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ENDOPLASMIC RETICULUM AMINOPEPTIDASE 2 HAPLOTYPES PLAY A ROLE IN MODULATING SUSCEPTIBILITY TO HIV-1 INFECTION

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SOMMARIO

ERAP2 è una proteina associa al reticolo endolasmatico coinvolta nella presentazione di peptidi antigenici al complesso maggiore di istocompatibilità di classe I (MHC I). Si distinguono due differenti aplotipi del gene ERAP2, l'aplotipo A e l'aplotipo B, caratterizzati dalla presenza di numerosi polimorfismi a singolo nucleotide (SNPs) in linkage disequilibrium tra loro. In particolare nell'aplotipo B la presenza di uno SNP a livello dell'introne 10 determina uno slittamento del sito classico di splicing generando un mRNA maturo che determina, in fase di traduzione, la prematura interruzione della sintesi proteica e la conseguente produzione di una proteina tronca. Al contrario, l'aplotipo A, genera un trascritto full-lenght (ERAP2-FL) che codifica per la proteina canonica di ERAP2(Andres et al., 2010). Recentemente la presenza dell'aplotipo A e B di ERAP2, è stata correlata ad una diversa suscettibilità all'infezione da HIV-1. In particolare, è stata rilevata una maggiore frequenza dell'aplotipo A in una coorte di soggetti Italiani che, nonostante ripetute esposizioni al virus dell'HIV, tramite rapporti sessuali non protetti con partner sieropositivi (HIV exposed seronegative,HESN), non si infettano e non mostrano i segni clinici dell'AIDS (Cagliani et al., 2010). Alla luce di queste osservazioni, lo scopo del mio progetto di ricerca è stato quello di approfondire la correlazione esistente tra le varianti alleliche del gene *ERAP2* e la naturale resistenza all'infezione da HIV-1 analizzando l'influenza che tali varianti esercitano nel meccanismo di presentazione antigenica.

Metodi: ERAP2 è stato genotipizzato in una coorte di 104 HESN Spagnoli tossicodipendenti esposti al virus tramite scambio di siringhe infette (IDU_HESN) e in 130 maschi bianchi sani utilizzati come controllo. Parallelamente, su 139 controlli sani (HC) suddivisi in funzione del genotipo di ERAP2 (AA, BB, AB) è stato condotto un saggio di infezione con HIV-1 BaL R5 tropico e sono stati analizzati i seguenti parametri: 1) livelli di espressione di ERAP2-FL, ERAP2-SA ed ERAP2-Tot e di 84 geni coinvolti nella via di presentazione antigenica; 2) concentrazione dell'antigene virale p24; 3) intensità media di fluorescenza (MFI) dei complessi MHC di classe I su cellule CD45; 4) espressione delle proteine derivate dalla forma FL e SA di ERAP2 con Western Blot. Infine abbiamo analizzato la co-segregazione dell'aplotipo HLA-B57 con quello di ERAP2 in una coorte Italiana di HESN esposti al virus tramite rapporti eterosessuali non protetti.

Risultati: I risultati ottenuti hanno confermato una maggior frequenza del genotipo A nei soggetti HESN Spagnoli rispetto al gruppo controllo a sostegno di un modello recessivo di protezione nei confronti di HIV-1. La meta analisi condotta su dati precedentemente pubblicati relativi ad una coorte di soggetti HESN italiani conferma l'associazione tra il genotipo omo A e la naturale resistenza all'infezione. Inoltre è stata individuata la co-presenza dell'aplotipo HLA-B57 con quello omozigote per l'aplotipo A. I dati

ottenuti sui 139 controlli sani, dopo il saggio di infezione con HIV-BaL, mostrano un aumento statisticamente significativo dell'mRNA di ERAP2-FL/TOT, di numerosi geni coinvolti nelle vie di presentazione antigenica e di molecole HLA-ABC sulla superficie cellulare leucocitaria in soggetti omo A.Questi risultati correlano con una riduzione statisticamente significativa dei livelli dell'antigene virale di HIV p24 in cellule periferiche mononucleate del sangue ma non in linfociti T CD4^{+}. Infine la forma tronca della proteina non è stata rilevata in nessuno dei soggetti presi in esame.

Conclusioni: I risultati ottenuti suggeriscono che negli HESN il genotipo omoA di ERAP2 è correlato alla naturale esistenza all'infezione da HIV-1 indipendentemente dalla via di esposizione al virus; che i livelli di espressione di ERAP2-FL ed SA differiscono in soggetti con diverso genotipo di ERAP2 e correlano con una diversa espressione sia di molecole HLA-ABC sulla superficie cellulare che di geni implicati nel pathway di presentazione antigenica. Complessivamente, i risultati ottenuti lasciano presupporre che il genotipo A di ERAP 2 sia in grado di controllare la resistenza all'infezione da HIV-1 tramite il potenziamento dell'immunità cellula mediata.

ABSTRACT

Background: The endoplasmic reticulum aminopeptidase type 2 (*ERAP2*) undergoes haplotype-specific alternative splicing which results in the production of either full-length (FL, from haplotype A) or alternatively spliced (AS, from haplotype B) mRNA Resistance to HIV infection in a cohort of Italian sexually HIV-exposed seronegative individuals (HESN) is recently associated with a variant in *ERAP2* that tags haplotype A. As the protein product of this gene trims peptides loaded on MHC class I, shaping the quality of viral peptides that are presented to CD8$^+$ T cells, we further analysed its role in HIV-1-susceptibility.

Methodology: *ERAP2* was genotyped in a cohort of 104 Spanish HESN individuals exposed to HIV through injection drug use (IDU-HESN). In parallel PBMCs isolated from 139 healthy controls (HC) grouped according to their *ERAP2* genotype were infected with HIV-1$_{Ba-L}$ and analysed for: 1) expression levels of mRNA specific for *ERAP2* and for 84 genes involved in the antigen presentation pathway; 2) MHC class I MFI on CD45$^+$ cells; 3) susceptibility to HIV-1 infection; 4) ERAP2 protein expression. Finally, we analyzed the co-segregation of *HLA-B*57* with *ERAP2* haplotype in a previously described cohort of Italian sexually-exposed HESN.

Results: Genotype analysis in IDU-HESN mirrored the previous association between *ERAP2* haplotype A and protection from HIV infection; meta-analysis with the Italian cohort yielded a *p* value of 7.6 x10^{-5}. Co-segregation analysis indicated that the *HLA-B*57* allele is significantly more common among HESN homozygous for haplotype A (homo A) compared to non-homo A (p=0.01), suggesting genetic interaction.

Data obtained in HC showed that following in vitro HIV infection the expression of mRNA for ERAP2-FL and a number of genes involved in antigen processing and presentation as well as of MHC class I on the surface of CD45$^+$ cells are significantly increased in homo A cells; notably, homoA peripheral blood mononuclear cells, but not isolated CD4 cells, were less susceptible to HIV-1 infection. Only the expression of ERAP2-FL could be detected by western blot analyses.

Conclusions: haplotype A of *ERAP2* confers resistance to HIV infection independently of the route of exposure; such variation possibly affects MHC presentation and immune response.

LIST OF ABBREVIATIONS

AIDS: acquired immune deficiency syndrome

A-LAP: adipocyte-derived leucine aminopeptidase

APOBEC: apolipoprotein b mrna-editing enzyme-catalytic polypeptide-like

ART: anti-retroviral therapy

ARTS: aminopeptidase regulating type i tnf receptor

AS: ankylosing spondylitis

ASP: asparagine

ATP: adenosine tri-phosphate

CCR5: C-C chemokine receptor type 5

cDNA: complementary deoxyribonucleic acid

CDK: cyclin-dependent kinase

CRF: circulating recombinant forms

CSW: commercial sex worker

Ct: threshold cycle

CTL: cytotoxic t lymphocytes

CTLA: cytotoxic t-lymphocyte antigen,

CXCR4: C-X-C chemokine receptor type 4

DCs: dendritic cells

DNA: deoxyribonucleic acid

dsRNA: double-stranded ribonucleic acid

EC: endothelial cells

ER: endoplasmic reticulum

ERAAP: endoplasmic reticulum aminopeptidase associated with antigen processing

ERAP: endoplasmic reticulum aminopeptidases,

ERAP2-AS: endoplasmic reticulum aminopeptidases alternative splicing

ERAP2-FL: endoplasmic reticulum aminopeptidases alternative splicing - full length

ESN: exposed seronegatives

FC: fold change

FGT: female genital tract

GALT: gut-associated lymphoid tissue

Gp: glycoproteins

GWAS: Genome-wide association study

HCMV: human cytomegalovirus

HCV: hepatitis C infection

HeLa cells: uterine cervix carcinoma-derived cells

HESN: HIV-exposed sero-negatives

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

HPC5: HLA complex P5

HPV: human papillomavirus

HTLV: human T-lymphotropic virus -

IDU-HESN: injection drug users-HIV exposed seronegative

IFN: interferon

Ig: immunoglobulin

IL: interleukin

LAG: lymphocyte activation gene

LCMV: lymphocytic choriomeningitis virus

LD: linkage disequilibrium

LNPEP: leucyl-cystinyl aminopeptidase

LPS: lipopolysaccharide

L-RAP: leukocyte-derived arginine aminopeptidase

LTNP: Long Term Non Progressor

MACS: Multicenter AIDS Cohort Study

MEU: multiple exposed uninfected

MHC: major histocompatibility complex class

MIP: macrophages inflammatory protein

miRNA: microRNA

MSM: man that have sex with man

Nef: negative factor

NK:.natural killer

NMD: non-sense mediated decay

OPD: ortho- phenylenediamine-HCl

PBMCs: Peripheral Blood Mononuclear Cells

PCR: polymerase chain reaction

PD: programmed death

PDB: Protein Data Bank

PDI: protein disulfide isomerase

PDK: phosphatidylinositol-dependent kinase

PILS-AP: puromycininsensitive leucine-specific aminopeptidase,

PLC: peptide loading complex

pMHC: peptide-MHC

PRR: pattern recognition receptors

RNA: ribonucleic acid

RT: retro-transcriptase

RT-PCR: real time - polymerase chain reaction

SNP: single nucleotide polymorphisms

SSP: sequence specific primer amplification

ssRNA: single-stranded RNA

HRP: horseradish peroxidase

TAP: transporters associated with antigen processing

TCRs: T cell antigen receptors

Tim: T cell immunoglobulin domain and mucin domain

TLRs: Toll-like receptors,

TNF: tumor necrosis factor

Tpn:,tapasin

VEGF: vascular endothelial growth factor

Vif: virion infectivity factor

Wt: wild-type

CHAPTER 1. INTRODUCTION

1.1 Origin and history of the HIV/AIDS

The human immunodeficiency virus (HIV) infection became evident in 1981 by association of mortal cases of *Pneumocystis cariini* interstitial pneumonia among male homosexuals in Los Angeles who also characterized an intense state of immunodeficiency and complete loss of CD4$^+$ T lymphocytes from their peripheral blood.

The viral etiology in 1983, was identified for the first time in a new retrovirus particle by Fracoise Barré-Sinoussi and Luc Montignier (for which they won the Noble Prize in 2008) and confirmed in 1984 by the team of Robert C. Gallo to be the etiological agent of acquired immunodeficiency syndrome (AIDS) (Vicenzi at al., 2013).

In 1984, HIV was found to be present in central Africa (Van de Perre P., 1984. Piot Pet al., 1984).

