed and re-suspended in medium containing IL-2 (15ng/ml)(R&D systems, Minneapolis, Minnesota, USA), RPMI 1640 containing 20% FBS with/without 100 ng/mL of recombinant ERAP2 FL (R&D) or with/ without 100 nM/mL of ERAP2 inhibitor (DG013A) and incubated at 37°C and 5% CO_2. Every two days cells were supplemented with IL-2, recombinant ERAP2 FL or ERAP2 inhibitor. Six days post-infection $0.5x10^6$ PBMCs were analyzed by FACS for CD8+ T cells mediated response.

2.4 p24 ELISA

As index of HIV infection, an HIV-1 p24 Elisa assay kit (XpressBio, Frederick, MD, USA) was used to measure viral p24 in PBMC culture supernatants after 6 days of infection according to the manufacturer's protocol. Not in detail, after plating samples directly into the monoclonal antibody-coated microplate wells, the captured antigen is complexed with biotinylated polyclonal antibody to HIV-1 p24, followed by a streptavidin-HRP (horseradish peroxidase) conjugate. The detection of the resulting complex is been done adding ortho- phenylenediamine-HCl (OPD) which produces a yellow colour directly proportional to the amount of HIV-1 p24 captured. Plates were read at 450 nm, using the IMark microplate reader equipped with Microplate Manager® 6 software (Biorad, Hercules, CA, USA). The absorbance of each microplate well was calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cut-off factor were considered initially reactive and were retested in duplicate to determine whether the reactivity was reproducible. The concentration of 1.7 pg/mL is the assay detection's limit.

2.5 RNA extraction, DNAse treatment and retrotranscription

RNA was extracted from basal and cultured PBMCs, using the acid guanidium thiocyanate–phenol–chloroform method. After RNAzol addition samples were centrifuged at 12,000g for 15 minutes at 4°C and aqueous phase, containing RNA, was collected. After isopropanol precipitation and 75% Ethanol precipitation, pure RNA was extracted and quantified with Nanodrop 2000 Spectrophotometer (Thermo Scientific). RNA in RNase-free water was purified from genomic DNA with TURBO DNAse (Applied Biosystems). One Unit of DNasi and TURBO DNasi buffer was used every 1µg and the result reaction mix was incubated at 37°C for 30 minutes. After inactivation with DNase inactivation reagent (Applied Biosystems), 1 µg RNA was reverse transcribed following the manufacturer instructions in a 20-µl

final volume by adding 1 μM random hexanucleotide primers and 1 μM oligo. The reaction was heated at 70 °C for 5 minutes. Immediately after, 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) and incubated at 42 °C for 60 minutes. Five minutes incubation at 95 °C was used to inactivate the RT.

2.6 Real Time PCR

cDNA quantification for ERAP1, TAP1 and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, MN). Reactions were performed using a SYBR Green PCR mix (Finnzymes, Espoo, Finland). This fluorogenic reagent emits a strong fluorescent signal upon binding to double-stranded DNA. The thermal profile used was: 15 minutes at 95°C, followed by 40 cycles of 15 sec at 95 °C, 1 min at 62 °C and 20 seconds at 72 °C. By recording the amount of fluorescence emission at each cycle, the PCR reaction was monitored and melting curve analysis for amplicon identification was performed. Threshold line was set above baseline activity. The Ct parameter (Threshold Cycle) is defined as the cycle number at which the fluorescence signal passes the fixed threshold. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct value. Ct values ≥ 35 were excluded from the analysis. Results were expressed as ΔΔCt and presented as ratio between the target gene and the GAPDH housekeeping mRNA. All the samples were analysed in triplicate. Table 3.2 shows list of primers used.

Gene	Primers	Sequence
GAPDH	5'	CGGATTTGGTCGTATTGGG
	3'	GCTTCCCGTTCTCAGCCTTG
TAP1	5'	GCTGCCCACCAATGTAGAGGA
	3'	GGCGAAGCCCAGAAGTTTAGG
ERAP1	5'	TCCAAACCAGCGGAAACCC
	3'	CTGCTCCACAAGTCATCATTC

Table 3.2 List of primers used to assess the expression of genes TAP1, ERAP1 and GAPDH. This latter housekeeping is used as a reference for assessing the increases.

2.7 Cytofluorimetric analysis

Flow cytometric analysis was performed at 0 (basal), 4 and 7 days post *in vitro* HIV-1 infection, staining $0.25x10^6$ PBMCs for 15 minutes with fluorescent directly labelled antibodies: HLA-ABC FITC, CD8 Pecy7, granzymes PE, perforin APC. (Beckman Coulter, CA, USA). As negative control, we used cells stained with the appropriate isotype-matched Ig. After staining, cells were fixed in 1% paraformaldehyde (PFA, Sigma-Aldrich). The expression was detected by mean intensity fluorescence (MFI). Analyses were carried out blindly with respect to genotypes. Cytometric analysis was performed using a FC500 flow cytometer (Beckman-Coulter CA, USA). Flow data were analysed by first gating on the leukocyte population as defined by forward and side light scatters and then on CD8+ expression.

2.8 Statistical analysis

Statistical analysis were performed using GraphPad Prism analysis software. Differences between the groups were assessed using nonparametric analyses (Mann–Whitney U test). All P values are two-tailed.

3. Trimming function of ERAP2 variants

3.1 ERAP2 DNA construct generation

3.1.1 Site Directed Mutagenesis (SDM)

To generate ERAP2-pcDNA3 DNA constructs with two different stop codons in frame, primers were designed for site directed mutagenesis (SDM), which utilises a PCR reaction to incorporate a single base change into the template DNA. The forward (5') and reverse (3') primers for both STOP1 (containing stop codon: TAA) and STOP2 (containing stop codon: TAG) consist of approximately 20 complementary nucleotide bases either side of the mutated nucleotide, and are detailed in Table 3.3. In addition, to generate the correct length ERAP2 polymorphism models, primers for STOP 1+9 and STOP 2+9 were also designed to incorporate 9 additional amino acids into the ERAP2-FL protein (Table 3.3 and Figure 3.1). To generate these constructs, 50µl PCR reaction was set up using KOD Hot Start polymerase and carried out according to manufacturers' instructions, using 5' and 3' primers specific for the single base change in the following reaction: 5ul 10x PCR KOD buffer, 25mM MgSO4, 2mM dNTPs, 10 µM of 5' and 3' primers, 1ul KODHot Start Polymerase, 0.5 µg DNA template and made up to 50 ul with dH_20 (Novagen, Merck). The reaction conditions were 95ºC for 2 minutes followed by 18 cycles of 95ºC x 20 seconds, 65ºC x 10 seconds and 70ºC x 3 minutes. After SDM, the product was digested with 1µl Dpn1 (10 units/µl; NEB) for 1 hour at 37ºC to digest any methylated adenine residues in the original ERAP2 DNA template, leaving only mutated ERAP2. DNA was stored at -20°C until further use.

Primer	Target Sequence
5' STOP1	CCAAGATGACAAGTAACATGTA**A**GCCTTTCTGGGGGAAAATGC
3' STOP 1	GCATTTTCCCCCAGAAAGGC**T**TACATGTTACTTGTCATCTTGG
5' STOP2	CCAAGATGACAAGTAACATGTA**G**GCCTTTCTGGGGGAAAATGC
3' STOP2	GCATTTTCCCCCAGAAAGGC**C**TACATGTTACTTGTCATCTTGG
5' STOP1+9	CCTTTCTGGGGGAAAATGCATA**A**GTCAAAGAGATGATGACTAC
3' STOP 1+9	GTAGTCATCATCTCTTTGAC**T**TATGCATTTTCCCCCAGAAAGG
5' STOP2+9	CCTTTCTGGGGGAAAATGCATA**G**GTCAAAGAGATGATGACTAC
3' STOP2 +9	GTAGTCATCATCTCTTTGA**C**CTATGCATTTTCCCCCAGAAAGG

Table 3.3 Primers for the generation ERAP2 polymorphisms constructs. Primers were designed to have a GC content of 55-60% and Tm of 60°C-70°C. Underlined and bold base's change.

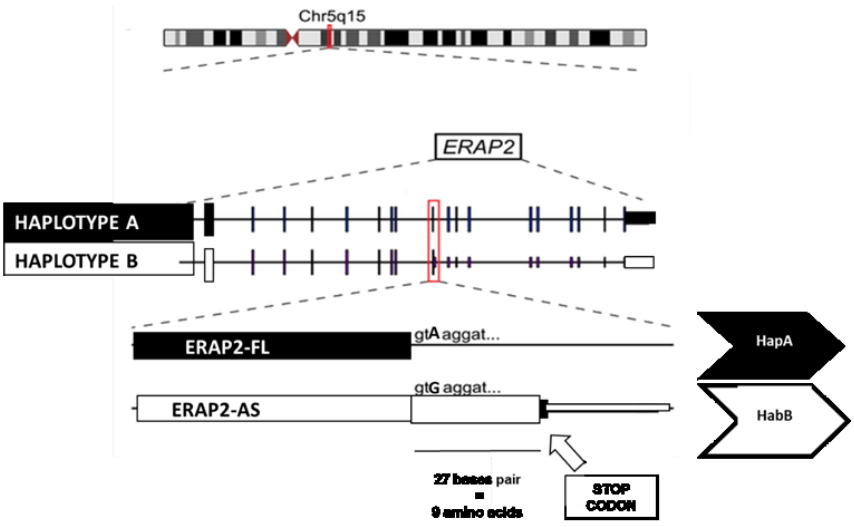
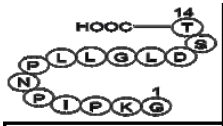


Figure 3.1 Schematic representation for the models construction.

3.1.2 V5-tag 2 step SDM

Due to the lack of commercial antibodies specific for truncated ERAP2, and after analysis of preliminary results, we used a V5 epitope tag sequence at the N-terminus of ERAP2 in order to detect in a clearer way the predicted protein product for both Hap A and Hap B-derived transcripts. The V5 epitope tag is derived from a peptide present at the C terminus of the P and V proteins of simian virus 5 (SV5). It contains 14 amino acids: N-GKPIPNPLLGLDST-C. To add the 14 amino acid tag to all ERAP2 constructs generated as in 3.1.1, we performed a 2 step SDM reaction, using the first step SDM to add the first 7 amino acids (GKPIPNP) and, after a sequencing confirmation, the second step SDM added the last 7 amino acids (LLGLDST). The 5' and 3'primers were designed to match in N term sequence of ERAP2 in pcDNA3 plasmid (Table 3.4). The 50µl SDM PCR reaction and thermo-reaction conditions were carried out as above (See 3.3.1.1).



	Target	Target Sequence
SDM 1 step (GKPIPNP)	5' E2 V5 (1)	CTCAGTGCCATCTAGTTATCACGG TAAGCCTATCCCTAACCCTTTCAC TGAGGATCCTGGGGCTTTC
	3' E2 V5 (1)	GAAAGCCCCAGGATCCTCAGTGA AAGGGTTAGGGATAGGCTTACCG TGATAACTAGATGGCACTGAG
SDM 2 step (LLGLDST)	5' E2 V5 (2)	GGTAAGCCTATCCCTAACCCTCTC CTCGGTCTCGATTCTACGTTCACT GAGGATCCTGGGGCTTTC
	3' E2 V5 (2)	GAAAGCCCCGGATCCTCAGTGAA CGTAGAATCGAGACCGAGGAGA GGGTTAGGGATAGGCTTACC

Table 3.4 Primers designed to tag plasmids in N terminus with the V5 epitope.