Later HIV seroposivity among two thirds of Nairobi prostitutes were described, demonstrating the presence of the epidemic in East Africa (Kreiss JK., et al., 1986). In 2012, the World Health Organization established that 2.5 million people became newly infected with HIV and that there were 34 million people living with the virus worldwide. It is estimated today that more than 34 million persons have been infected and, in large proportions, died of HIV infection or of AIDS-associated diseases. (Vicenzi at al., 2013). In fact, the state of profound immunodeficiency caused by HIV infection leads to the co-infection by other opportunistic pathogens, like Mycobacterium tuberculosis, hepatitis viruses, degeneration of central nervous system as well as the development of tumors mostly Kaposi's Sarcoma and B-cell lymphoma.

1.2 HIV Virology

1.2.1 HIV classification and structure

HIV belongs to the viral Retroviridae family and the genus Lentivirus. HIV group M can be subdivided into 11 major clades (A1, A2, B, C, D, F1, F2, G, H, J and K). Circulating recombinant forms (CRF) also contribute to the extensive diversity of HIV subtypes. Clades A1, D and CRFs are predominant in Kenya, whereas clade B is prevalent in North America and Europe (Thomson MM et al., 2005).

Two different types of HIV cause the same syndrome but differs for genome structure and antigenicity: HIV-1 is responsible for the AIDS syndrome in America, Europe and Central Africa, while HIV-2 is more prevalent in West Africa and in Asia (Clavel et al., 1986).

HIV is an enveloped virus containing two identical positive sense single-stranded RNA (ssRNA) genomes that colocalize in the icoshaedral core. The HIV genome encodes the envelope (Env) glycoproteins (gp120 and gp41), the internal structure core (Gag) proteins (p24, p9, p7 and p17), the enzymatic polymerase (Pol) proteins (reverse transcriptase (RT), integrase (IN) and protease (PR)) and the accessory proteins Vif, Vpr, Rev, Vpu, Tat and Nef (Turner et al., 1999).

Nef is a virion–associate protein, originally considered "negative factor" for virus replication. Deletion of nef gene resulted in an asymptomatic infection in macaques that also showed a significant resistance to super-infection by wild-type SIV (Daniel et al., 1992).

Vif is the acronym of "virion infectivity factor". Indeed, Vif can be considered the prototype of viral antagonist of host-cell factors capable of restricting or preventing virus infection or replication.

The accessory protein Vpu is present only in HIV-1. It functions like Nef and gp120 Env, contributes to the down-regulation of CD4$^+$Tcells from the plasma membrane of infected cells (Dube et al., 2010). In addition, Vpu

counteracts a host cell protein, named BST-2 or Tetherin, which plays a role in the final step of the virus life cycle, i.e. budding and release of new progeny virions (Andrew A, et al., 2010).

Like Tat the virion–associated protein Vpr was early identified as a transcriptional activator of HIV-1, particularly effective in non-dividing cells such as mature macrophages (Subbramanian et al., 1998). In addition, Vpr has been described as modulator of several additional steps in the virus life cycle, including reverse transcription. Vpr makes part of the pre-integration complex (PIC) that shuttles the viral genome to the cellular nucleus while undergoing conversion from RNA into DNA as result of the virion–associated reverse transcriptase enzyme. At cellular level, Vpr induces cell cycle arrest in the G2/M phase, but also apoptosis.

The virion-associated protein Vpx is present only in HIV-2, and in many SIV strains. Its expression facilitates virus replication in both T cells and macrophages. Recently Vpx was demonstrated to interact with host cellular target SAMHD1 that hydrolyzes the deoxynucleoside triphosphates required for the synthesis of HIV-1 and 2 DNA (Lahoussa H et al., 2012).

1.2.2 HIV life cycle

HIV enters target cells by binding of the viral envelope protein gp120 to CD4$^+$ cells, which is expressed on cellular surface of different cell types. A conformational change allows gp120 to bind either of the coreceptors (CCR5 or CXCR4), depending on viral tropism (Greene WC et al., 2002). Transmission of viral infection generally occurs in a CCR5-dependent manner, as has been demonstrated by viral tropism determination of transmitted isolates.

Following receptor binding, HIV enters in the cell by fusion between the viral envelope and in the cell membrane. Reverse transcription results in a single stranded complementary DNA copy, which is replicated to produce

the double stranded DNA proviral genome (Liu R et al., 1996). The process of reverse transcription is error-prone, leading to extensive diversity of progeny genomes. The proviral genome is transported to the nucleus and linear form of the double stranded DNA viral genome is inserted into the host chromosome. The integrated viral genome can remain latent for the lifetime of the host cell. However, binding of host factor to the 5' long terminal repeat (LTR) region can lead to transcription of the viral genome. Translation of HIV transcripts produces polyproteins that are subsequently cleaved by viral and cellular proteases into individual viral proteins. Assembly occurs at the plasmamembrane and is followed by budding of progeny virions out of the host cell (Greene et al., 2002) (Fig.1.1)
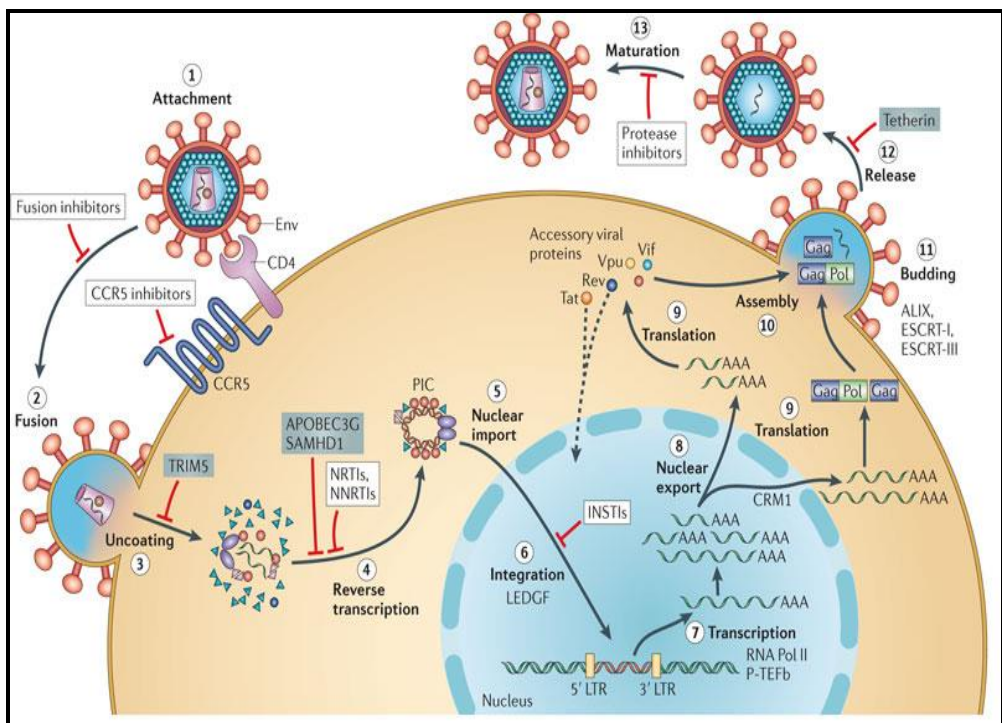
Figure 1.1 *HIV life cycle* (Engelman & Cherepanov *Nature Reviews Microbiology 2012*)

1.3 HIV pathogenesis

1.3.1 HIV transmission

HIV transmission is due to contact with infected body fluids. This may occur through sexual transmission (heterosexual or homosexual), parenteral transmission (injection drug use, receiving contaminated blood products), or vertical transmission (transmission of HIV from mother to child in utero or trough breast-feeding). Although transmission dynamics vary by population, heterosexual transmission accounts for the majority of incident HIV cases worldwide (Simon V et al., 2006).

1.3.2 Disease progression

The characteristic passage of HIV disease progression in the absence of ART is shown in Figure 1.2. After transmission, acute HIV infection typically lasts up to three months. Viremia peaks within a few weeks, and is followed by a sharp drop in viral load that coincides with the emergence of HIV-specific $CD8^+$ T cells. This low point in viral load is termed the viral set point (Helz et al., 2006) and is an indicator of HIV disease progression, such that individuals with low viral set points tend to exhibit slower progression to AIDS (Mellors et al., 1996). During acute infection, $CD4^+$ T cell levels decline in the periphery but rebound as viral replication is contained. $CD4^+$ T cells in the gut-associated lymphoid tissue (GALT) are extensively depleted early in infection, leading to prompt dysregulation of gut immunity. Acute infection is followed by a much longer chronic phase, in which there is excessive $CD4^+$ T cell turnover and progressive decline of $CD4^+$ T cells in the periphery. The onset of AIDS occurs when the $CD4^+$ T cell count falls below 200 cells/µl of blood (Stevenson et al., 2003). At this point, the immunity system is unable to cope with infectious challenges, and the host eventually succumbs to opportunistic infections.
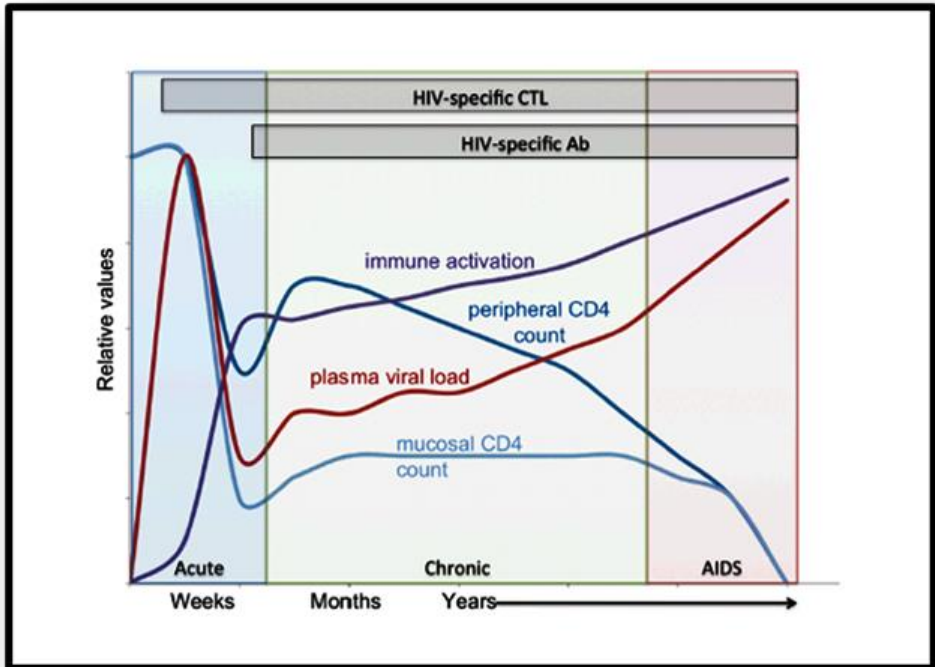
Figure 1.2 *Clinical and immunologic events associated with disease progression in untreated HIV-1 infection*

1.4 HIV immunology

1.4.1 Innate response to HIV infection

The first line of defense against infection is the innate immune system and consists of innate immune cells, which are able to recognize and respond to infections quickly through the recognition of pathogens by pattern recognition receptors (PRR). These receptors include Toll-like receptors (TLRs), which recognize conserved motifs unique to microorganisms; in viral infections, they can detect double-stranded RNA (dsRNA) and single-

stranded RNA (ssRNA), as well as certain viral proteins (Diebold et al., 2004). It has been shown that HIV-1 ssRNA encodes for multiple TLR7/8 ligands that can mediate direct activation of the immune system *in vitro*. Stimulation of TLR7/8 induces the production of several antiviral and immunomodulatory cytokines. In particular, interferon (IFN) production after TLR stimulation has been shown to be up regulated during acute infection with HIV-1 or SIV (Stacery et al., 2009). These early cytokines and the innate cells that produce them, such as dendritic cells (DCs), are pivotal in shaping the immune responses that develop in acute or early HIV-1 infection. Recently, the cascade of cytokine production in the periphery has been thoroughly documented, showing an initial rapid increase and peaking of IFN-α production, followed by secondary tumor necrosis factor (TNF) α, and interleukin (IL) 18 secretion, and finally IL-10 and IFN-γ production, which was associated with the rise in virus-specific adaptive immune response (Norris et al., 2006).

The comprehensive mechanism of IFN-α inhibition of HIV-1 is not well characterized, but in vivo elevation of IFN-stimulated genes (ISGs) has been observed in both gene expression profiling of HIV-1 and SIV infection. The *in vitro* inhibitory effects of IFNα on HIV-1 replication have been described in macrophages, monocytes, and humanized mouse models of HIV-1 infection (Lapenta et al., 1999). In addition to the activation of plasmacitoid dendritic cells (pDCs) through innate pattern recognition receptors, acute HIV-1 infection also results in the activation and expansion of natural killer cells (NK). In part, this activation of NK cells might be caused by high levels of proinflammatory cytokines secreted by DCs and monocytes, including IL-15 and IFNα. Initial studies demonstrated a significant expansion of NK cell numbers in acute HIV-1 infection, in particular before the development of any detectable antibody responses (Alter et al., 2007). After this initial expansion of highly activated NK cells,

NK cells become increasingly impaired, with persisting viral replication and disease progression.

1.4.2 HIV-specific B cells and antibodies
The role of B cells in a protective immune response to HIV is contentious. Neutralizing antibodies are not correlated with control of viremia. However, non-human primate (NHP) studies have demonstrated passive protection of neutralizing monoclonal antibodies against virus challenge (Mascola JR et al., 2000). In addition, recent studies have identified several highly potent antibodies with broad neutralization coverage of HIV (Walker et al., 2011), leading support for vaccine strategies aimed at inducing neutralizing antibodies.

Spontaneous activation of immunoglobulin G (IgG) secreting B cells and hypergammaglobulinemia were among the first immune dysfunctions reported in AIDS patients. A wide variety of B cell pathologies have been noted since then, recently reviewed in (Nicole et al., 2006)In the past year, B cells of HIV-infected patients were shown to have an abnormal distribution of various subsets: a decreased frequency of a CD27+B220− memory B subset and an increased frequency of CD20lo/−CD27hiCD38hi plasmablasts compared to uninfected controls. Tissue-like memory B cells, which have properties of exhausted cells (e.g. reduced proliferation and a truncated replication history) and the unusual phenotype CD27−CD20hiCD21lo, are shown to be increased in HIV-infected patients compared to uninfected controls. In addition, B cells of HIV-infected patients have an altered chemotactic capacity. Some changes in the B cell compartment, such as the proportions of immature/transitional cells, normalize to some extent during antiretroviral therapy although decreased frequencies of IgM memory B cells may persist. The majority of gp140-labelled B cells are class-switched memory cells. In addition, although

plasmablasts are increased in HIV-infected patients, only a small fraction are observed to stain with gp140. Thus, it is possible that a large fraction of the plasmablasts that are increased in chronic HIV is not HIV-specific unlike the situation in acute responses to influenza vaccination (Nicole et al., 2006).

1.4.3 HIV-specific T cells

HIV-specific CD8^{+} T cells arise within the first few weeks of infection as peak viremia declines, and correspond with the emergence of viral escape of mutation, implicating CD8^{+} T cells in initial control of viral replication. In line with this, CD8^{+} T cells isolated from HIV-infected individuals can inhibit viral replication in vitro (Saez-Ciriòn et al., 2007), and depletion of CD8^{+} T cells from SIV-infected macaques results in a loss of viral control and rapid disease progression (Jin X et al., 1999). The earliest CD8^{+} T cell responses are often specific for HIV Env and Nef proteins, although responses directed at Gag are associated with viral control later in infection (Turnbull et al., 2009). There are a large number of studies that convincingly demonstrate a requirement for HIV-specific CD8^{+} T cells in control of viral replication (Betts et al., 2006), but effective CD4^{+} T cell help is also required (Sun et al., 2003).

HIV infects and progressively depletes CD4^{+} T cells, and HIV-specific memory cells are particularly susceptible to HIV-1 infection (Douek et al., 2002). However, early administration of Anti-Retroviral Therapy (ART) can rescue strong HIV-specific CD4^{+} T cell responses. Robust HIV-specific CD4^{+} T cell responses are associated with better control of HIV viral replication (Potter et al., 2007) and HIV undergoes mutational escape from CD4^{+} T cell epitopes (Rychert et al., 2007).

During chronic infection, exposure to HIV-specific T cells to persistent *viremia* leads to functional exhaustion. T cell exhaustion is characterized by

loss of proliferation capacity as well as polyfunction and cytotoxic ability, followed by apoptosis. Functional exhaustion is reflected by up-regulation of different inhibitory molecules on the cell surface, including programmed death 1 (PD-1), Cytotoxic T-Lymphocyte Antigen (CTLA)-4, lymphocyte activation gene (LAG)-3, T cell immunoglobulin domain and mucin domain (Tim)-3, 2B4 (CD244) and CD160 (Wherry 2011). PD-1 has been identified as a critical negative regulator in HIV infection (Zhang et al., 2007), and Tim-3 has been linked to elevated sensitivity to Treg-mediated suppression (Elahi et al., 2011).

1.5 Models of Natural Protection

1.5.1 HIV-exposed seronegative individuals (HESN)

A characteristic feature of infectious diseases is that only a percentage of exposed individuals do become infected. Despite this awareness, during the first years after the explosion of the AIDS epidemics, little consideration was paid to the observation that parenteral exposure to HIV-1 does not consistently induce infection, and to the possibility that such putative non-infectious exposure might be secondary to inheritable factors.

Early epidemiologic studies from the UCLA Multicenter AIDS Cohort Study (MACS) (Inagawa et al., 1989) indicated that in a small percentage of homosexual men, frequent unprotected sexual contacts did not necessarily result in HIV sereoconversion. Interestingly, using peripheral blood leukocytes, HIV-specific T helper cell responses are shown to be present in these repeatedly exposed seronegative individuals, suggesting immunologic and/or genetic protection (Clerici et al., 1992). Shortly thereafter, other investigators reported the presence of a differential susceptibility to HIV infection in repeatedly-exposed female sex workers in Kenya (Plummer et al., 1993). Resistance to HIV infection was subsequently described in intravenous drug users, health care workers

accidentally exposed to HIV, homosexuals or heterosexual subjects that have unprotected sex with their seropositive partners, and hemophiliacs who had received HIV-contaminated anti-clotting factors. A particularly interesting cohort is represented by uninfected newborns of HIV-infected mothers. These infants are exclusive in that most of them are uninfected at birth (65-75%) despite having been continuously exposed to a HIV-saturated environment during pregnancy; and are HIV seropositive, due to passively transferred maternal anti-HIV antibodies. During the first two decades after the discovery of this phenomenon, HIV-exposed uninfected individuals were referred to as exposed seronegatives (ESN), exposed uninfected (EU) or multiple exposed uninfected (MEU). Since the early 90s, numerous studies examined such cohorts of ESN in the attempt to define immunological and/or genetic correlates of protection to HIV infection, but none of the identified mechanism has so far been able to completely shed light on this phenomenon. Furthermore, efforts to compare results obtained in different cohorts of ESN and in different laboratories have been problematic for many reasons, including the lack of a codified and clear definition of who should be classified as ESN.

In November 2009 the main investigators that had contributed to define the ESN/EU phenotype organized the first *International Symposium on Natural Immunity to HIV* which was held in Winnipeg, Canada, to definitely delineate the inclusion criteria of the ESN individuals and to compare the results of studies performed on different ESN cohorts. This first convention was followed by a second meeting that was held in Rockville, Maryland, in June 2010. At the second conference the decision was made to refer to these individuals as HESN, an acronym for HIV-Exposed Sero-Negatives. The results of these meetings were gathered in a supplementary number of the Journal of Infectious Diseases that summarizes the state of the art, as of the end of 2010 (JID Volume 202 Supplement 3 November 1, 2010)

While several HESN cohorts have been identified worldwide and are currently being studied by different groups, key information are still lacking. For instance, the relative contribution of genetic and immune factors to determine the phenomenon of resistance to HIV is not yet clarified. Notably, the possibility to study the early dynamic of this phenomenon is hindered by the definition of the resistance phenotype itself. In fact, the HESN status is assigned only after an individual has been able to efficaciously resist infection -typically over a period of years- despite multiple, frequent, and repeated documented exposures to the virus. Thus, researchers can only observe and compare the consequences of potentially important immunological changes that likely occurred years before the HESN status could be defined. Even so, if a genetic profile is at least partially responsible for this phenomenon it should be possible to identify this/these factor(s) at any time after exposure. Furthermore, once a putative protective variant gene is associated to the resistant phenotype, analyses can be easily performed using ex vivo infection assays on cells isolated from healthy controls carrying that specific mutation; it is therefore worthwhile to concentrate energy in this research field.

Luckily, the integration of candidate gene approach with genome wide studies has allowed to identify a considerable number of genetic variants responsible for HIV-infection and the control over AIDS progression (Fig. 1.3).
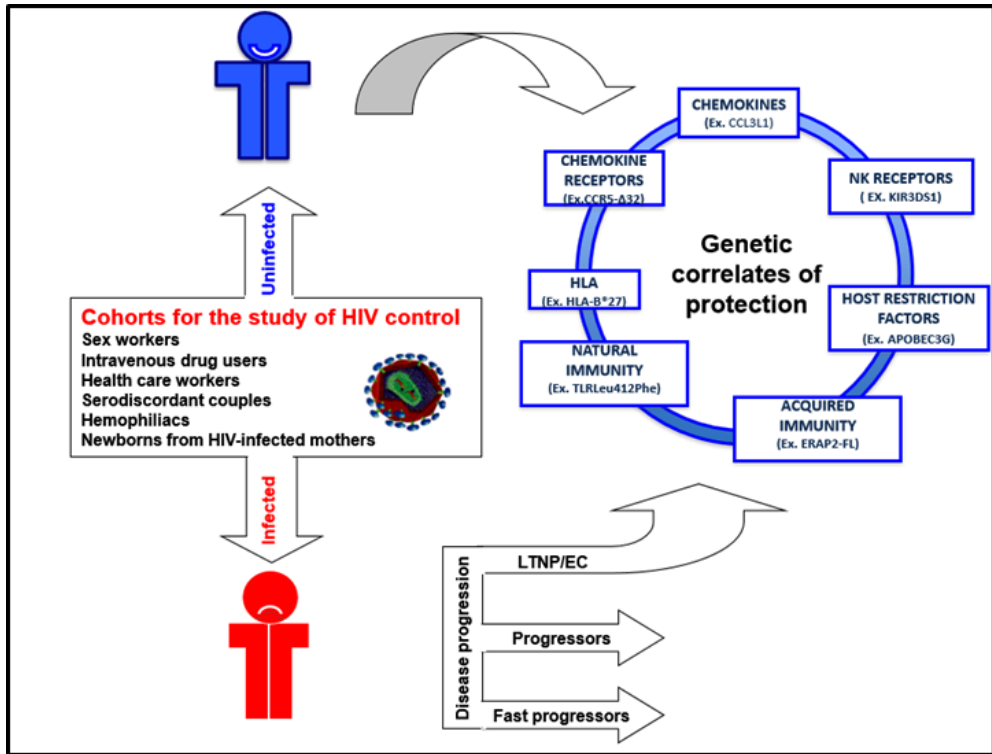
Figure1.3 *Different cohorts that control HIV infection are used to assess the impact of hereditably factors on HIV-1 infection and/or progression. The main genetic correlates of protection to HIV infection so far identified by candidate gene approach are summarized*

1.5.2 Genetic correlates of protection

1.5.2.1 Candidate-gene studies

Candidate-gene studies imply an a priori knowledge of the gene function in HIV-1 pathogenesis (Shea et al., 2012). This approach selectively takes into consideration only variants in those genes known to be directly implicated in the HIV-1 life cycle or in the immune response to the virus, thus excluding still unidentified genetic factors potentially able to interfere with HIV-infection. Despite this apparent limit, most of the AIDS restriction genes identified so far with this approach have been subsequently confirmed by genome wide studies and essentially fall within the human leukocyte antigen (HLA) class I locus (An et al., 2010). The protective effect

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of HLAB57, for example, has been confirmed in different studies in which the authors highlighted the ability of HLAB57-restricted cytotoxic T lymphocytes (CTL) to target multiple HIV peptides. The B5701 and the B5703 alleles (present mostly in Caucasians and Africans, respectively) exhibit a broad cross-reactivity against both common and rare variants of dominant gag epitopes, and have been associated with delayed progression of HIV disease (Makadzange et al., 2006; Boutwell et al., 2009). Likewise, the expression of HLAB*27 has been shown to exert a protective effect by maintaining a high steadiness between the HLA B pocket and the arginine in position 2 of the gag peptide. In fact, mutations in this position lead to both instability of the B27-gag complex and faster progression to AIDS (Kelleher et al., 2007).