3.1.3 Ethanol Precipitation

To precipitate SDM mutated DNA, the 50ul dpn1 digested SDM product was made up to 100µl with dH_2O, before the addition of 0.1x 3M sodium acetate (pH5.5) and 2.5x 100% ethanol (-20ºC). The DNA was incubated at 37ºC for 1 hour, and centrifuged at 13,000rpm (4°C) for 20 minutes. The supernatant was removed and the DNA washed with 200µl of 70% ethanol (-20°C) by centrifuging as above for 5 minutes. The supernatant was removed and the DNA pellet left to air dry for 10mins before re-suspending in 10µl H_2O.

3.2 Overlap extension PCR cloning: ERAP2 AS

In order to create the fully truncated sequence for ERAP2-AS, we used the intermediate construct ERAP2wt + 9aa. Briefly, the alternatively spliced ERAP2 mRNA (ERAP2-AS) with an in-frame stop codon results in an alternative 27 bases down to the stop codon compared with ERAP2-FL (Figure 3.1). To insert these 27 bases (resulting in 9 amino acids) within ERAP2 FL sequence, we performed a 2 step overlap extension PCR [249]. ERAP2 FL-pcDNA3 (0.5 µg) was amplified using KOD Hot Start polymerase (Novagen, USA) and specific primers incorporating the alternative 9 amino acids (Table 3.5). The 50ul PCR reactions (1 and 2) were set up as in section 3.3.1.1 and PCR cycling conditions as follows: 95°C for 2mins followed by 35 cycles of 95°C for 20 seconds, 56°C for 10 seconds and 70°C for 32 seconds for PCR1 and 26 seconds for PCR2. The PCR product from both PCR reactions (1 µl) was used as template for PCR 3, with PCR1 5' and PCR2 3' primers (Table 3.5) used in this reaction. The PCR 3 reaction was set up as before and condition followed the manufacturers' instructions; 95ºC for 2 minutes followed by 18 cycles of 95ºC x 20 seconds, 65ºC x 10 seconds and 70ºC x 3 minutes. A small sample of PCR product was run on a 1% agarose electrophoresis gel to confirm the PCR had been successful and the correct size of DNA was present. When successful, the PCR product was purified using Qiagen PCR Purification kit (Qiagen, UK) according to

manufacturers' instructions and eluted in 30µl H_2O. The amount and concentration of DNA present were determined using spectrophotometer nanodrop (Thermo Scientific).

	Target	Target Sequence
PCR 1	5' E2wt + 9aa (1)	GACAAGCTTATGTTCCATTCTTCTGCAATG
	3' E2wt + 9aa (1)	CTACTCTGTGACTCTCTTTATCCTTACCATGTTACTTG TCATCTTGG
PCR 2	5' E2wt + 9aa (2)	GTAAGGATAAAGAGAGTCACAGAGTAGGTCAAAGAG ATGATGACTACATGGACT
	3' E2wt + 9aa (2)	GACCTCGAGTTAAGTATTAACCATTAGCC

Table 3.5 Primers used for intermediate sequence ERAP2wt + 9aa.

3.2.1 Restriction enzyme digestion

The pcDNA3 plasmid construct and PCR3 product were digested in standard 30µl reactions consisting of dH_2O, 1x BSA, 1x buffer, 3µg DNA, 1µl Hind III (12 units/µl; Promega) and 1µl Xho1 (10units/µl; Promega) for ERAP2wt + 9aa (intermediate of ERAP AS). As negative control, a single restriction digest were carried out in 20ul reaction volumes; 10ul dH_2O, 1x BSA, 1x buffer, 3µg DNA and 1µl restriction enzyme Xho1. Both single and double restriction digest reactions were incubated at 37ºC for one hour before being run on 1% agarose electrophoresis gel. The DNA was excised from the gel and purified according to the QIAquick Gel Extraction kit (Qiagen) following manufacturers instructions.

3.2.2 DNA ligation

A ligation between HindIII and XhoI digested PCR3 product (ERAP2wt + 9aa) and pcDNA3 vector was carried out using a molar ratio of 3:1 of DNA to vector. T4 DNA ligase (NEB, UK) and 10X ligase buffer were used for

ligation in a final reaction of 15µl. The reaction was incubated overnight at 16°C.

3.3 Cloning of DNA constructs

3.3.1 Bacterial transformation

JM109 competent cells were used to transform construct DNA generated by site directed mutagenesis (See 3.1.1, 3.1.2) and TOP10 bacteria (Invitrogen) used to transform cloned ERAP2 (See 3.3.2). One ul SDM product or 5µl of ligated plasmid DNA was added to 50ul bacterial cells and incubated for 30 minutes on ice. After this time, the bacteria were heat shocked at 42°C for 35 seconds and placed back on ice before the addition of 250µl SOC media (Table 3.6). The bacterial culture was incubated for 1 hour at 37°C with shaking at 220rpm before spreading 30 μl on agar plates containing ampicillin (100µg/ml) and incubated overnight at 37 °C.

Medium	Components
LB	0.5% yeast extract
	2% Tryptone
	10mM NaCl
SOC	2.5 mM KCl
	10 mM MgCl2
	10 mMMgSO4
	20mM Glucose

Table 3.6 Components of LB and SOC medium.

3.3.2 Screening of bacterial colonies

To screen the bacterial colonies, a selection of colonies were picked incubated in 2 ml LB medium containing ampicillin (100µg/ml) at 37°C with 220rpm shaking for 16 hours. Plasmid DNA was purified from bacteria using

QIAprep Spin Miniprep Kit (Qiagen) following the manufacturers' instructions. To determine the successful incorporation of addition or mutated nucleotides within the gene sequence, 100 ng/ μl (10 μl) of miniprep plasmid DNA product was sent for sequence verification using Source BioScience LifeSciences laboratories (Nottingham, UK). Sequencing primers used are shown in Table 3.7.

Primer	Target Sequence
T7 Forward	TAA TAC GAC TCA CTA TAG GG
SP6 Reverse	ATT TAG GTG ACA CTA TAG
BGH reverse	TAG AAG GCA CAG TCG AGG
ERAP2 seq	AAG ATG ACA AGT AAC ATG CTC

Table 3.7 Primers used for sequencing.

3.3.3 Maxiprep

The bacterial culture containing the correct DNA sequences was amplified overnight in 150ml LB medium containing ampicillin (100µg/ml) at 37°C with 220rpm shaking. The QIAfilter Plasmid Midi and Maxi kit (Qiagen) was used to purify a greater quantity of plasmid DNA for use in functional assays, resuspending DNA in TE buffer before determining the concentration of purified DNA using the Nanodrop.

3.4 Cell based functional assessment of ERAP2 constructs

3.4.1 Cell culture and maintenance

293T ERAP1-ERAP2 -/- cells were maintained at 37°C/5% CO2 in DMEM culture medium (Sigma) and B3Z T cell hybridoma (Nilabh Shastri, University of Berkeley, California) maintained in RPMI 1640 culture medium (without glutamine, Lonza, UK). Both the media (RPMI 1640 and DMEM) were supplemented with 10% heat inactivated (56 °C for 30min) fetal bovine serum

(FBS, PAA, UK), 2mM L-glutamine (Lonza, UK), 50U/ml Streptomycin (Lonza, UK), 50U/ml Penicillin (Lonza, UK), 1% Hepes (1M, PAA, UK). RPMI medium was also supplemented with 500nM β2-mercaptoethanol (Sigma). Adherent cell lines were harvested using 1mM EDTA and removed from the cell culture vessel by pastette.

3.4.2 Transfection of variant model constructs for ERAP2

Genetic model constructs were introduced into cells using Fugene6 transfection reagents (Promega, UK). 293T ERAP1-ERAP2 -/- cells were seeded at a concentration of 10^5 cells/ml in 2ml DMEM per well of a six well cell culture plate and incubated overnight to achieve 50-80% confluency on day 2. Transfection of cells was carried out as follows: 97 µl of serum free RPMI was added to 1.5ml eppendorf tubes followed by Fugene6 at ratio 3:1 with plasmid (3µl Fugene 6 to every 1µg plasmid DNA) and incubated for 5 minutes at room temperature. After this, 1µg of the plasmid DNA constructs were added (DNA per reaction described in Table 3.8) and incubated at room temperature for 15 minutes. After the incubation, the transfection mix was added drop wise to cells and maintained at 37°C/5% CO2. At the time points of 24 and 48 hours, $1x10^6$ cells and cellular supernatants were harvested for subsequent protein analyses.

Plasmid (0.5 µg)	H-2Kb (0.25µg)	R-SHL8 (0.25 µg)	SHL8 (0.25 µg)
ERAP2 wt	+	+	-
ERAP2 STOP1	+	+	-
ERAP2 STOP1+9	+	+	-
ERAP2 STOP2	+	+	-
ERAP2 STOP2+9	+	+	-
ERAP2 wt + 9 aa	+	+	-
V5-ERAP2 wt	+	+	-
V5- ERAP2 STOP2	+	+	-
V5- ERAP2 STOP1+9	+	+	-
V5- ERAP2 wt + 9 aa	+	+	-
pcDNA3	+	-	+

Table 3.8 List of plasmid used during transfection assay. H-2kb is a molecule of MHC class I complex requested for antigen presentation. R-SHL8 and SHL8 are peptides, the first needs to be cleaved before be presented instead the SHL8 is ready to be loaded to the antigen presentation complex (used as positive control for the assay).

3.4.3 T cell activation assay

After 24hours transfection, 293T ERAP1-ERAP2 -/- cells were harvested, counted and $2x10^5$ cells added into the first well of a 96 well plate. The cells were titrated, to achieve a 1:2 dilution across the plate, with $1x10^5$ cells in 100ul the starting well. The LacZ inducible B3Z T cell hybridoma were counted and added to the 293T ERAP1-ERAP2 -/- at 10^5 cells/100µl per well. After co-culture overnight incubation at 37°C, plates were centrifuged at 1500rpm for 2 minutes, the supernatant discarded and replaced with 100µl per well of chlorophenol red-beta-D-galactopyranoside (CPRG; 91mg CPRG, Roche) and incubated at room temperature. CPRG is a substrate for β-galactosidase and the response is generated through the activation of T cells by the recognition of SHL8 peptide in complex with the H-2Kb which transcribes the LacZ reporter gene generating β- galactosidase. In the presence of β-galactosidase, CPRG is cleaved releasing a substrate causing colour change from yellow to increasing intensities of red upon cleavage.

This colour change direct relates to the number of SHL8-H-2Kb complexes at the cell surface and is determined by a Biorad 680 microplate reader. Readings were taken every hour at a reference wavelength of A595nm with an additional wavelength of A695nm used to subtract background levels from the result. The data was then analysed using GraphPad Prism software. Statistical analysis was undertaken using an unpaired T test with 99% confidence interval, or with one-way ANOVA with Welch correction with a 99% confidence interval.