Conversely, the B*35 allele was associated with a more rapid disease progression (Gao et al., 2001). Specifically, the B*35-Cw*04 haplotype is shown to be significantly associated with disease development in Caucasians but not in African-American (Carrington et al., 1999); suggesting that this variation exerts a negative influence on HIV pathogenesis (Huang et al., 2009).

More recently, the HESN phenotype was associated with another gene involved in the antigen presentation pathway: the endoplasmic reticulum aminopeptidases 2 (ERAP2) (Cagliani et al., 2010). This enzyme trims peptides to optimal size for loading onto HLA I molecules and shapes the antigenic repertoire presented to CD8^{+} T lymphocytes. The haplotype network of ERAP2 is highly structured with two differentiated haplogroups: haplotype A and haplotype B. Haplotype A has a higher frequency in HESN and leads to the expression of a 960 amino acid full-length protein. Conversely, haplotype B, because of an alternative splicing, generates a truncated protein of 534 amino acids (Andres et al., 2010).

The association between NK activation and resistance to HIV infection has been established by diverse genetic studies. Jennes reported that African HESN sex workers are characterized by a higher frequency of inhibitory KIR genes in the absence of their associated HLA genes, and a higher rate of the AB KIR genotype, which includes more activating KIR. The evidence that NK activation exerts a defensive effect in HIV infection derives also from studies on the activating receptor allele KIR3DS1. Hence, Guerini observed an increase of homozygosis for this receptor in HESN compared to HIV-infected patients (Guerini et al., 2011), while Carrington reported that the presence of this allele is strongly protective in the presence of its presumed HLAB ligand(s) (Carrington et al., 2008). In addition, it was recently shown that copy number variations of activating and inhibitory KIR influence the peripheral expansion of protective KIR3DS1^{+} NK cells, which in turn may determine differences in HIV-1 control post infection (Pelak et al., 2011). Another key molecular family associated to the HESN phenotype is the one that includes chemokines and chemokine receptors. Contradictory results have been reported for: 1) polymorphisms in the regulatory region of the CCR5 gene 2) a genetic variant of CCR2 (CCR2-64I), that is in strong linkage disequilibrium with a mutation in the CCR5 regulatory region 3) an A-to-G substitution at position 801 within the 3' untranslated region of the SDF-1 chemokine gene; and 4) promoter polymorphisms responsible for increased levels of CC-chemokine production (RANTES, macrophages inflammatory protein (MIP)-1α, MIP-1β and MIP1αP) that can block R5-HIV-1 infection either by competition or by inducing receptor internalization (Biasin et al., 2012).

More recently, analyses of the relationship between CCL3L1 copy number and susceptibility to HIV-1 infection showed that CCL3L1 levels are inversely correlated with CCR5 expression on CD4^{+} T lymphocytes (Gonzalez et al., 2005). Additional results performed in Human T-

lymphotropic virus (HTLV)-2-infected individuals indicated that the median copy number of CCL3L1 and the CCL3L1/CCL3 mRNA ratios are increased in HESN and in Long Term Non Progressor (LTNP) compared to those seen in HIV-unexposed healthy controls.

Notably, the APOBEC3 locus is present in the middle of chromosome 22 and include 7 members (A, B, C, DE, F, G and H) which display different rate of anti-HIV activity. Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (A3G) (APOBEC3G) provokes G to A mutation on viral genome. Despite the fact that the HIV viral infectivity protein (VIF) can counteract A3G, a number of data suggest that A3G may play a role in altering the natural course of HIV infection.

Among the other members of APOBEC family, attention has been recently focused on APOBEC3H. A case-control association analysis indicated that one of its haplotype, Hap I, is associated with protection from sexually transmitted HIV-1 infection. Hap I carries a protein-destabilizing variant as well as a residue conferring resistance to Vif-mediated degradation. These results suggest that lower protein stability might have been traded-off with a higher ability to circumvent Vif-mediated hijacking (Cagliani et al., 2011)

Among host endocellular factors, modulating post-entry events of HIV replication, great attention has been focused on TRIM5α an antiviral factor that can restrict the replication of a wide range of retroviruses by interacting with the viral capsid. TRIM5α controls retroviruses in a species-specific manner; for example, TRIM5 α in the Rhesus Monkey completely blocks HIV-1 infection. As it was suggested that overexpression of human TRIM5α weakens HIV replication, polymorphisms in the TRIM5α gene were investigated as they could be associated with a higher anti HIV activity. One study found that the 136R (R136Q polymorphism) and the 43H (H43 polymorphism) alleles do indeed correlate with in vitro resistance to HIV infection. Conversely, the 136Q allele was shown to be significantly more

frequent in HIV-infected individuals compared to HESN of Caucasian ethnicity, but this was not replicated in a second study (Speelmon et al., 2006). Indeed, the same allele was observed to be more frequent in an African American HESN compared to HIV-infected patients (Nakajima et al., 2009). A different genetic background and diverse routes of exposure might justify the inconsistencies in these results. Single Nucleotide Polymorphisms (SNP) 43Y, was shown to be associated with reduced susceptibility to HIV-1 infection in Asians. This result nevertheless contrasts with in vitro evidences showing an accelerated disease progression in individuals who were homozygous for the 43Y genotype as compared to individuals who were heterozygous or homozygous for the 43H genotype. Other results indicated that the co-presence of the 136Q and of the -2GG alleles localized in the 5' UTR of TRIM5.

Candidate gene approaches have also been used to investigate polymorphisms in genes directly involved in the immune response to the virus. Thus, variants in genes codifying for cytokines or cytokines receptors as well as factors involved in signal transduction pathways have been taken into consideration. Cytokines are, indeed, crucial players in controlling the immune system and in fighting pathogens. Nevertheless, studies of polymorphisms in these effector molecule genes are often controversial, and the majority of these results indicate associations with susceptibility rather than resistance. More remarkable results stemmed from the study of Toll-Like receptors (TLR) a family of pattern-recognition receptors for bacteria and viruses. Interestingly, stimulation of PBMCs with agonists specific for TLR3 (poly I:C), TLR4 lipopolysaccharide (LPS), TLR7 (imiquimod), and TLR7/8 (ssRNA40) resulted in higher cell responsiveness in HESN compared to healthy controls (Biasin et al., 2010). The ultimate mechanism responsible for this protection is to be better investigated but the activation of TLR pathway in HESN has recently been associated to an

increased expression of the full-length form of the adaptor protein MyD88, which acts as an intracellular negative regulator of this pathway. Furthermore, recent data obtained in two independent cohorts of HESN showed that a common TLR3 allele (rs3775291, Leu412Phe) confers immunologically-mediated protection from HIV-1 infection.

1.5.3 Results from Genome-wide Association Studies (GWAS)

Genome-wide association study (GWAS), a hypothesis-free approach, has led to the identification of new associations between genetic variants and infectious diseases, as well as to the validation of previously reported variants discovered by candidate-gene studies. This approach needs a very large sample size in order to have a sufficient statistical power to detect associations (the statistical significance at genome-wide level is a combined p value < $5x10^{-8}$) (Chapman et al., 2012).

In the context of HIV-1 infection, different GWASs have been reported and associated with HIV-1 viral load and/or disease progression, but also with HIV-1 acquisition, replication and resistance. In 2007, Fellay investigated, in an European cohort, the presence of polymorphic variants that could correlate with viral load during the asymptomatic set-point period. Results demonstrated the presence of high levels of association of three different single nucleotide polymorphisms (SNPs) with a reduced viral load (Fellay et al., 2007; Shianna KV, Ge D et al.,). A whole-genome association study of major determinants for host control of HIV-1. One SNP, rs2395029, located in the HLA complex P5 (HPC5), is in high linkage disequilibrium (LD) with the HLA B*5701 allele, an allele identified by candidate gene approach as being protective toward progression to AIDS and associated with lower viral loads. The second SNP rs9264942, located upstream of the HLA-C gene and telomeric to the HPC5 gene, is correlated with differences in HLA-C expression levels. Finally, the third SNP, rs9261174 was located in a

regulatory 5' region near the ZNRD1 gene and encodes a RNA polymerase I subunit, probably related to HIV-1 progression. In 2009, the same researchers published an extension of the first work. They first confirmed the strong association of the two main SNPs, rs2395029 and rs9264942, with variations in viral load and disease progression. In 2010, Pelak performed the first GWAs study to identify genetic correlates of protection against HIV-1 infection in a cohort of African American patients (Biasin e al., 2012). Results: 1)showed a strong association with an intronic SNP of HLA-B gene-rs2523608- tagging for HLA-B*5703; 2) confirmed the results obtained studying the European cohort for rs9264942; 3) did not confirm the presence of associations between the G allele of rs2395029 and protection, probably because of the low frequency of this allele in the African American population. Another goal of this research was to correlate significant SNPs to specific amino acidic positions within the HLA region, in particular, those involved in the interaction between viral peptides and HLA class I molecules. Results identified Arg^{97}, Cys^{67}, Gly^{62} and Glu^{63} in HLA-B; Ser^{77} in HLA-A; and Met^{304} in HLA-C as independent markers associated with HIV-1 control. These residues, with the exception of Met^{304} which is in the transmembrane domain of HLA-C, are located in the peptide binding groove of HLA class I, thus suggesting a role of the presentation of class I-restricted epitopes in host control (Talenti et al., 2012).

As summarized above GWASs have mostly highlighted the importance of the HLA region (mainly of HCP5/HLA-B*57) in controlling viral replication, in 2010 nevertheless Limou presented a non-HLA-replicated association obtained by GWAS approach. Interestingly the results showed a strong association of rs2234358SNP with long-term non-progression. This variant maps within the CXCR6 gene and it seems to be correlated with its gene expression modulation.

As for resistance to HIV infection, results of only few GWASs have been published so far, but they did not succeeded in the identification of any SNP associable with the HESN phenotype. Petrovsky studied 848 HESN and 531 HIV-1 seropositive individuals but despite the sufficient power of the study, no association is found. Lingappa performed a similar study in two cohorts of African HIV-1 sero-discordant heterosexual couples; again no SNPs are found to be associated with HIV-1 acquisition at genome-wide significance. More recently, GWAS performed in the attempt to identify additional genetic factors that could contribute to HIV-1 acquisitions, genotyping data of HIV-1 infected patients and uninfected control of the same ancestry from two European AIDS progression cohorts are compared. Results showed a strong association of the rs6996198SNP (chromosome 8) with a reduced risk of HIV-1 infection. This variant, which was more frequent in HESN than in HIV-infected patients, was previously associated with a higher transcriptional level of CYP7B1, a gene that plays a role in inflammation and in the synthesis of IgA. Another important GWAS study performed in two independent HESN cohort identified a correlation between resistant phenotype and a SNP minor allele rs1552896, in an intron of FREM1, an extracellular protein that can interact with many key factors for HIV-1 infection, establishment, and dissemination (Biasin et al., 2013).

Taken together, GWAS have confirmed the important role of HLA class I and CCR5 loci in the pathogenesis of HIV1 but these finding explain only a little portion of the inter-individual variability observed in HIV-1 control. Notably, the limited success of GWASs in studying HIV-1 resistance/susceptibility is mostly due to the difficult in recruiting HESN cohorts sufficiently numerous for an adequately powered study.

1.5.4 Immune correlates of protection

Innate immunity is likely to contribute to protection upon primary exposure to HIV in HESN before adaptive responses have developed.

Accumulating functional evidence supports a role for NK activation in resistance to HIV infection (Montoya et al., 2006). Additionally, TLR stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with TLR agonists resulted in higher cell responsiveness in HESN as demonstrated by elevated production of proinflammatory cytokines and chemokines (Biasin at al., 2010).

Secreted factors have also been associated with reducing susceptibility to mucosal transmission of HIV. The β-chemokines macrophages inflammatory protein (MIP)-1α, MIP-1β and RANTES bind the HIV co-receptor CCR5, thereby reducing HIV infection of target cells by outcompeting the virus for receptor usage. Peripheral production of β-chemokines was associated with resistance to HIV-1 infection (Tomescu et al., 2011), and elevated salivary of β-chemokines were associated with oral sexual behavior in HESN MSM (Hasselrot et al., 2010). Elevated IL-22 has also been associated with resistance to infection in HESN, and is thought to act by induction of acute–phase serum amyloid A, which downregulates CCR5 expression on target cells (Missè et al., 2007).

Multiple lines of evidences suggest that HIV-specific T cell responses are associated with resistance against HIV infection. HIV-specific T cells have been detected in PBMCs from commercial sex workers (CSW), men that have sex with men (MSM) discordant couples, occupationally exposed healthy care workers, injection drug users-HIV exposed seronegative (IDU-HESN) and prenatally exposed infants. Both CD4$^+$ and CD8$^+$T cell responses have been linked to HIV infection in HESN. HIV-specific CTLs have been described in several different HESN cohorts and many authors claim that these cells make a fundamental contribution at modulating resistance to HIV infection. HIV-specific CTLs have been observed and

characterized in the Pumwani Kenyan cohort of sex workers both at systemic and mucosal levels; in injecting drug users; and in sexual partners of HIV-infected patients. These studies analyzed approximately 100 HESN individuals; HIV-specific CTLs were observed in the majority (>70%) of such HESN. The detection of HIV-specific CTLs in HESN individuals raises important scientific questions: only the successful infection of host cells, that is, infection resulting in at least one complete cycle of viral replication, allows the effective presentation of viral peptides within a binary complex with a human leukocyte antigen (HLA) class I molecule. The detection of HIV-specific CTLs in HESN individuals thus seems to indicate that HIV has managed to infect the host, but that its further propagation has been contained by immune mechanisms. The recent description of an alternative mechanism of processing and presentation by HLA class I molecules of exogenous antigens known as cross-priming could, nevertheless, explain the presence of CTLs in HESN individuals in the absence of actual infection. According to this mechanism, dendritic cells can process the virus and present it to CTL precursors in the absence of viral replication. If this were the case, the presence of HIV specific CTLs in HESN individuals would be the consequence, not necessarily of an infection, but of a different and presumably more efficient processing pathway of HIV antigens within dendritic cells.

In particular, HIV-specific CD8+ CTLs of the HESN individuals recognize HIV epitopes that are different from those recognized by cells of HIV-infected patients 32 and Gag-stimulated CD8 T lymphocytes isolated from HESN individuals are characterized by higher levels of intracellular perforin and granzymes than those isolated from the HIV-infected partners (n ¼30 HESN individuals studied) (Miyazawa et al., 2009, Kaul et al., 2001).

HIV-specific IgA have been isolated from the female genital tract (FGT) of HESN CSW as well as uninfected partners of discordant couples (Devito et

al., 2002). However, similar antibodies were not detected in Gambian HESN CSW cohort and in Pumwany cohort there were HIV-specific IgA but no association was found between HIV-specific IgA and virus neutralization or shedding (Horton et al., 2009).

1.6 Antigen processing and presentation

Cytotoxic T lymphocytes (CD8^{+}) monitor the surface of 'professional' and 'nonprofessional' antigen presenting cells for the presence of viral infections. During infection, peptides from intracellular pathogens are generated and presented on the cell surface. This process is achieved through the major histocompatibility complex class I (MHC I) molecules. MHC I molecules bound to peptides (pMHC I) serve in fact as a flags for CD8^{+} T cells whose T cell antigen receptors (TCRs) recognize pMHC I and will eventually eliminate the infected or tumor cells. The formation and presentation of pMHC I complexes at the cell surface is a multistep process (Fig.1.4). First, endogenous proteins (viral proteins in case of virus-infected cells) are trimmed to peptides in the cytosol by the multicatalytic complex of the proteasome and several cytosolic peptidases. This step is followed by active translocation of peptides of 8 to 16 amino acids length across the endoplasmic reticulum (ER) membrane via proteins known as transporters associated with antigen processing (TAP1 and TAP2). While peptides of 8-10 amino acids may directly fit in the MHC I binding groove, longer peptides need to undergo further trimming to produce mature N-termini (Brouwenstijn et al., 2001; Fruci et al., 2001; Lauvau et al., 1999). In mouse this step is catalyzed by ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing) (Serwold et al., 2002), while in human by two endoplasmic reticulum aminopeptidases, ERAP1 and ERAP2 (Saric et al., 2002; Saveanu et al., 2005; York et al., 2002). Finally, peptides are assembled with MHC I molecules and the pMHC I complex translocate to

the cell surface for the recognition by specific CD8^{+} T cells (Hammer et al., 2007; Lehner and Cresswell, 1996).
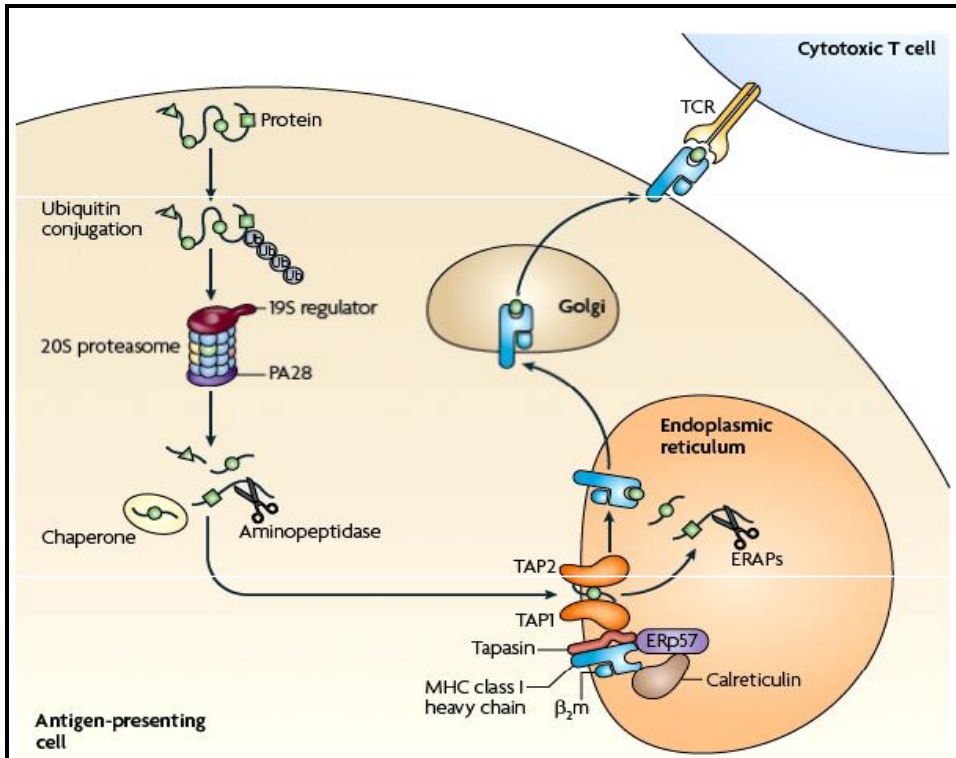
Figure 1.4 *MHC I antigen processing and presentation. Cytosolic and nuclear proteins are degraded by the proteasome. Peptides undergo further trimming by cytosolicaminopeptidases. The transporter for antigen processing (TAP) then translocates peptide into the lumen of the ER. The MHC I molecule folds with the assistance of the chaperones calnexin and calrecticulin, the adaptor molecule tapasin and the oxidoreductase ERp57. Protein disulfide isomerase (PDI) helps in formation of the disulfide bond in the a2 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 goes to the cell surface. (Fruci, et al., 2013)*

1.6.1 The MHC class I

The MHC I genes encode polymorphic membrane glycoproteins that present peptides of foreign or self-origin to CD8$^+$T cells. These molecules play critical roles in immune responses to viruses, MHC I proteins are encoded by three highly polymorphic genes, HLA-A, HLA-B and HLA-C, that are expressed in all eukaryotic cells (Little and Parham, 1999), and three less polymorphic genes, HLA-E, HLA-F and HLA-G that are expressed in a tissue-restricted fashion.

In human, MHC I consist of two polypeptide chains, a heavy or α chain encoded in the MHC locus and a smaller non polymorphic light chain, ß2-microglobulin, which is encoded elsewhere.

The MHC-encoded polypeptide folds into three separate domains called α1, α2 and α3. ß2-microglobulin is non-covalently associated with the α3 domain (Ohnishi, 1983). The α3 domain and ß2-microglobulin have a folded structure that closely resembles that of an immunoglobulin domain. The most remarkable feature of MHC I molecules is the structure of the folded α1 and α2 domains in which site of polymorphism are present. These two domains fold to make up a region bounded by a ß-pleated sheet on the bottom and two α helices on the sides. This groove, capable of binding via non-covalent interactions a small peptide of about 8-10 amino acids, is the peptide-binding site. The small peptide presented defines the antigenic epitope that can be recognize by CD8$^+$ T-cell. MHC class I molecules are expressed by almost all nucleated cells, however, MHC class I molecules can also bind and present peptides from extracellular proteins to CD8$^+$ T cells, a process known as cross-presentation (Cresswell et al., 2005). Once the TCR of CD8$^+$ T cells recognizes peptide/MHC class I complexes at the cell surface, a T-cell signaling cascade is initiated and promotes the elimination of abnormal or virus-infected cells.

1.6.2 The ubiquitin-proteasome system

Cytosolic protein degradation is mostly carried out by a large, multicatalytic protease complex (700 kDa) called the proteasome. The proteasome is found in the nucleus and cytosol of eukaryotic cells, where it is involved in non-lysosomal protein degradation of cytosolic proteins (Fehling et al., 1994). The ubiquitinated proteins enter into the core of the proteasome where they are broken down into short peptides of 2 to 20 amino acids. The 20S core of this complex has barrel shape made of four rings. The two outer rings are identical and each is composed of seven distinct α subunits. The two inner rings are also identical. Each contains seven different ß subunits, which surround a central chamber where proteolysis occurs. The activity is exerted by three of the ß subunits, namely ß1, ß2 and ß5. The 20S core proteasome can associate with two regulatory complexes. Association with the 19S cap produces the 26S proteasome particle, which is able to degrade ubiquitin-conjugated proteins, whereas association with the P28 complex is thought to increase its catalytic activity (Tanaka and Kasahara, 1998). The major role of the proteasome is the degradation of intracellular proteins. The products of this degradation represent a major source of peptides for MHC I presentation.