3.5 Molecular Biology

3.5.1 Cellular Supernatants concentration

During the transfection assays, at 24 and 48 hours cellular supernatants were collected for protein analyses. To concentrate the extracellular proteins within supernatants, Amicon Ultra15 centrifugal filters 30K (Millipore) were used to centrifuge samples (2ml) for 30 minutes at 2500 rpm (4 °C). After this step, the concentrated supernatant (~200 µl. 10X) was collected and transferred to an eppendorf for subsequent protein analyses.

3.5.2 Immunoprecipitation (IP) for V5 tag

To reduce the non-specific binding and improve the detection of ERAP2 protein from the supernatants, immunoprecipitation assay of V5-tag transfected supernatants was performed after the concentration step above. Dynabeads protein G (Invitrogen, UK) were resuspended in solution and 50µl was transferred to a tube, placed on the supplied magnet to separate the beads from the solution, and the supernatant discarded to leave only the magnetic dynabeads. Five µg of V5 antibody (ThermoFisher) was diluted in 200 µl PBS and added to the beads, incubating with rotation at room temperature for 10 minutes in order to bind the antibody to the dynabeads via the Fc region of the antibody. The beads-antibody complexes were placed on the magnet and the supernatant removed. 200 µl of concentrated

supernatant (See 3.5.1) was mixed with antibody-bead complex and incubated with rotation at room temperature for 10 minutes to allow the bond antibody-target protein complex. The tubes were placed on the magnet and the supernatant removed and retained for further analysis. The dynabeads-antibody-protein complex was washed three times in 200µl PBS 0.1% Tween for each wash step. Finally the complex was re-suspended in 100µl PBS 0.1% Tween and transferred into a clean tube to avoid co-elution of proteins bound to the tube wall. To elute the target proteins, the complexes were mixed with 20µl 1x NRSB, gently re-suspended by pipetting, and heated for 10 minutes at 70°C to dissociate the dynabead complex. After heating, the sample was placed on the magnet and the supernatant remaining was used to analyse by immunoblotting.

3.5.3 Immunoblotting

3.5.3.1 Preparation of cell lysates

To create protein lysates from cells after 24 and 48 hours after transfection, $2x10^6$ cells (previously counted) were centrifuged at 1200rpm for 5 minutes and washed in 500µl PBS and transferred to an eppendorf and centrifuged at 2,000rpm for a further 3 minutes, in order to generate a pellet of cells. The PBS was discarded, and the pellet of cells re-suspended in NP40 lysis buffer; 150mM NaCl (Sigma), 5mM EDTA (Fisher), 20mM Tris-HCl pH7.4 (Sigma) and 1% Nonidet-P40 (NP40, US Biological, USA). Protease inhibitors iodoacetamide (IAA, 5%, Sigma) and phenylmethyl sulfonyl fluoride (PMSF, 5%, Sigma) were added to the lysis buffer. After 30 minutes of incubation on ice, the tubes were centrifuged at 13,000rpm for 20 minutes at 4°C to pellet the unwanted cell debris. The resulting supernatant was collected and stored at -20°C until required.

3.5.3.2 SDS-PAGE gel

Protein lysates were separated on a 10% SDS-PAGE gel, consisting of 10% resolving gel followed by 5% stacking gel for loading of protein samples (Table 3.9). 3x Non reducing sample buffer containing 50% glycerol, 1M Tris pH 6, 10% SDS and H_2O, was added to each sample (protein extracts and supernatants) and loaded onto the gel, along with 1ul Magic Marker (Sigma) and 3µl Pro sieve marker (Invitrogen). After 1 hour of run in 1X SDS running buffer at 200V, the proteins were transferred from resolving gel to a nitrocellulose membrane hybond C (Amersham, UK) at 23V for 1 hour at room temperature. Layers of sponges, paper, hybond C, resolving gel, paper and sponges were assembled. 5x transfer buffer (72.05g glycine, 15.15g Tris base made to 1litre with H_2O) diluted to 1x using 50ml 5x transfer buffer, 150ml H_2O and 50ml ethanol, were added to the tank.

Reagents	10% Resolving Gel (ml)	5% Stacking Gel (ml)
dH_2O	4.6	2.1
30% Acrylamide/Bis (37:5:1,BioRad, UK)	3.3	0.5
1.5M Tris HCl (pH 8.8)	2.5	--
0.5M Tris HCl (pH 6.8)	--	0.38
10% SDS	0.1	0.3
10% APS	0.1	0.3
TEMED (Sigma, UK)	0.004	0.003

Table 3.9 Contents of the resolving and stacking gels for Western Blot analysis.

3.5.3.3 Blocking and immunodetection

To block non-specific binding sites the nitrocellulose membrane was incubated, in agitation, at 4°C overnight in blocking buffer (5% milk (Marvel) in PBS with 0.1% Tween 20 ,Sigma). The membranes were washed (3x 10

minutes) with 10ml fresh wash buffer (PBS with 0.1% tween 20). Primary antibody was added and incubated for 1 hour at room temperature, followed by 3x 10 minutes washes before 1 hour of incubation in secondary antibody. Before the detection, furthermore 3 washes have been made. The primary and secondary antibodies were diluted in blocking buffer (5% milk in PBS 0.1% Tween 20) (Table 3.10). In order to detect the presence of proteins, the membrane was developed using equal volumes of super signal enhancer and super signal stable peroxide (ThermoScience) were mixed and incubated for 5 minutes on the membrane then exposed and imaged with Fluor-S Multi-imager (Biorad, UK).

Antibody	Specificity	Species	Assay/Diluition	Source
ERAP2	Human Aminopeptidase LRAP/ERAP2	Mouse	WB, 1:500	R&D
Anti-V5 HRP	V5 Epitope	Mouse	WB, 1:5000	Invitrogen
Anti-V5	V5 Epitope	Mouse	IP: 5 µg/50µl beats	Invitrogen

Table 3.10 Antibodies used in immunodetection (WB) and IP.

RESULTS

1. Role of ERAP2 variants in HIV1-infected treated patients (ICONA Cohort)

1.1 Clinical parameter analysis of the ICONA cohort subjects

Studies on HESNs carried out in collaboration with the Infectious Diseases Unit of SM Annunziata Hospital in Florence, have led us to the definition of a group of genes (TLR3, MX2, APOBEC3H and ERAP2) whose haplotypes are related to the immunologic advantage of these subjects. Nevertheless, it is not yet known whether the combination of protective gene variants may correlate to different responses to therapy in HIV-positive subjects undergoing ART. For all these reasons, 300 HIV-infected patients from ICONA cohort undergoing a first ART regimen were enrolled in this study with the aim of evaluating the frequency of single and combined SNPs within the four previously mentioned genes. In addition, we investigated a possible correlation between the frequency of single and combined polymorphisms ("supergenotype") and the course of infection in HIV-positive patients receiving ART, using the viral load slope and CD4+ cell count as indexes of response to therapy.

Demographic and clinical parameters of enrolled subjects are summarised in Table 4.1, in which age, gender, mode of HIV transmission, AIDS diagnosis, presence of coinfections, CD4+/CD8+ cell counts and viral load are reported.

Characteristics	Total
	N= 300
Age, years	
Median (IQR)	38 (32, 45)
Gender, n (%)	
Female	31 (10.3%)
Mode of HIV Transmission, n(%)	
IDU	13 (4.4%)
Homosexual contacts	198 (66.4%)
Heterosexual contacts	77 (25.7%)
Other/Unknown	10 (3.4%)
AIDS diagnosis, n(%)	
Yes	24 (8.0%)
HBsAg, n(%)	
Negative	228 (76.0%)
Positive	3 (1.0%)
Not tested	69 (23.0%)
HCVAb, n(%)	
Negative	229 (76.3%)
Positive	21 (7.0%)
Not tested	50 (16.7%)
Calendar year of starting ART	
Median (IQR)	2013 (2012, 2014)
Time from HIV diagnosis, months	
Median (IQR)	12 (1, 50)
CD4 count nadir, cells/mmc	
Median (IQR)	354 (242, 450)
CD4 count at ART, cells/mmc	
Median (IQR)	385 (268, 496)
CD8 count at ART, cells/mmc	
Median (IQR)	1047 (775, 1396)
Viral load set point, log10 copies/mL	
Median (range)	4.5 (3.8, 5.1)
Viral load at cART, log10 copies/mL	
Median (range)	4.6 (1.6, 7.0)
Time to VL suppression, months	
Median (IQR)	4 (3, 7)

Table 4.1 Clinical and demographic characteristics of HIV-infected patients enrolled in the ICONA cohort.

1.2 Analysis of allelic variants in the ICONA cohort

The ICONA cohort genotyping results and the prevalence of different polymorphisms in our immune genes patients are summarised in Table 4.2. No differences in SNP frequency between HIV-infected cohort and both World and European population were observed. In addition, the co-presence of single and combined polymorphisms previously mentioned is reported in Table 4.3. The analysis did not show any significant result in terms of prevalence of genetic variant combinations in the enrolled individuals.

	ERAP2		
	GG	**GT**	**TT**
Freq	60	157	83
%	20	52.33	27.67

	APOBEC3H		
	CC	**CG**	**GG**
Freq	63	140	97
%	21.00	46.67	32.33

	MX2		
	AA	**AG**	**GG**
Freq	135	134	31
%	45	44.67	10.33

	TLR3		
	CC	**CT**	**TT**
Freq	155	120	25
%	51.67	40	8.33

Table 4.2 Genotyping results of prevalence and frequency in subjects of the ICONA cohort. Protective SNPs are underlined.

N° Protective variants	N° HIV+ subjects
4 all/aplot prot (4+)	2
%	0,67
3 all/aplot prot (4+)	10
%	3,3
2 all/aplot prot (4+)	85
%	28,33
1 o nessuno all/aplot prot (4+)	203
%	67,67

Table 4.3 Number/Sum of protective alleles in subjects of the ICONA cohort.

1.3 Correlation between single allelic variants and clinical parameters (CD4+/CD8+ T cell count and Viral Load)

One of the aims of the project was to explore whether there was a correlation between protective gene variants and a different response to therapy in HIV-positive subjects undergoing a first regimen of combination antiretroviral therapy (cART). As expected, we did not find any association between the analysed allelic variants and the clinical characteristics of HIV-infected patients enrolled at baseline time-point (data not shown). Similarly, no association between allelic variants within APOBEC3H, TLR3 and MX2 genes or HIV viral load and CD4+ /CD8 + T cell count slopes was observed in our cohort 12-months post cART administration (data not shown). Conversely, we observed a significant association between the frequency of the GG allelic variant of ERAP2 and response to therapy. Indeed, for rs2549782 G/T there is evidence that the GG genotype (protective form) is associated with a steeper decline in HIV-1 viral load after adjusting for age, gender, CD4+/CD8+ T cell count and VL at cART, use of INSTI or >3 drugs, ART-naive and year of starting cART Figure 4.1).