When cells are exposed to IFNγ the three catalytic subunits, ß1, ß2 and ß5 are replaced by their IFNγ-inducible homolog LMP2 (ß1i), MECL1 (ß21) and LMP7 (ß5i), respectively. In vitro studies, performed with viral epitopes, showed that the proteasome equipped with LMP2, LMP7 and MECL1 is more efficient in processing a number of immunogenic peptides (Cerundolo et al., 1995). For this reason, it is called 'immunoproteasome' as opposed to the constitutively expressed standard proteasome. When tested in vitro on fluorogenic substrates the catalytic activity of the immunoproteasome is characterized by a reduced cleavage after acidic amino acids and an increase in the cleavage after hydrophobic and basic residues. As the latter residues are those most frequently binding to MHC I molecules, this

reinforced the notion that the immunoproteasome is more efficient than the standard proteasome for antigen presentation. It has been noted that changes in subunit composition sharpen the quantitative and qualitative ability of the proteasome to generate peptides, and thereby limit the production of self-peptides (Groettrup et al., 1996). Immunoproteasomes are constitutively expressed in immune tissues and expressed at much lower levels in other cell types where they can be induced by exposing cells to IFNγ or TNFa, cytokines released in the early stages of viral infections. The vast majority of the peptides produced during protein degradation by the cytosolic proteasome-ubiquitin system are consecutively hydrolyzed to single amino acids by multiple cytosolic peptidases. The small fraction of peptides surviving the aggressive cytosolic environment can be recruited for presentation by MHC I molecules (Kloetzel, et al., 2004).

1.6.3 Peptide transport across membranes.

The peptides generated in the cytoplasm are translocated into the ER through the Adenosine Tri-Phosphate (ATP)-dependent TAP1- TAP2 heterodimer complex (Neefjes et al.,1993). Both TAP1 and TAP2 are spanning proteins with an ATP-binding cassette. The peptide transporters are selective for peptide length and the N- and C-terminal amino acids (Momburg and Hammerling, 1998). The translocation of peptides by TAP is tightly coupled to hydrolysis of ATP, so much so that TAP mutants unable to bind to ATP were also unable to bind, their peptide substrate. In an interesting example of functional convergent evolution, peptide binding and ATP hydrolysis are each targeted by several different viral immunoevasin proteins (Hansen et al., 2009). The higher the affinity of a peptide for TAP, the greater its likelihood of begin transported and the better the probability that it will be recognized by T cells during infection. In humans, a hydrophobic C-terminal is favored by TAP although basic residues can also

be accommodated. The peptides transported by TAP are generally between 8 and 16 residues in length, however, longer peptides can be transported, albeit with lower efficiency (Herget et al., 2011).

1.6.4 The N-terminal trimming

The peptides, after proteasome trimming, may have to be adapted to the strict MHC I-binding requirements by one or several N-terminal-trimming steps. An accepted model proposes that peptides of 15 or more residues are shortened by cytosolic tripeptidyl peptidase II. The cytosolic aminopeptidases including leucine aminopeptidase, leucine aminopeptidase, bleomycin hydrolase and puromicin-sensitive aminopeptidase, shown to contribute to the generation of individual epitopes while begin overall destructive and therefore for bulk antigen presentation. Nardilysin and thimet oligopeptidase have also been implicated in the generation of three defined CTL epitopes (Saunders et al., 2011). However, such peptides can directly translocate into the ER lumen by TAP. In the ER, the NH2 terminus of these peptides is further trimmed by ER aminopeptidases, i.e., ERAP1 in mice and ERAP1 and ERAP2 in humans, to the proper length for binding to MHC class I molecules and presentation on the cell surface for recognition by NK cells and specific CD8$^+$ T cells (Savenou et al., 2005).

1.6.5 The peptide-loading complex

The peptide loading complex (PLC) is a multisubunit structure in the ER containing TAP at its center assembled with MHC I molecules, ß2-microglobulin, tapasin (tpn), calrecticulin, ERp57, and possibly protein disulfide isomerase (PDI) (Cresswell et al., 2005; Elliott and Williams, 2005). Tpn, ERp57 and PDI are required for the stabilization of TAP and regulation of the redox state of a disulfide bound in the peptide-binding

groove of the MHC I heavy chain (Cabrera, 2007). Upon peptide loading, the PLC dissociates and the complex consisting of the MHC I and β2-microglobulin is released and transported via the trans-Golgi to the cell surface to be exposed to CD8+cytotoxic T lymphocytes. Deficiency in components of the PLC can have dramatic consequences on the expression and quality of the pMHC I repertoire on the cell surface (Garbi et al., 2000).

1.7 Endoplasmic Reticulum Aminopeptidases

The endoplasmic reticulum (ER) aminopeptidase 1 (ERAP1) and the closely related ER aminopeptidase ERAP2 are zinc-metallopeptidases of the oxytocinase M1 subfamily, which share consensus zinc-binding motifs essential for their enzymatic activity (Hattori et al., 1999). The human ERAP1 and ERAP2 genes are located on chromosome 5q15 in the opposite orientation, likely to share regulatory elements. Human ERAP2 has no analogues in rodents (e.g., mouse, rat, rabbit and guinea pig) and evolution studies suggest that it originates from a relatively recent duplication of ERAP1 (Andres et al., 2010). These enzymes are normally present in many tissues and are strongly induced after stimulation with type I and type II interferons (IFNs) (Servold et al., 2002; Tanioka et al., 2003) and tumor necrosis factor-alpha (TNF-a) (Forloni et al., 2010). ERAP enzymes trim aminoacid residues from the NH2 terminus of polypeptides playing an important role in various biological processes. In the ER, ERAP1 and ERAP2 cleave precursors to generate or destroy MHC class I binding peptides (Savenou et al., 2005; Servold et al., 2002). ERAP1 has also been involved in the regulation of innate immune and inflammatory responses by increasing the shedding of cytokine receptors (Cui X et al., 2002-2003). In addition to these immunological functions, ERAP1 and ERAP2 have been implicated in the regulation of angiogenesis and blood pressure (Sato et al., 2004). According to these multifunctional properties, ERAP1 is also termed

endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), 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). The terms ERAP1 and ERAP2, have been approved by the Human Genome Organization Nomenclature Committee.

1.7.1 ERAP1 structure and its variants

The ERAP1 human gene is located on the long arm of chromosome 5 (5q15) and consists of 20 exons spanning about 47 kilo-bases (Hattori et al., 2001). The zinc-binding motif HEXXH is encoded in exon 6, while the essential glutamic acid (E) residue is encoded in exon 7. The GAMEN motif, which is shown to be important for the enzymatic action of gluzincin aminopeptidases, is also encoded in exon 6. Two major ERAP1 protein isoforms are generated depending on differences in exon 20: the longer isoform a (ERAP1-a) and the shorter isoform b (ERAP1-b). Recently, Kim et al. found that the isoform ERAP1-b is more abundant than that of the isoform ERAP1-a mRNA in various cell lines, and that the amount of the isoform ERAP1-b accounts for most of the cellular content of ERAP1 (Kim et al., 2011)

These crystal structure of ERAP1 reveals an open conformation with a large internal cavity that can accommodate long peptide substrates and has features that explain ERAP1 broad specificity for antigenic peptide precursors. It has been hypothesized that binding of long peptide substrates in the catalytic and regulatory sites induces a conformational arrangement that leads to enzyme activation and efficient trimming (Kochan et al., 2011).

Potential effects on the biological functions of some of these variants can be inferred from the location in the ERAP1 structure: rs2287987 (M349V) is located in the active site and rs17482078 (R725Q) and rs27044 (Q730E) are exposed on the inner surface of the C-terminal cavity, which could affect substrate sequence or length specificity. Other polymorphisms, such as rs26653 (R127P), rs30187 (K528R), and rs10050860 (D575N) at domain junctions could indirectly affect either specificity or enzymatic activity by altering the conformational change between open and closed conformations. rs30187 and rs27044 have been repeatedly confirmed by nearly all population studies to confer strong susceptibility to disease. Both polymorphisms have been shown to cause a significant reduction in aminopeptidase activity towards a synthetic peptide substrate and several antigen precursor peptides (Evnouchiodou et al., 2011)

1.7.2 ERAP2 structure and its variants

The ERAP2 gene is located on chromosome 5q15, residing between ERAP1 and leucyl-cystinyl aminopeptidase (LNPEP) and contains 19 exons, spanning about 45 kilo-bases. The GAMEN and HEXXH motifs are encoded in exon 6 while the essential E residue, located 19 amino acids downstream from the HEXXH motif, is encoded in exon 7. Exon 19 contains the coding sequence for the last 47 aminoacids, stop codon and the 3′UTR region. A recent genetic analysis revealed that ERAP2 contains a variant that influences MHC class I antigen presentation. There are two different haplotypes of ERAP2 A and B, that correlate with the co-presence of different polymorphisms all in linkage disequilibrium between them. In particular, the single nucleotide polymorphism (SNP), Lysina 392 Asparagina (K392N), determines the non-synonymous substitution of a polar basic residue, Lys (haplotype A), with neutral polar residue, Asp (haplotype B). In haplotype B the presence of the SNP rs2248374 (A to G),

located within the 5′ canonical splice site of exon 10, results in an alternatively spliced ERAP2 mRNA that contains a part of intron 10 that could be translated in a ERAP2-alternative spliced truncated protein of 60kDa (ERAP2 AS) or degraded by non-sense mediated decay (NMD) (Andres et al., 2010). Heterozygous AG and homozygosity for the major A allele result in the expression of a 120KDa ERAP2-full length protein (ERAP2-FL), whereas homozygosity for the minor G allele results in the production of ERAP2-AS protein and reduced surface MHC class I expression in human lymphoblastoid cell lines (Andres et al., 2010) (Fig. 1.5).
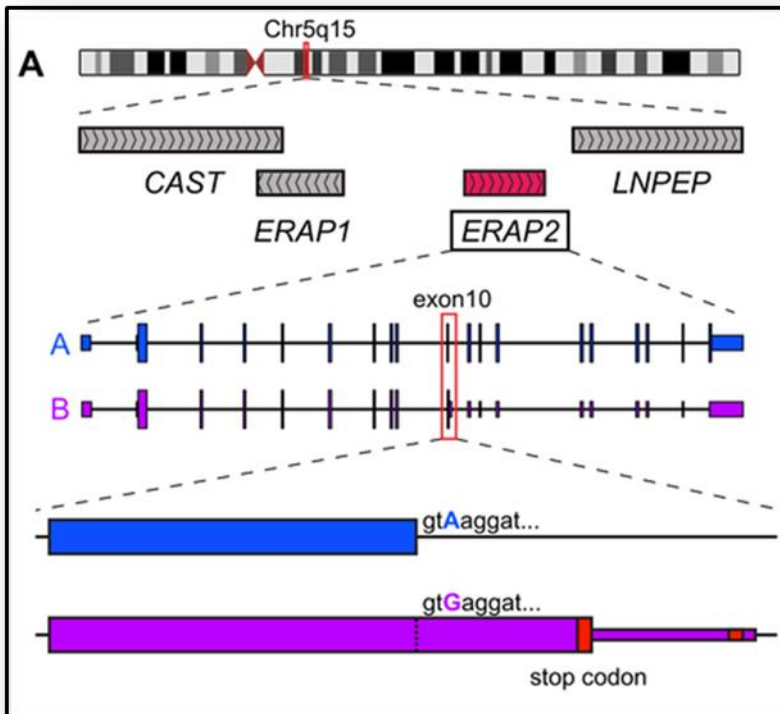
Figure1.5 The genomic organization of the human chromosome 5q15 region containing ERAP1and ERAP2 is included at the top. The two haplotype specific ERAP2 spliced forms are shown for Haplotype A (in blue) and Haplotype B (in purple). The different alleles of rs2248374 are shown as a blue or purple base position, respectively. The red boxes represent the premature stop- codons in the Haplotype B mRNA (Andres et al.,2010)