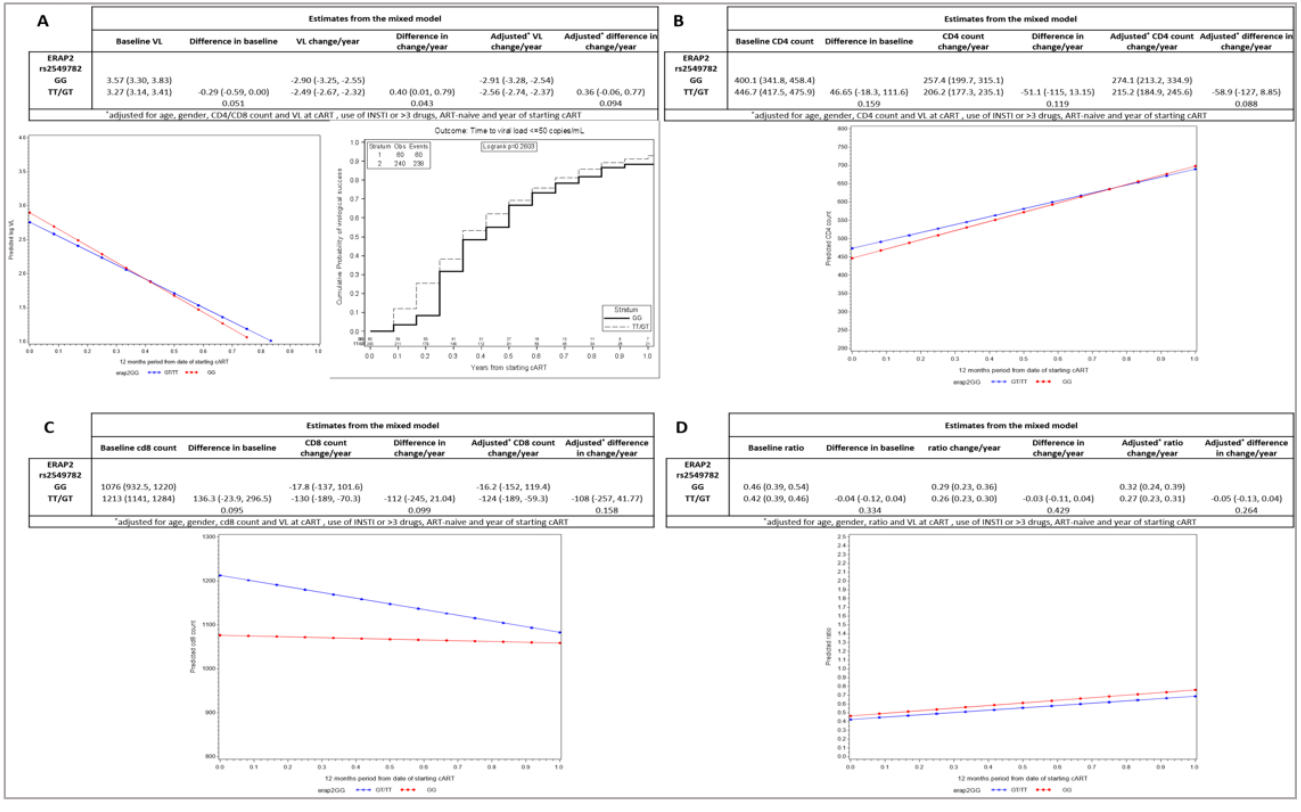


Figure 4.1 Mean intercept and slope of log10 Viral Load (**A**), CD4+ T Cells (**B**) and CD8+ T cells (**C**) from fitting a mixed-linear model in ICONA subjects. Panel **D** shows the ratio of CD4/CD8 in ICONA subjects. All the data are adjusted for age, gender, ratio and VL at cART , use of INSTI or >3 drugs, ART-naive and year of starting cART.

Notably, the association with a steeper decline in HIV-1 viral load is even stronger (p=0.042) when the analysis is restricted to people of Caucasian ethnicity (251 subjects) (Figure 4.2 A). Patients carrying this genotype also show a trend towards a steeper increase in CD4+ T cell count (Figure 4.2 B). As for CD8+ data in GG patients, we observed no differences in CD8+ T cell count over 1 year-treatment. Conversely, TT/GT genotyped patients show a decrease of CD8+ T cell count over the year (Figure 4.2 C). This results in a significant association with a steeper decline in CD8+ T cells in TT/GT patients but it could be explained by a no difference in change/year in CD8+ T cell count over time in GG patients. Further analyses concerning this aspect are not possible in this observational study, however these data support the necessity to deepen the role of this rs2549782 G/T allelic variant in a case/control study. Nevertheless, no differences in CD4+/CD8+ cell ratio have been observed over time in our cohort after controlling for age, gender, CD4+/CD8+ T cell count and VL at cART, use of INSTI or >3 drugs, ART-naive and year of cART start (Figure 4.2D). These results are particularly relevant considering the key role played by ERAP2 in the orchestration of the immune system. Indeed by trimming the N-terminal end of peptide loaded on MHC class I molecules, it is able to shape the CD8+ T cell repertoire that controls viral replication. Therefore, it is possible to speculate that peptides generated by homozygous G subjects are qualitatively and/or quantitatively more protective against viral replication and this hypothesized difference in antigen processing could have a role in terms of a better response to ART treatment in HIV+ patients.

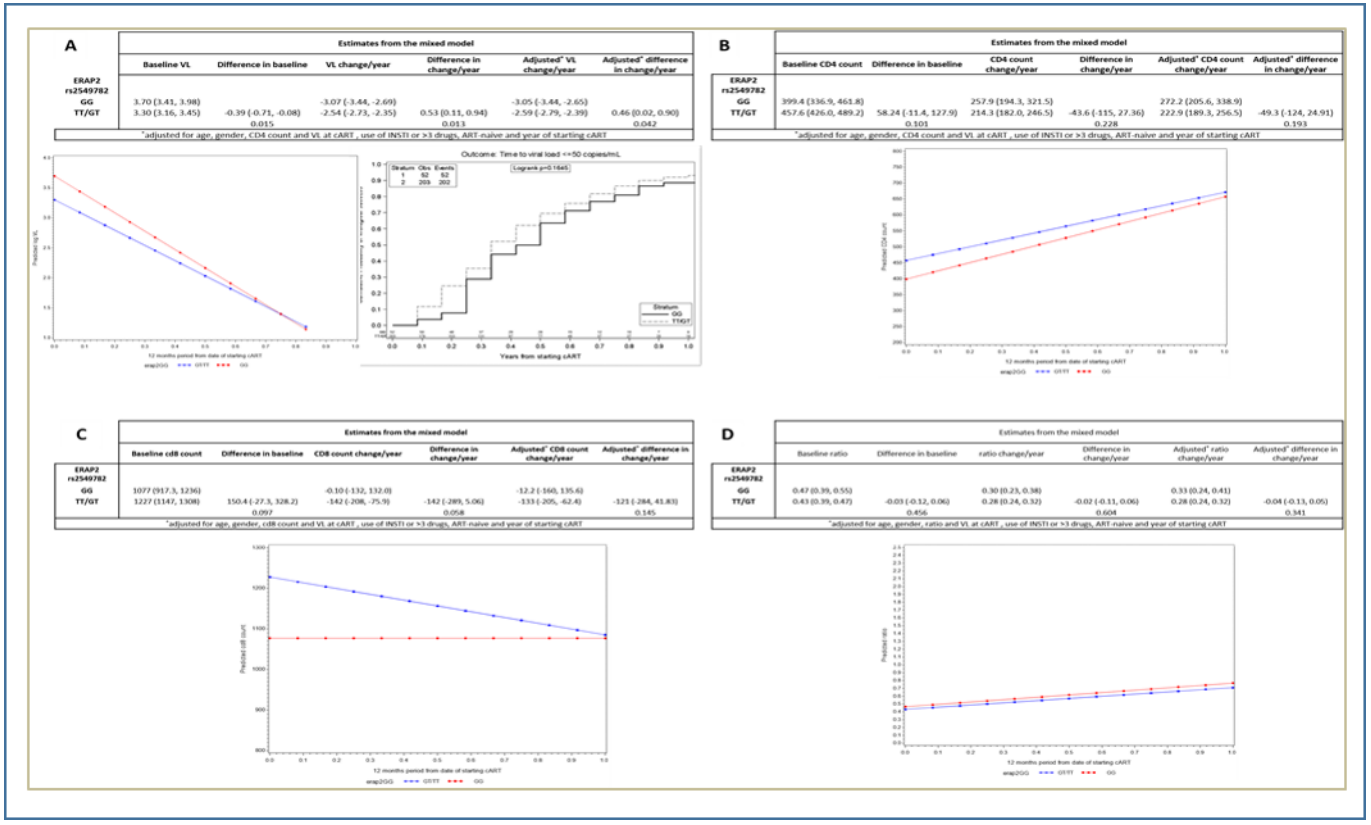


Figure 4.2 Mean intercept and slope of log10 Viral Load (**A**), CD4+ T Cells (**B**) and CD8+ T cells (**C**) from fitting a mixed-linear model in Caucasian ICONA subjects. Panel **D** shows the ratio of CD4/CD8 cells in Caucasian ICONA subjects. All the data are adjusted for age, gender, ratio and VL at cART , use of INSTI or >3 drugs, ART-naive and year of starting cART.

1.4 Correlation between combined allelic variants and clinical parameters (CD4+ or CD8+ T cell count and Viral Load)

One of the aims of this study was to identify combinations of polymorphisms (a “supergenotype”) in genes that are considered to be protective in viral infection / replication control and response to ART therapy by monitoring the clinical parameters of interest (VL and CD4/CD8 lymphocyte counts). Specifically, a score (SNP score) is assigned to each SNP variant (Table 4.3 A) and all the subjects are grouped following the score assigned (Table 4.3 B). With the latter, we performed a statistical analysis in order to identify a combination of variants that could provide an immunological benefit in terms of infection and response to therapy. The analysis, however, did not show any significant correlation between variant combinations and viral load or CD4+ or CD8+ cell counts in either all subjects or just Caucasian subjects of ICONA cohort (Figure 4.3). This result was not unexpected because during single variant analyses, only the ERAP2 related variant correlated with a better response to ART treatment in terms of VL and CD4+ cell count.

A

	ERAP2		
	GG	GT	TT
SNPscore	1	0	0

	APOBEC3H		
	CC	CG	GG
SNPscore	0	0.5	1

	MX2		
	AA	AG	GG
SNPscore	0	0	1

	TLR3		
	CC	CT	TT
SNPscore	0	1	1

B

snp_score	Frequency	Percent	Cumulative Frequency	Cumulative Percent
0	19	7.45	19	7.45
0.5	49	19.22	68	26.67
1	43	16.86	111	43.53
1.5	63	24.71	174	68.24
2	59	23.14	233	91.37
2.5	14	5.49	247	96.86
3	7	2.75	254	99.61
4	1	0.39	255	100.00

Table 4.3 SNP score assigned to each variant (**A**) and frequency-percent of Caucasian subjects related to the score (**B**).