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The crystal structure of ERAP2 has been recently resolved and shown to have the same domain organization of ERAP1 (Birtley et al., 2012) with which a high homology (49% structural identity) is shared. The internal cavity, similar to that of ERAP1, extends from the catalytic site of the enzyme and can accommodate large precursor antigenic peptides. Moreover Birtley et al., demonstrate that ERAP2 is able to make a homodimer complex

1.7.3 Trimming of antigenic peptides by ERAP1

ERAP1 efficiently trims peptides of 9–16 amino acids, the length of peptides efficiently transported into the ER by TAP, but spares the longer ones (Chang et al., 2005). Of note, its activity is substantially reduced for peptides with proline at position 2 (X-P-Xn) (Servold et al., 2002), or for peptides with a size of 8 or 9 amino acids, the optimal length for binding to MHC class I molecules (Chang et al., 2005). ERAP1 shows a strong preference for peptides with large hydrophobic COOH-terminal residues (Chang et al., 2005). ERAP1 activity appears to be also affected by the nature of the internal residues of peptides. In particular, positions 2, 5 and 7 (with position 1 defined as the N-terminal residue of the peptide) were found to be the most important for the peptide sensitivity to ERAP1 degradation (Evnouchidou et al., 2008). Based on the analogies with TAP and MHC class I preferences, Chang et al. proposed the "molecular ruler" model for ERAP1 (Chang et al., 2005), that suggests its role in facilitating antigen processing and presentation by trimming precursors transported by TAP to MHC class I binding peptides. The substantial contribution of ER peptide trimming to MHC class I antigen processing and presentation has been confirmed in mice lacking ERAAP generated independently in four laboratories (Hammeret al., 2007). Although loss of ERAAP had a relatively modest effect on the cell surface expression of most MHC class I

molecules (a reduction of 20–40% for Kb and Db class I molecules) (Hammer et al., 2006). Immunization of ERAAP-/- mice with wild-type (wt) cells or vice versa, resulted in potent CD8^{+} T cell responses, suggesting that loss of ERAAP alters the peptide-MHC (pMHC) class I repertoire not only quantitatively but also qualitatively (Hammer et al., 2007).

1.7.4 Trimming of antigenic peptides by ERAP2

ERAP2 has been shown to cooperate with ERAP1 to trim a large variety of precursor peptides to generate mature epitopes for binding to MHC class I molecules (Savenou et al., 2005). ERAP2 is found to have distinct specificities for the N-terminal residue of the peptide substrates of ERAP1. In fact, the aminopeptidase 2 shows a strong preference for the basic residues Arg and Lys. ERAP2 crystallizes as a homodimer through head-to-head interactions of the N-terminal domain that map an extended dimerization interface. Each monomer has an overall domain organization (domains I−IV) similar to that of the closed structure of ERAP1 [Protein Data Bank (PDB) entry 2YD0] with domain IV coming into the proximity of domain II, the catalytic site, and the S1 pocket (the latter defined as the specificity pocket accommodating the peptide side chain N-terminal to the scissile bond, according to the usual convention of defining peptidase specificity sites; subsequent specificity sites will be named S1′, S2′, etc.). This domain configuration generates an internal cavity that is of sufficient size to accommodate large peptides, similar to ERAP1. The shape and electrostatic potential distribution in that cavity, however, are distinct from the latter, suggesting that ERAP2 may apply distinct selective pressures in the antigenic peptide repertoire. Asp198 in ERAP2 is very important of N-terminal specificity. Furthermore, same structure studies suggest that specificity differences between ERAP1 and ERAP2 may extend throughout the peptide-binding cavity. We map a common SNP of ERAP2 (rs2549782,

N392K), which has been linked with predisposition to disease, to a location adjacent to the active site of the enzyme. The ERAP2 homodimer in our structure is stabilized by an extended interface mediated by residues largely conserved in ERAP1 and may therefore constitute a useful model of the ERAP1−ERAP2 interaction.

ERAP2 monomer is made of four structural domains (I−IV) forming a closed hollow structure. The N-terminal domain I (residues 54−271) exhibits a β-sandwich structure. Domain II (residues 272−546) has a thermolysin α/β-fold and contains the HExxHx18E zinc-binding motif as well as the GAMEN aminopeptidase motif. Domain III (residues 547−647) has a β-sandwich structure and acts as a link between domains II and IV. Domain IV (residues 648−960) is an all α-helical domain interacting closely with domain II, essentially capping the Zn (II) catalytic site (Fig. 1.6).
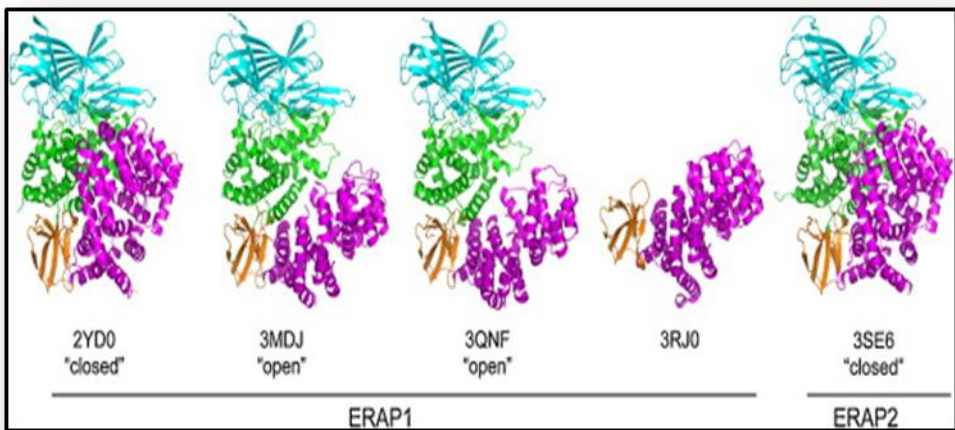
Figure 1.6 *Cartoon representations of known ERAP1/2 crystal structures. Domain I is shown in cyan, domain II in green, domain III in orange and IV in magenta. Note the repositioning of domain IV relative to domains I and II in the "open" versus the "closed" conformations (Efstratios Stratikos et al., 2013).*

The contacts between domains II and IV define a large internal cavity, adjacent to the catalytic site, which has almost no access to the external solvent but exhibits numerous water molecules trapped inside it (Efstratios Stratikos et al., 2013).

1.8 Alteration of ERAP function in human diseases

ERAP1/2 are highly polymorphic with strong linkage disequilibrium evident across the gene. Recent GWAS have identified numerous ERAP1/2 single nucleotide polymorphisms (SNPs) strongly associated with different human diseases. Because of the role of these enzymes in MHC class I antigen presentation and in all other biological processes, it is conceivable that variations impairing the presentation of pathogen-derived peptides might lead to inadequate immune responses and development of disease.

1.8.1 Hypertension

By screening for 33 polymorphisms in the human ERAP1 gene, Yamamoto et al. identified the association of ERAP1 variant rs30187 (K528R) with essential hypertension and hypothesized that the R528 form of ERAP1 was less active than the K528 form, leading to hypertension due to reduced bradykinin formation and/or lower inactivation of angiotensin II (Yamamoto et al., 2002). This variant is shown to reduce by 60% the efficiency of ERAP1 to cleave angiotensin II to angiotensin III and by 70% to convert kallidin into bradykinin (Goto et al., 2006). The ERAP1 rs30187 variant is also found to determine the degree of regression of left ventricular hypertrophy during anti-hypertensive treatment in patients with essential hypertension (Hallberg et al., 2003).

More recently, variants of ERAP1 and ERAP2 have been found to be associated with an increased risk of preeclampsia, a heritable pregnancy specific disorder characterized by new-onset hypertension and proteinuria

(Hill et al., 2011). Of note, ERAP2 expression is previously found altered in first trimester placentas of women prone to develop pre-clampsia (Founds et al., 2009).

1.8.2 Bacterial and Viral infections

ERAP1 plays an important role in immune response to viruses, either enhancing or reducing CD8^{+} T-cell responses to particular viral epitopes. ERAP1-deficient or wt mice infected with lymphocytic choriomeningitis virus (LCMV) showed profound differences in the frequency of CD8^{+} T cells specific for particular LCMV peptides (York et al., 2006). In wt mice the magnitude of T-cell responses to different LCMV epitopes followed a hierarchy of immunodominance that is markedly changed in the ERAP1-deficient mice (York et al., 2006).

Draenert et al. reported the first evidence that escape mutations arising in flanking regions of a human immunodeficiency virus (HIV) epitope alter antigen processing mediated by ERAP1 (Draenert et al., 2004). The authors showed that in HLA-B57[+]-HIV infected individuals, immune selection pressure leads to a mutation from alanine to proline at residue 146 of HIV Gag protein immediately before the NH2 terminus of a dominant HLA-B57-restricted CTL epitope. This mutation was found to prevent the NH2-terminal cleavage by ERAP1, resulting in decreased CTL responses (Draenert et al., 2004). Of note, it was demonstrated that antigen processing shapes CTL response hierarchies, and that HIV evolution modifies cleavage patterns influencing proteasomal cleavage and, hence, the likelihood of CTL responses toward all epitopes (Tenzer et al., 2009). Interestingly, some variants in ERAP2 have been shown to confer resistance to HIV-1 infection possibly via the presentation of a distinctive peptide repertoire to CD8^{+} T cells (Cagliani et al., 2010).

Similarly, in cervical carcinoma induced by persistent infection and malignant transformation of the uterine cervical epithelium by human papillomavirus (HPV), increased cancer metastasis and decreased survival have been reported to be associated with several variants of ERAP1. In this case, down-regulation of ERAP1 may lead to the preferential loading and presentation of non-tumor-associated or non HPV-associated peptides, thereby yielding a less immunogenic phenotype and facilitating tumor growth and progression (Mehta et al., 2009).

Recently, ERAP1 has been identified as a host target of human cytomegalovirus (HCMV) microRNA miR-US4–1, demonstrating a previously unknown miRNA-based immunoevasion strategy. Viral miR-US4–1 interferes with MHC class I-mediated antigen presentation by targeting ERAP1, thereby influencing the production of many HCMV-derived antigenic peptides during viral infection, which results in immunoevasion of the recognition of viral antigen by CD8^{+} T cells during the host immune response (Kim et al., 2011).

1.8.4 Autoimmune diseases

Recent genome-wide association studies (GWAS) have proven the importance of ERAP1 and ERAP2 genes in conferring susceptibility of individuals to different autoimmune diseases and their linkage with particular MHC class I alleles (Fierabracci et al., 2011).

The association between ankylosing spondylitis (AS) and ERAP1 variants has been founded in different population like Caucasian and Mongolian persons (Maksymowych et al., 2009; Davidsonet al., 2009; Choi et al., 2010; Harvey et al., 2009). More recently, the association with the variant rs30187 has been exclusively found in a cohort of HLA-B27-positive AS patients (Evans et al., 2011). Moreover, this functional polymorphism confers susceptibility to MS in Italian populations (Guerini et al., 2012). Of

note, the disease-associated ERAP1 variant rs30187 (K528) had faster rate of trimming of peptide precursors than protective ERAP1 variant (Evans et al., 2011). Taken together, these findings support a model in which aberrant peptide trimming by ERAP1 and, as consequence, impaired peptide presentation by HLA-B27 are involved in the AS pathogenesis (Evans et al., 2011).