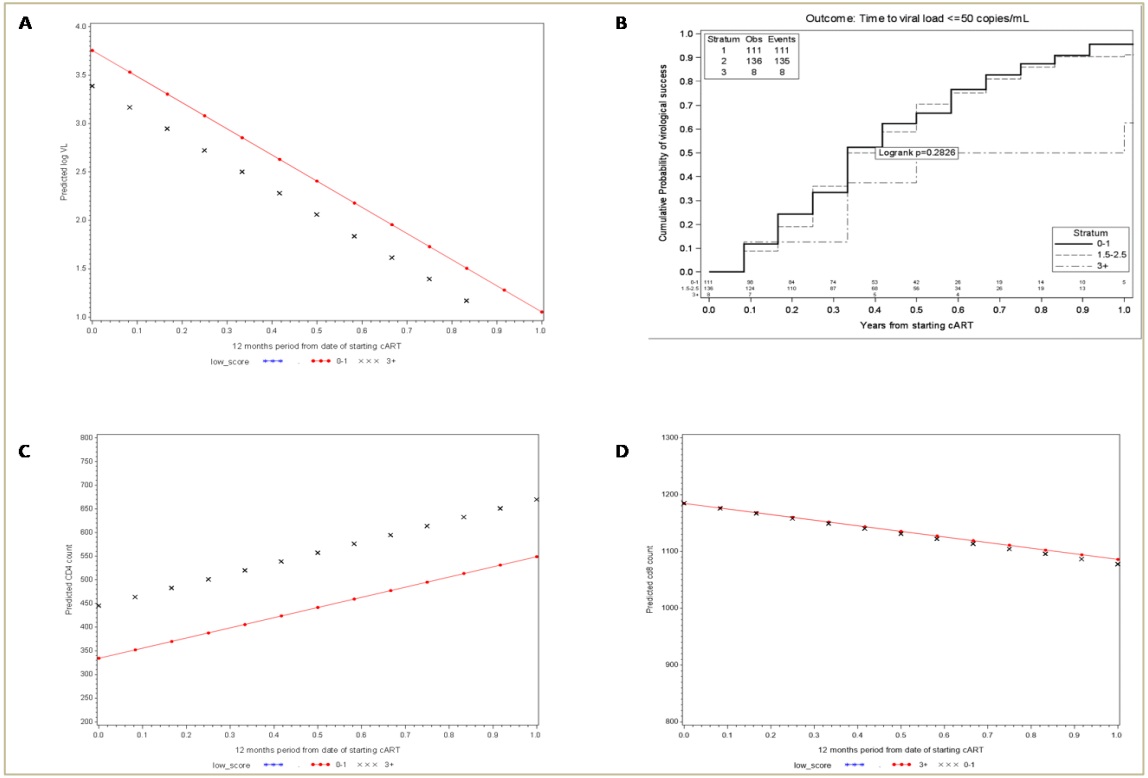


Figure 4.3 Mean intercept and slope of log10 Viral Load (**A-B**), CD4+ T Cells (**C**) and CD8+ T cells (**D**) from fitting a mixed-linear model in Caucasian ICONA subjects. All the data are adjusted for age, gender, ratio and VL at cART , use of INSTI or >3 drugs, ART-naive and year of starting cART.

2. Role of ERAP2 in *in vitro* HIV-1 infection

Interestingly ERAP2 Hap A has been found to be significantly overexpressed in HIV-1-exposed seronegative individuals (HESNs), following a recessive model and it also correlates with resistance to HIV-1 infection [122]. Results obtained above show an involvement of ERAP2 in HIV-1-progression, modulating the response to therapy in patients undergoing a first HAART regimen. The aim of this part of project was to better characterize ERAP2 role in HIV-1infection resistance.

2.1 ERAP2 Inhibition increases susceptibility to *in vitro* HIV-1 infection

To confirm the involvement of ERAP2 in HIV-1 susceptibility, PBMCs isolated from 30 healthy donors, genotyped for ERAP2 allelic variants, were *in vitro* infected, with an R5 tropic HIV-1 strain. Cell cultures were made in the presence or absence of DG013A phosphinic peptide, which is able to bind to the ERAP2 catalytic site and inhibit enzyme activity [248]. Notably, under basal conditions, cytofluorimetric analysis showed a significant reduction in MHC class I (HLA-A, B,C) in Hap B genotype samples compared to the Hap A samples ($p<0.05$) (Figure 4.4). This data indicates a correlation between the Hap A genotype and the generation of a greater abundance of HLA-binding peptides that are presented at the cell surface. This result is further supported by experiments showing a correlation between transient knock-down of ERAP2 with a reduction in cell surface MHC class I expression [137].

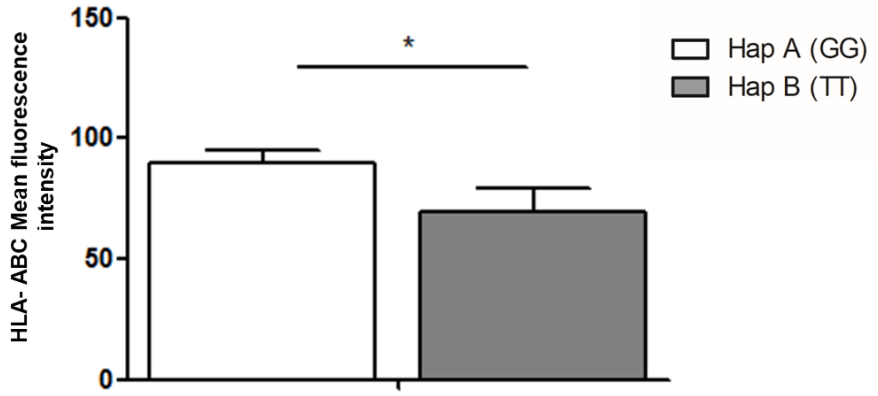


Figure 4.4 Human leukocyte antigen (HLA)-ABC mean fluorescence intensities in HapA and Hap B donor cells at baseline. Mean values and standard error are shown. *P<0.05

Six days after infection, p24 levels were lower in cells from Hap A compared to Hap B individuals, a result suggestive of a more general restrictive role of ERAP2 in HIV (and likely other) infection. Addition of DG013A caused a dose-dependent increase in HIV-1 infection susceptibility, resulting in higher p24 levels, in both the haplotypes (Figure 4.5). Unfortunately, when the cohort was increased (20 more subjects), we were unable to confirm the result obtained. A possible explanation for these opposing results may be due to the newly synthesised DG013A, used in the latter experiments, showing lower activity and specificity. Therefore, further analyses are required to confirm the consequences of ERAP2 inhibition by DG013A

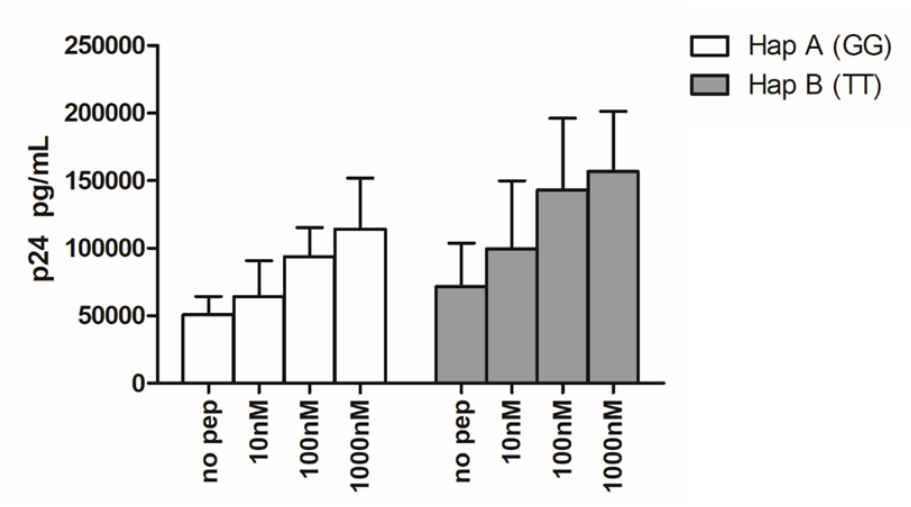


Figure 4.5 p24 concentration in PBMCs of subjects with different ERAP2 diplotype 6 days after HIV-infection in presence/absence of DG013A peptide inhibitor at different concentrations (10nM, 100nM and 1000nM). Mean values and standard error are shown.

Four days post in vitro HIV-1 infection in the presence of DG013A, we observed an increased mRNA expression of ERAP1 (GG: $p<0.05$; TT $p<0.01$) and TAP1 (GG: $p< 0.005$;TT: $p< 0.05$), two further genes involved in antigen presentation with a peak effect at 1000nM (Figure 4.6). This result could be explained by compensation mechanisms occurring within cells; indeed trying to maintain a canonical rate in antigen presentation, in a condition where ERAP2 is inhibited, it is plausible to hypothesize an enhanced activation in other aminopeptidases and transporter expression, such as ERAP1 and TAP1.

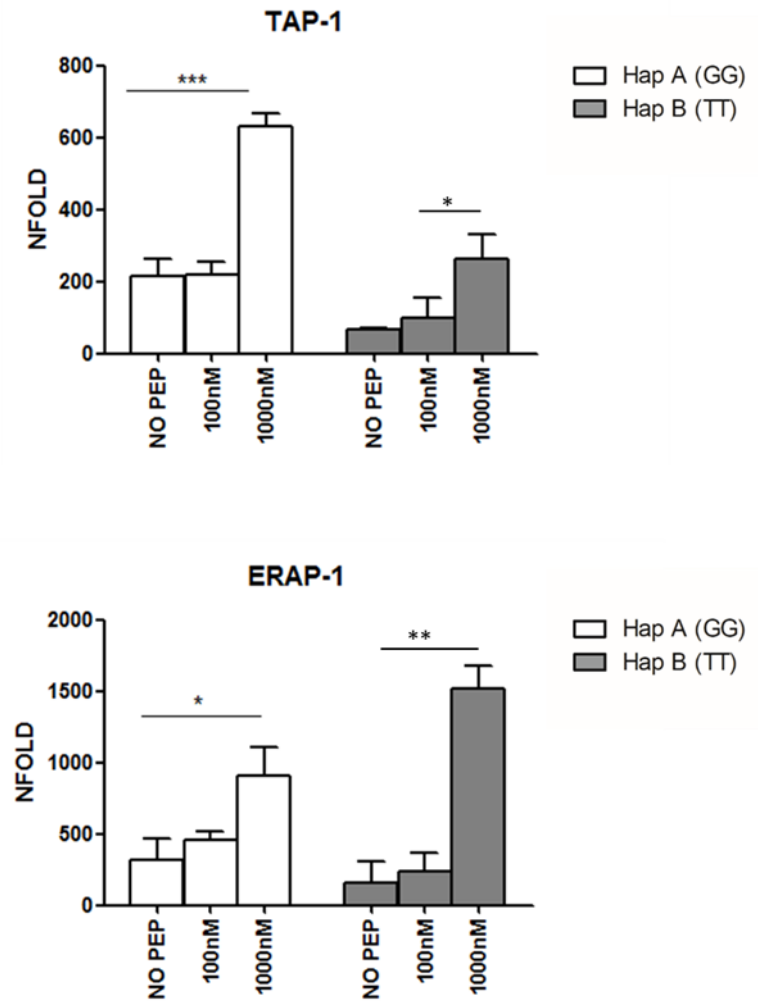


Figure 4.6 mRNA expression of transporter associated with antigen processing 1 (TAP1) and endoplasmic reticulum aminopeptidase type 1 (ERAP1) 4 days post HIV-1-infection in presence or absence of 100nM or 1000nM of DG013A peptide inhibitor. Mean values and standard error are shown. * P<0.05, * P<0.01, *** P<0.005.

2.2 rhERAP2 addition in the extracellular milieu reduces viral infection and/or replication

Secretion of ERAP1 and ERAP2 from plasma membrane in response to activation has been recently reported [212-214]. We therefore decided to investigate if, once released, ERAP2 still retains its antiviral function. PBMCs isolated from 30 healthy donors were *in vitro* infected with an R5 tropic HIV-1 strain in presence or absence of recombinant (rh) ERAP2 (100 ng/ml). Addition of rhERAP2 to cells did not affect cell viability during HIV infection. As previously shown, 6 days post *in vitro* infection, Hap A subjects were less susceptible to HIV-1. Notably the addition of rhERAP2 to cell cultures resulted in a reduction of viral replication in both Hap A and Hap B individuals (Figure 4.7). These differences reached statistical significance when the analyses were performed independently of the ERAP2 genotype, ($p<0.01$) (Figure 4.8). Furthermore, this protective effect was independent of modulation of HLA-ABC and/or perforin and granzyme expression by CD8+ T lymphocytes (data not shown). The role and the targets of ERAP2 in the extracellular milieu are still unknown and needs further investigation. However, data herein suggests that once added to cell culture, ERAP2 provides a protective function against HIV-1 infection and, presumably, this defensive feature is mediated through an unconventional mechanism, distinct from immune system modulation.

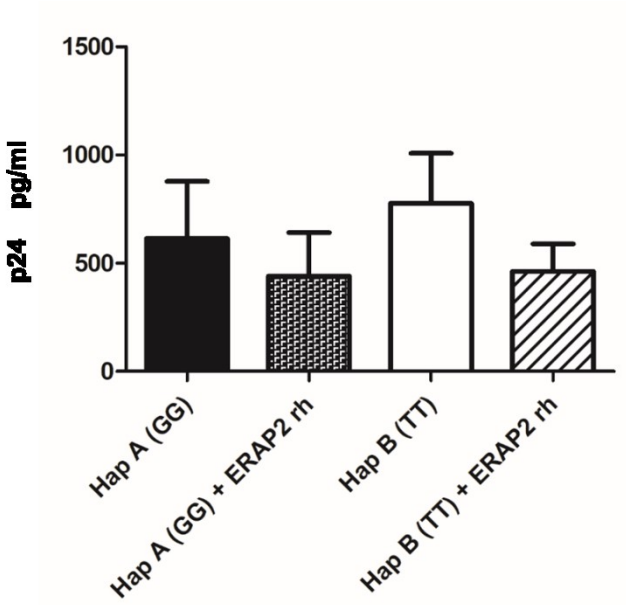


Figure 4.7 p24 concentration in PBMCs of subjects with different ERAP2 diplotype 6 days post *in vitro* HIV-1 infection in presence/absence of 100 ng/ml of recombinant (rh) ERAP2. Mean values and standard error are shown.

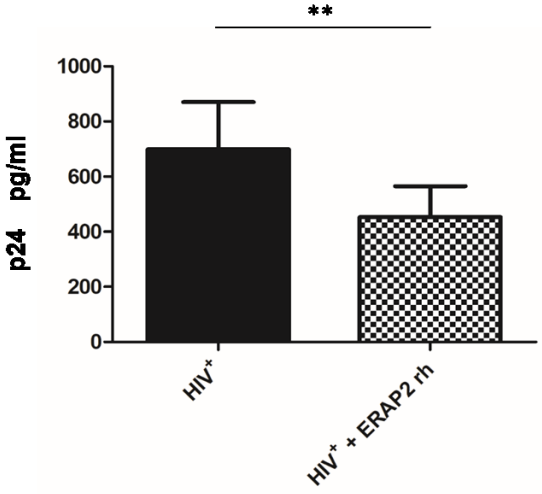


Figure 4.8 p24 concentration in 6-day post *in vitro* HIV-1 infected PBMCs of Hap A plus Hap B subjects in presence or absence of 100 ng/ml of recombinant (rh) ERAP2. Mean values and standard error are shown. **p <0.01.

3. Trimming function of ERAP2 variants

The aim of this last part of the project was to investigate and clarify the different trimming phenotypes of the two haplotypes, to provide a greater understanding of the molecular mechanism underpinning the observation of HIV infection susceptibility phenotype mediated by the two haplotypes.

3.1 Hap B models show a reduced trimming activity compared to ERAP2 wt (Hap A)

To investigate the trimming activity of ERAP2 haplotypes, ERAP1-ERAP2-/- 293T cells were transiently transfected with different DNA constructs containing either Hap A or Hap B ERAP2 (See 3.1 and 3.2). At the same time, cells were transfected with minigenes containing a specific ERAP2 substrate R-SIINFEHL (R-SHL8) and the relevant MHC class I molecule (H-2Kb). As a positive control, a minigene containing SIINFEHL (SHL8) final peptide was used. This peptide does not require ERAP2 trimming to be loaded onto and presented at the cell surface by H-2Kb. By comparison, the R-SHL8 peptide requires trimming of the N-terminal Arg amino acid to be presented. To detect the generation of the final 8mer SHL8 peptide and the subsequent formation of SHL8/H-2Kb complex, we used the B3Z T cell hybridoma. These cells express a specific TCR at the cell surface that recognises the modified peptide SIINFEHL (original SIINFEKL), bound and presented on H-2Kb molecule. Recognition of SHL8-H-2Kb complex by the TCR receptor at the cell surface of B3Z, allows the transcription of a β-galactosidase (lacZ) reporter gene construct in the cells. To detect the presence of β-galactosidase, a colorimetric substrate called chlorophenol red β-galactopyrannoside (CPRG) is used resulting in a colour change upon production of β-galactosidase. This colour change reaction is an indirect method to measure the lacZ activity of B3Z cells and directly correlates with the amount of SHL8 peptide presented by H-2Kb at the cell surface and, consequently, can determine the level of N-terminal processing. As

described above, the SHL8 minigene is used as a positive control and an R-SHL8 minigene transfected together with an empty pcDNA3 vector (no ERAP2) is used as the negative control for the assay. Transfection of ERAP1-ERAP2-/- cells with ERAP2 haplotypes showed that all the models for Hap B ERAP2 have a reduced trimming function compared to the full length Hap A ERAP2 (Figure 4.9).

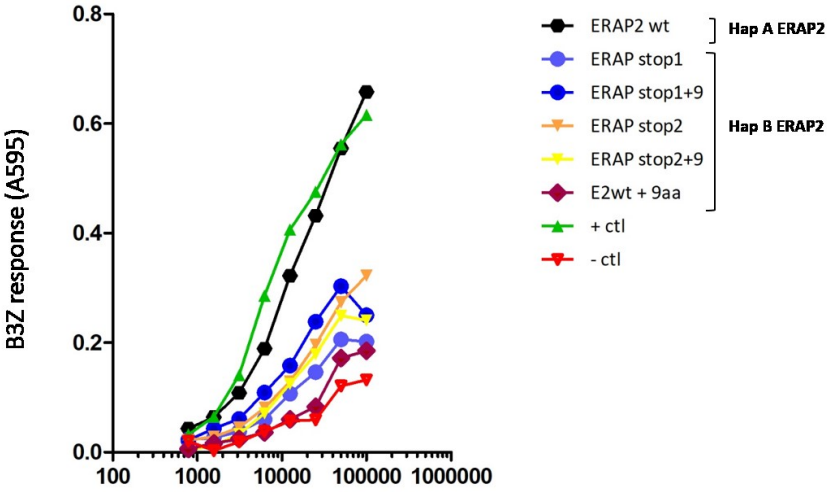


Figure 4.9 Trimming activity assay in ERAP1/2 deficient cells. ERAP1/2-deficient 293T cells were transfected with Hap A or Hap B ERAP2 together with an N-terminally extended R-SHL8 minigene and assayed for trimming by the stimulation of the LacZ-inducible, SHL8/Kb-specific B3Z T cell hybridoma. As a control for ERAP trimming, ERAP1/2 deficient cells were transfected with SHL8 only (+ ctl) and as control for the assay cells were transfected with empty pcDNA3 vector and R-SHL8 minigene (- ctl). Hap A is designated for ERAP2 wt, whereas remaining models (*stop1, stop1+9, stop2, stop2+9 and E2wt+9aa*) refer to Hap B variant of ERAP2.

There are no commercially available antibodies specific for ERAP2-AS, therefore in order to confirm the presence of ERAP2 protein in transfected cells, we created N-terminally V5 tagged Hap A and Hap B constructs. Figure

4.10 shows the comparison of trimming activity between the DNA constructs with or without N-terminal V5 tag. As expected, we did not observe any differences in the trimming activity between the two groups of DNA constructs, because the N-terminal sequence portion of the ERAP2 gene is not related to the aminopeptidase activity of the protein [207]. This result is also important for future detection studies, because the V5 tag construct allows the detection of predicted protein products for both Hap A and Hap B-derived transcripts in a clearer way.

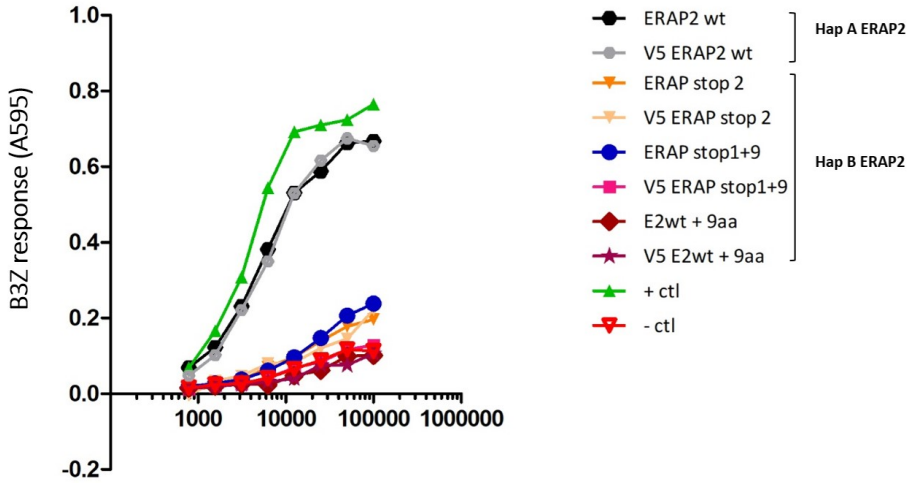


Figure 4.10 Trimming activity assay to compare DNA constructs models with or without the V5tag. ERAP1/2-deficient cells were transfected with Hap A or Hap B ERAP2 together with an N-terminally extended R-SHL8 minigene and assayed for trimming by the stimulation of the LacZ-inducible, SHL8/Kb-specific B3Z T cell hybridoma. As a control for ERAP trimming ERAP1/2 deficient cells were transfected with SHL8 only (+ ctl) and as control for the assay cells were transfected with empty pcDNA3 vector and R-SHL8 minigene (- ctl). *ERAP2 wt* and *V5 ERAP2 wt* refer to Hap A ERAP2; remaining datasets (*stop2, V5 stop2, stop1+9, V5 stop1+9, V5 E2wt+9aa and V5 E2wt+9aa*) refer to Hap B.

3.2 The different trimming activity of Hap A and Hap B results in a different T cell response

The generation of peptides to present onto MHC I molecules is fundamental for recognition and activation of the CD8+ T cell response and the subsequent immune surveillance process. Our transfection results revealed a significant reduction ($p<0.005$) in percentage of T cell response (B3Z) in Hap B constructs compared to the ERAP2 FL (Hap A) (Figure 4.11 A). The same result was also observed with the N-terminal V5 tag constructs ($p<0.01$; $p<0.05$) (Figure 4.11 B). These results are interesting since the difference in trimming activity may help explain the different haplotype-mediated phenotype in HIV infection. Indeed, it was observed that Hap A expression was higher in HESNs [121], subjects who remain seronegative despite repeated exposures to the virus, and therefore a greater ability to induce and activate a T cell response by Hap A ERAP2 may contribute to a natural resistance to infection. Moreover, our results obtained in *in vitro* HIV infection, where GG (Hap A) PBMCs presented a reduction of p24 level (HIV replication index) compared with TT genotype (Hap B), agrees with a qualitatively and/or quantitatively different peptide repertoire generated by the two haplotypes.

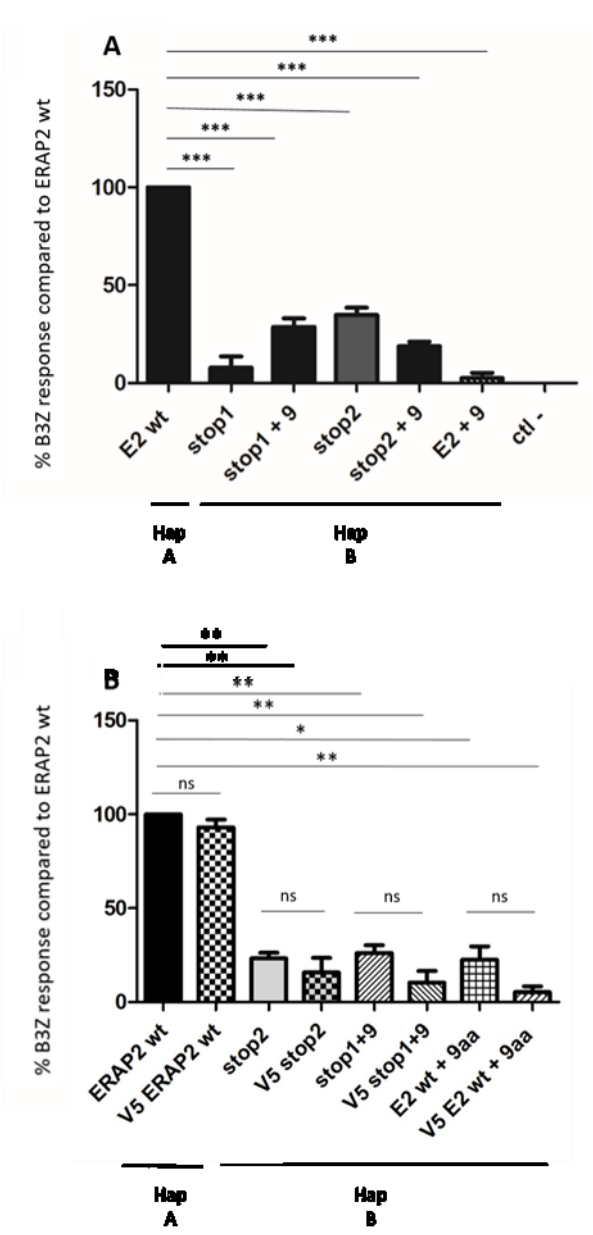


Figure 4.11 Percentage of T cell response (B3Z) using DNA constructs for Hap B compared to the ERAP2 FL wt (Hap A). Panel **A** shows the reduction of T response using DNA constructs without V5-tag, whereas **B** shows the comparison in T cell activation between vectors with and without N-terminal V5 tag. Mean values and standard error are shown. *P<0.05, **P<0.005, ***P<0.001.

3.3 ERAP2 localization (preliminary results)

Evidence that ERAP1 and ERAP2 play a fundamental role in ER proteolysis and the subsequent ER localisation are well established. However, recently, the subcellular localisation of the two aminopeptidases has been questioned [194-195]. To investigate the localisation of the derived protein for both Hap A and Hap B transcripts, we collected $2x10^6$ cells and supernatants from cell cultures used in trimming activity assays, at 24 and 48 hours after transfection. Cells that had been transfected with ERAP2 FL showed the presence of a clear full-length ERAP2 protein band (120 kDa) in Hap A model samples at 24 and 48 hours post transfection. In Hap B samples, weak signals at 120 and 60 kDa (size of predicted truncated ERAP2 protein) have been detected at 24 hours (Figure 4.12 A). By contrast, protein analysis performed on cell lysates derived from V5 tag transfections did not show the 60 KDa band, whereas the 120kDa ERAP2-specific V5 signal was detected in both Hap A and Hap B (Figure 4.12 B). This result suggests that protein translation run through leading to a full-length ERAP2 protein even in constructs that possess a stop codon. Interesting, the result is consistent with the trimming data (see 3.1), with greater trimming activity observed in Hap A transfectants and a clear 120 kDa band in lysates. Whereas in Hap B models, a very low ERAP2FL signal is consistent with the reduced (almost zero) trimming activity.

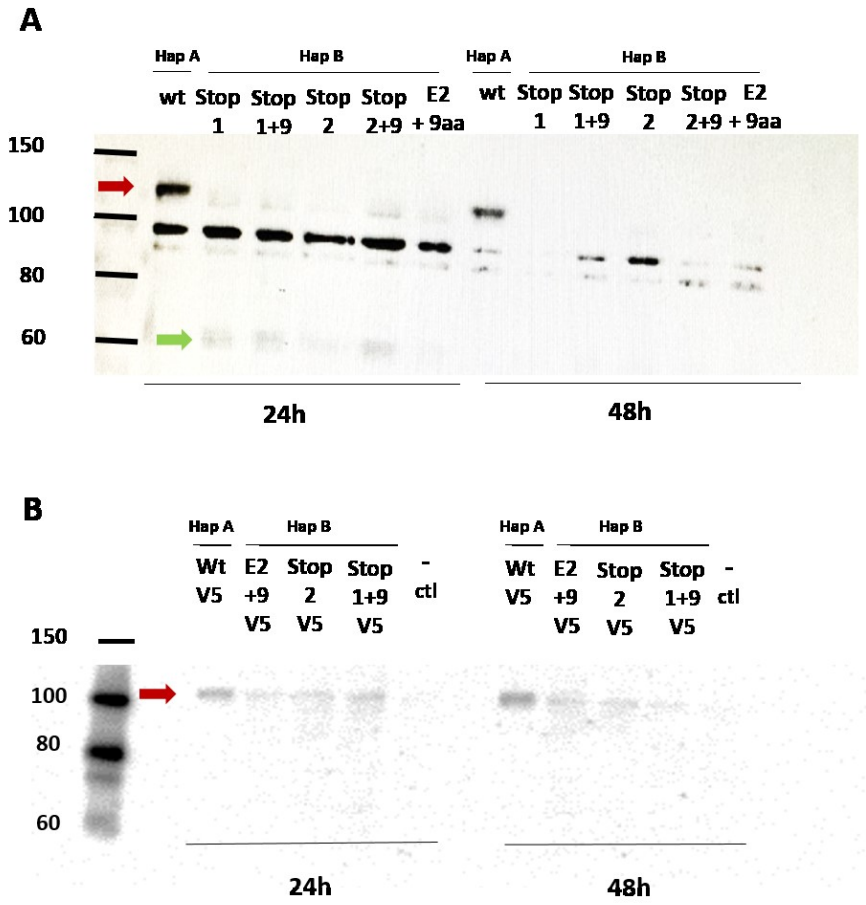


Figure 4.12 WB on cell lysates from transfected cells with pcDNA3 constructs (**A**) and V5 tag constructs (**B**). In **A** protein analysis shows the presence of a clear FL ERAP2 protein (120 KDa) in Hap A samples (indicated by red arrow), whereas weak signals at 120 and 60 kDa (predicted truncated ERAP2 protein HapB-derived; indicated by green arrow) are detected in Hap B samples at 24 hours post transfection. The 60kDa signal is lost in V5 blot (**B**) whereas a specific V5 signal at 120 kDa is still detectable. – *ctl* (cell lysis without V5 tag) is used as negative control.

An extracellular localisation of ERAP1 has been previously described by several studies, more recently data about extracellular ERAP2 has been published [212-214]. For this reason, we decided to analyse the protein

content also in cellular transfection supernatants, however analyses did not detect any ERAP2 protein (data not shown). However, this result is not unexpected since the secretion of ERAP1 and ERAP2 are related to a stimulation condition with LPS and IFNγ [212-213], stimuli not introduced in these experiments. Future analyses will be performed to characterise both ERAP2 FL and ERAP2 AS and whether they are secreted.

These protein data, even if not completely defined, are consistent because they suggest a possible presence of a skip-stop codon mechanism in Hap B, derived from the detection of an ERAP2 FL signal in V5 in cells transfected with Hap B ERAP2. These data contradict the assumption that ERAP2-AS (Hap B) mRNA undergoes a non-sense-mediated decay [120], however previous transfection experiments performed in our laboratory suggest Hap B mRNA might be translated [250]. Interesting this plausible skip-stop codon process could explain the intermediate frequency of the haplotype in most human populations. Moreover, future specific experiments will investigate the function and localization of the predicted protein products of Hap A/Hap B-derived transcripts and assess their impact on infection of cells with HIV.

DISCUSSION AND CONCLUSIONS

The immune response during HIV-1 infection and replication is quite complex because many viral and host factors, beyond individual variables, are involved at different levels [85]. Many efforts have been made to evaluate the influence of these factors on HIV-1 infection and disease progression, focusing mainly on the identification of genetic variants that can play a role in HIV-1 infection susceptibility. These studies have led to the concept of "immunological advantage", which would confer resistance to HIV to those individuals who possess it. The best prototype of such condition is represented by HIV exposed seronegative (HESN) subjects, whose phenotype has been consistently associated with a number of genetic correlates protecting them from repeated exposure to HIV-1. Some preliminary data suggest that peculiar genetic variants can also determine an optimal response to ART in HIV-infected subjects. Indeed, in a patient with 4 "protective" SNPs in APOBEC3H, TLR3, ERAP2 and MX2, enrolled at Santa Maria Annunziata Hospital in Florence, viremia declined rapidly and a significant recovery in CD4 + T cell count was observed after treatment. A similar trend was detected in a patient with 3 “protective” SNPs (APOBEC3H, ERAP2 and MX2) (data not shown). We must consider that since HIV-infected subjects contracted the infection, despite the presence of these protective haplotypes, the "immunological advantage" alone is not sufficient to confer a complete shield from HIV-1 infection. However, one or more haplotypes, considered to be "protective", could allow for a better control of disease progression if compared with a “susceptible" ones. Given these interesting findings, the first aim of this study was to determine whether there was a correlation between response to ART and the immune genetic profile of HIV-1-infected patients. The investigation was performed considering those immune genes known to be protective in viral infection/replication control in order to optimize pharmacological intervention in HIV-1-infected subjects. However, the results obtained in HIV-1-infected patients, from the

ICONA cohort, undergoing ART therapy did not show any significant correlations between the genotypes of TLR3, APOBEC3G and MX2 (single or combined) and the progression of HIV-1 infection, evaluated in terms of viral load and CD4+ or CD8+ T cell count. The only exception is represented by ERAP2 gene: in HIV-1-infected patients with a GG homozygosis conditions for SNP rs2549782 (Hap A), a significant reduction in viral load and a recovery in CD4+ T cells were observed compared to heterozygous or homozygous TT patients (Hap B). Analyses for CD8+ T cells showed no differences in CD8+ T cell count over 1 year-treatment in Hap A patients, conversely, Hap B patients showed a therapy related decrease in CD8+ T cell count over the year. This could be explained by a no difference in change/year in CD8+ T cell count over time in Hap B patients. The data is particularly significant taking into consideration the key role that ERAP2 plays in the activation of the immune response against viruses. In fact, ERAP2 enzyme trims the N- terminally extended residues to generate correct length peptides, which are subsequently loaded on MHC class I molecules and presented on the cell surface. This, in turn, modulates the antigen repertoire available for CD8+ T cell clone stimulation. Therefore, as ERAP2 diplotype status determines the abundance of the full length protein product of ERAP2, it is possible to speculate that peptides generated by homozygous Hap A subjects are “better” in terms of immunogenicity or quantity. The absence of correlations between the presence of a “supergenotype” and the progression of the disease is unexpected but plausible. First, the allelic variants considered to be "resistance genes" have only recently been correlated with susceptibility to HIV-1-infection in HESN subjects. Hence, we are not able to foresee if the same variants are able to control/block viral replication in HIV-infected patients, even if present in combination. In addition, the allelic variant associated with the TLR3 gene is related to an increase in receptor responsiveness to specific stimuli in *in vitro* infection assay, resulting in pro-

inflammatory cytokine production (IL-6, IL-1b, CCL3). This state of hyper-responsiveness has been linked to a reduced susceptibility to infection by many authors [251-253] and may therefore have a protective significance during exposure to the virus. However, it is commonly accepted that in HIV-infected patients the presence of a state of immune activation condition is correlated with an increased disease progression [254]. It is therefore likely that the same allelic variant plays a diametrically opposite role according to the phase of the considered viral cycle. Finally, these first results demonstrate that it is not possible to associate the existence of a "super genotype" (characterized by the presence of several “protective” allele variants) to the control of the infection progression or to a better ART therapy response. Nevertheless, positive data obtained in relation to the presence of GG genotype for ERAP2 (Hap A), support the necessity to deepen the role of this allelic variant in a case/control study, both in the progression of the infection and in response to various therapeutic treatments.

Another aim of this PhD project was to further the understanding into the mechanism of action of ERAP2 haplotypes in HIV-1 infection susceptibility. We performed an *in vitro* HIV-1 infection assay in PBMCs with different ERAP2 diplotype, analyzing ERAP2 role in two different experimental conditions: 1) by using an ERAP2 inhibitor; and 2) by adding a recombinant (rh) ERAP2 protein in cell cultures. As expected from published data, homozygous (Homo) Hap A subjects under HIV-1 infection, presented lower viral replication compared to homozygosis Homo Hap B ones in both experiments. Moreover, inhibition data showed a dose dependent increase of infection, evaluated by p24 levels, in both the haplotypes. This result seems to confirm a direct involvement of ERAP2 in resistance of HIV-1 infection. Remarkably, as increased viral replication was observed also in homo Hap B PBMCs treated with the inhibitor peptide we speculate that a

basal expression of ERAP2 FL protein is present even in Hap B subjects and/or that ERAP2-AS (Hap B) mRNA undergoes a non-sense-mediated decay (NMD) only partially [120]. Anyway, our data are in line with previous observations suggesting that high dose of gentamicin antibiotic, a natural inhibitor of NMD, do not affect the ERAP2-AS mRNA expression [250]. Further analyses are needed to ascertain if ERAP2-AS is expressed even at low doses in Homo Hap B subjects and which is its possible role in antigen presentation. Nevertheless data obtained herein confirm the presence of a correlation between ERAP2 expression modulation and antigen presentation. Indeed, following ERAP2 inhibition, we observed a massive increase in ERAP1 aminopeptidase and transporter TAP1 expression, suggesting the presence of compensation mechanisms in antigen presentation pathway. In addition, increased basal HLAABC (MHC class I) expression observed in Homo Hap A subjects, confirms the role of ERAP2 variants in antigen processing and presentation pathways. In the next future it will be extremely interesting to verify if such increase is associated with specific HLA class I variants besides HLA B27, whose interplay with ERAP2 has already been at least partially dissected [98].

The final aim of the project was to investigate and clarify the different modulation of peptide production mediated by the two ERAP2 haplotypes and the subsequent impact on T cell activation. Therefore, we investigated the different trimming phenotypes in DNA model constructs for the two haplotypes (Hap A and Hap B) in order to describe a molecular mechanism responsible for what observed in HIV-1 infection susceptibility phenotype. Results have shown a reduction in trimming function in Hap B models compared to the normal Hap A trimming activity. Notably, this latter effect results in a reduction of CD8+ T cell response observed in Hap B. These data are relevant because can explain a better ability in antigen presentation

related to Hap A, compared to Hap B subjects, and this could explicate the reduced susceptibility reported in *in vitro* HIV-1-infection on subjects carrying different haplotypes. It should be noted that we did not observe zero reduction in trimming function and in cleavage activity in Hap B models and, notably, it is coherent with what we observed from protein analyses. Indeed, a very low detectable FL ERAP 2 protein also in Hap B samples has been detected. This observation could explain the very low trim rate obtained in our Hap B models and again, it could be explained by the assumption that ERAP2-AS (Hap B) mRNA undergoes a non-sense-mediated decay (NMD) only partially [120].

Our cleavage data are in line with other observations that have demonstrated that ERAP2 SNP rs2549782 is not a loss-of-function variant, but rather a "change-in-function" variant that leads to substrate-specific changes in enzymatic activity, essentially allowing the enzyme to alter its specificity profile for peptide substrates [255]. On the final note, further analyses are rquired to investigate the different trimming phenotypes and the resulting peptide extracts by using specific techniques, such as reverse-phase HPLC, in order to finally assess the different quality and/or quantity of peptide extracts derived from ERAP2 haplotypes.

Evidence that ERAP1 and ERAP2 regulates precursor peptide proteolysis in the ER is unquestionable. However, recently, the possibility of an alternative subcellular localization of the two aminopeptidases has been widely discussed [136; 214]. Indeed, a cytosolic and extracellular ERAP1 localisation has been described by several studies and, even more recently, data demonstrating an extracellular ERAP2 localisation in cancer cell secretome were reported by Wu et al., [212-214]. For these reasons, we decided to investigate if once released in the extracellular milieu ERAP2 still maintains its antiviral function. Notably, recombinant ERAP2 addition to *in*

vitro HIV-1-infected PBMCs, without affecting cell viability, resulted in a reduction of viral replication in cells isolated by both Hap A and Hap B. This protective effect is independent from an increase of HLA-ABC expression and/or of perforin and granzyme expression by CD8+ T lymphocytes. We, therefore, might speculate that this defensive feature is mediated through an unconventional mechanism, distinct from immune system modulation. However, further investigations are needed to clarify the "physiological" role of ERAP2 in the extracellular milieu as well as the molecular pathway leading to this unusual ERAP2 localization. Similarly to ERAP1, once secreted by antigen presenting cells, ERAP2 could interact and regulate different and unpredictable targets thus regulating mechanisms far away from the one exerted in the ER.

To summarise, ERAP2 diplotype status is associated with resistance/progression to HIV-1 infection; this phenotype is due to a secondary effect on the control in antigen processing/presenting machinery, resulting in quantitative and possibly qualitative changes in MHC class I complexes on target cells and in a CD8+ T cell immune response modulation. This anti-HIV-1 function is preserved even following ERAP2 secretion in the extracellular environment. Further analyses are needed to ascertain the ERAP2 antiviral involvement in natural resistance to and progression of HIV-1 infection. These results, nevertheless, suggest a possible basis for therapeutic interventions to control of HIV-1 infection.

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SCIENTIFIC PRODUCTS

List of scientific products produced during the PhD Course (October 2015-October 2018).

Abstracts

- **CROI 2019:** "ERAP2 ADMINISTRATION REDUCES IN VITRO PBMCs SUSCEPTIBILITY TO HIV-1-INFECTION" Irma Saulle, Salomè Valentina Ibba, Cecilia Vittori, Claudio Fenizia, Federica Piancone, Davide Minisci, Elisa Maria Lori, Daria Trabattoni, Mario Clerici, Mara Biasin (Submitted)

- **HIVR4P 2018**: "Evaluation of Protective Genetic Variants in HIV-1-Infected cART Treated Patients "Elisa Maria Lori, Alessandro Cozzi-Lepri, Mara Biasin, Vincenzo Mercurio, Sergio Lo Caputo, Francesco Castelli, Antonella Castagna, Andrea Gori, Giulia Marchetti , Carolina Venditti , Mario Clerici, Antonella D'Arminio Monforte

- **10th Annual Cancer Sciences Unit Conference 2018**: "Endoplasmic reticulum aminopeptidase 2 haplotypes modulate HIV infection susceptibility" Elisa Maria Lori, Emma Reeves, Mara Biasin, Edward James

- **Poster Showcase Southampton 2018**: "Endoplasmic reticulum aminopeptidase 2 haplotypes modulate HIV infection susceptibility" Elisa Maria Lori, Emma Reeves, Mara Biasin, Edward James

- **ICAR 2018:** "ANALYSES OF PROTECTIVE GENETIC POLYMORPHISMS IN HIVINFECTED CART TREATED PATIENTS" Elisa Maria Lori, Alessandro Cozzi-Lepri, Mara Biasin, Vincenzo Mercurio, Sergio Lo Caputo, Francesco Castelli, Antonella Castagna, Andrea Gori, Giulia Marchetti, Carolina Venditti, Mario Clerici, Antonella D'Arminio Monforte.

- **IAS 2017:** "INTERLEUKIN -21/microRNA-29 AXIS IN NATURAL RESISTANCE TO HIV-1 INFECTION" Paula Andrea Serna Ortega , Irma Saulle , Vincenzo Mercurio , Elisa Maria Lori , Claudio Fenizia , Daria Trabattoni1 , Sergio Lo Caputo , Francesca Vichi , Francesco Mazzotta , Mario Clerici , Mara Biasin

Publications

- **"ANALYSES OF PROTECTIVE GENETIC POLYMORPHISMS IN HIVINFECTED CART TREATED PATIENTS"** Elisa Maria Lori, Alessandro Cozzi-Lepri, Mara Biasin, Vincenzo Mercurio, Sergio Lo Caputo, Francesco Castelli, Antonella Castagna, Andrea Gori, Giulia Marchetti, Carolina Venditti, Mario Clerici, Antonella D'Arminio Monforte. (In submission)

- **The interleukin 21 (IL 21)/microRNA-29 (miR-29) axis is associated with natural resistance to HIV-1 infection**." Paula Andrea Serna Ortega , Irma Saulle , Vincenzo Mercurio , Elisa Maria Lori , Claudio Fenizia , Daria Trabattoni1 , Sergio Lo Caputo , Francesca Vichi , Francesco Mazzotta , Mario Clerici , Mara Biasin- AIDS (London, England) (2018)