A recent meta-analysis of six Crohn's disease GWAS identified ERAP2 as one of the most interesting candidate genes (Franke et al., 2010).

1.8.5 Cancer

ERAP1 and ERAP2 are expressed in all tumor cell lines examined (melanomas, leukemia-lymphomas and carcinomas of breast, colon, lung, chorion, skin, prostate, cervix, kidney and bladder) at highly variable levels and independently from each other (Fruci et al., 2006). MHC class I surface expression is significantly correlated with ERAP1, but not with ERAP2, suggesting that ERAP1 has a dominant role in the generation of MHC class I epitopes (Fruci et al., 2006). In a subsequent study, ERAP1 and ERAP2 are investigated in a large panel of surgically removed normal and neoplastic tissues. In approximately 150 neoplastic lesions, the expression of either or both enzymes was lost, acquired or retained as compared to the normal counterparts, depending on the tumor histotype (Fruci et al., 2008). Down-regulation of ERAP1 and/or ERAP2 expression is mainly detected in ovary, breast and lung carcinomas, whereas an up-regulation of these enzymes was observed in colon and thyroid carcinomas (Fruci et al., 2008). Of note, ERAP1 and MHC class I are coordinately expressed in normal and, to a lesser extent, neoplastic lesions. As expected, the altered expression of ERAPs results in abnormal cell surface expression of MHC class I molecules in tumor cell lines (Fruci et al., 2006). In the most aggressive type of neuroblastoma cells, ERAP1, ERAP2 as well as MHC

class I molecules are expressed at very low levels as consequence of a poor constitutive NF-kB nuclear activity (Forloni et al., 2007)

In a recent study, heterogeneous expression of ERAP1 and ERAP2, ranging from high to very low levels, is detected in 28 melanoma cell lines as compared to primary melanocytes (Kamphausen et al., 2010). In most cases, expression of these genes is enhanced by IFN-.treatment, suggesting that it is under control of regulatory mechanisms and that only in rare cases to abnormalities in their sequences (Kamphausen et al., 2010).

Expression of ERAP1 has been detected in 64% of endometrial carcinomas and correlated with CA-125 levels, suggesting a role of this enzyme in endometrial cancer cell growth and differentiation (Kazeto et al., 2003; Watanabe et al., 2003; Shibata et al., 2005). The authors also showed that ERAP1 suppresses angiogenesis and endothelial cell migration in human endometrial carcinoma by regulating the angiotensin II concentration (Watanabe et al., 2003).

A recent study demonstrated that interfering with ERAP1 expression ultimately leads to tumor rejection in syngeneic animals by boosting NK cell, and subsequently T cell-mediated cytolysis. This rejection was mainly due to NK cell response and depends on the MHC class I peptides presented by ERAP1-silenced tumor cells, because replacement of the endogenous peptides with high-affinity peptides was sufficient to restore an NK protective effect of MHC class I through the NK inhibitory receptor Ly49C/I (Cifaldi et al., 2011).

Of note, Wang et al., showed that there is a strong correlation between ERAP1 and tumor suppressor factor p53. In fact, p53 could directly activate ERAP1 influencing in this way the expression on cell surface of MHC class I molecules (Wang et al., 2013).

1.8.6 Cytokine Receptor Shedding

In addition to antigen processing, ERAP1 has been shown to promote the cleavage of several cytokine cell surface receptors (Cui X,et al., 2012). Cui *et al.* demonstrated that ERAP1 binds to the extracellular domain of the TNFR1, facilitating TNFR1 shedding through the formation of a TNFR1/ERAP1 complex. The authors showed that overexpression of ERAP1 produces soluble TNFR1 that competes with cell-surface TNF receptors, thereby attenuating TNFα bioactivity when the levels are elevated, and reconstituting TNFα when the levels have declined (Cui X et al., 2003). However, several evidences demonstrated that ERAP1 is not required to be catalytically active for this function. Coimmunoprecipitation experiments revealed that ERAP1 could bind to, but not cleave TNFR1. Moreover, ERAP1 does not possess endopeptidase activity and overexpression of ERAP1 catalytic mutants results in an increased TNFR1 shedding. Thus, ERAP1 does not directly catalyze TNFR1 shedding, but may instead promote the activity of a TNFR1 sheddase. Subsequently, the same authors showed that ERAP1 modulates the proteolytic cleavage of two other cytokine receptors, the type I IL-6 cytokine receptor (IL-6Rα) (Cui X et al., 2012) and the type II IL-1 decoy receptor (IL-1RII) (Cui X et al., 2003). Based on these functions, ERAP1 has been proposed to play an important role in regulating innate and inflammatory immune responses by increasing the shedding of these cytokine receptors.

1.9 Non immunological function

ERAP1 and ERAP2 are thought to play a role in the regulation of blood pressure through their involvement in the renin-angiotensin system. In vitro studies using Chinese Hamster Ovary cells demonstrated that ERAP1 efficiently cleaves angiotensin II to angiotensin III and IV (Hattori 2000), while ERAP2 cleaves angiotensin III to angiotensin IV (Tanioka et al., 2003). In the same system, both enzymes are shown to convert kallidin to

bradykinin (Tanioka et al., 2003) In addition, ERAP1 has been reported to control post-natal neo-angiogenesis, a physiological process involving the growth of new blood vessels from pre-existing microvessels, by regulating the proliferation and migration of endothelial cells (EC) (Miyashita et al., 2000). Functional studies revealed that ERAP1 is expressed in ECs during differentiation in vitro and at the site of angiogenesis in vivo upon stimulation with vascular endothelial growth factor (VEGF) (Miyashita et al., 2000). Suppression of ERAP1 expression in ECs inhibited VEGF-stimulated proliferation, migration, vessel network formation in vitro, and angiogenesis in vivo (Miyashita et al., 2000). The authors also demonstrated that ERAP1 regulates VEGF stimulated G1/S transition during EC proliferation by binding to phosphatidylinositol-dependent kinase 1 (PDK1). Formation of the ERAP1-PDK1-S6 kinase complex resulted in activation of cyclin-dependent kinase (CDK) 4/6 by phosphorylated S6K that promotes G1/S-phase transition leading to EC proliferation (Yamazak et al., 2004). It was also demonstrated that ERAP1 controls the spreading of ECs by activating endothelial integrins and focal adhesion kinase (Akada et al., 2002), increasing EC adhesion to the extracellular matrix via RhoA activation (Suzuki et al., 2007). More recently, ERAP1 has also been shown to bind pigpen, a nuclear-coiled body component protein involved in angiogenesis (Yoshidaet al., 2010; Abe et al., 2006). However, how pigpen interacts with ERAP1 to promote angiogenesis and whether pigpen is a substrate for ERAP1 remains to be established.

CHAPTER 2. THE AIMS OF THE STUDY

ERAP1 and ERAP2 play an important role in immune response to viruses, either enhancing or reducing CD8^{+} T-cell responses to particular viral epitopes. RNA interference experiments have produced contrasting results concerning the effect of ERAP1 and ERAP2 on MHC class I molecule expression and peptide presentation (Sovenau et al., 2005; York et al., 2002), suggesting that factors such as peptide sequence, cell-type and MHC class I alleles may determine whether the two aminopeptidases enhance or inhibit antigen presentation.

In addition, haplotype variations have already been associated with different autoimmune diseases and tumoral forms (Fruci et al., 2013). Moreover, haplotype A of ERAP2 was shown to have a higher frequency in a cohort of seronegative subjects repeatedly exposed to HIV trough unprotected heterosexual intercourses suggesting that ERAP2 haplotypes could play a role in resistance to HIV infection (Cagliani et al., 2010).

In this context, the present study aims at confirming the correlation between ERAP2 haplotypes and susceptibility to HIV-1 by verifying whether diverse ERAP2 alleles differently modulate antigen processing and presentation.

CHAPTER 3. MATERIAL AND METHODS

3.1 Cohorts enrolled in the study

Two different HESN cohorts were enrolled in this study. The first one included 69 Italian HESN subjects exposed to the virus through unprotected heterosexual intercourses. Inclusion criteria were a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrollment, with at least three episodes of at-risk intercourse within 4 months prior to study entry and an average of 30 (range 18 to 100) reported unprotected sexual contacts per year. 218 healthy Italian donors were used as controls.

The second cohort included 104 white males exposed to HIV-1 infection by injection drug use (IDU) from a Spanish cohort (Valme Hospital, Sevilla) who have been sharing infected needles for a period longer than 3 months and with a concurrent hepatitis C infection (HCV). A group of 130 HIV-1 and HCV-seronegative healthy white male blood donors from the Jaen Hospital was used as control.

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

Similarly, informed consent was obtained from 139 HIV-1 seronegative Italian healthy controls who gave their blood to perform functional analyses. Whole blood was collected from all participants, and DNA was extracted by phenol chloroform method (Promega, Madison, Wisconsin, USA).

3.1.2 Genotyping

Genomic DNA was used as template for PCR amplification using TaqMan probes specifically designed to perform a SNP genotyping assay for rs2549782 (G/T) (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, California, USA) and using the allelic discrimination real-time

PCR method. The polymorphic 32 base pair deletion at the *CCR5* locus is typed by PCR using specific primers (forward: 5′ TGGTGGCTGTGTTTGCGTCT-3′; reverse: 5′-ATGACAAGCAGCGGCAGGAC-3′). The PCR products is electrophoretically separated on 3% agarose gels.

3.2 HLA typing

Genomic DNA was isolated from peripheral blood by phenol-chloroform extraction using standard procedures. HLA typing of class-I HLA-B locus was performed by the Sequence Specific Primer amplification (SSP-PCR) method using Histo-Type DNA plates (BAG health care, Formedic s.r.l., Milano, Italy) according to the manufacturer's instructions. Detection of the alleles recognized by the specific primers was possible after amplification in a GeneAmp PCR 9700 thermocycler (Applied Biosystem, Foster City, CA U.S.A.) and gel electrophoresis on 2% agarose gel.

3.3 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were separated by whole blood on lymphocyte separation medium (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA). Briefly, whole blood was centrifuged for 25 minutes at 2300 RPM, without brake, on a Ficoll discontinuous density gradient
After the centrifugation step, the blood sample was separated showing the following layers from top to bottom plasma and platelets, the PBMCs band situated at plasma/Ficoll interface, Ficoll and red blood cells, covered by a granulocyte layer.
The PBMCs layer were carefully removed from the tube and transferred to a new conical tube. The PBMCs were then washed with phosphate buffered saline (PBS) and cell number and cellular vitality were determined.

3.4 CD4 T lymphocytes magnetic separation

CD4^{+} T lymphocytes were separated from PBMCs by direct magnetic labelling using the CD4-microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), according to manufacturer's protocol. Briefly, the CD4^{+} cells are magnetically labeled with CD4MicroBeads. Then, the cell suspension is loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD4^{+} cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD4+ cells. After removing the column from the magnetic field, the magnetically retained CD4+ cells can be eluted as the positively selected cell fraction.

3.5 Cell cultures

PBMCs were cultured at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% AB human serum and stimulated with different concentrations of IFNγ (12 ng/ml and 60 ng/ml) for 3h; 24h, 48h and 72h.
Moreover, 1x106 PBMCs were cultured for 20h with 300 µg/mL of gentamicin to block NMD complex.

3.6 HIV infections assay

PBMC and CD4^{+} T cells (10×106 cells/mL) were cultured for 2 days at 37°C and 5% CO_2 in RPMI 1640 containing FBS (20%), phytohemagglutinin (PHA) (7.5 µg/mL), and interleukin (IL)—2 (15 ng/mL). After viability assessment, 2.5×106 cells were resuspended in medium containing 1 ng of HIV-1Ba-L p24 viral input/10^6 PBMCs and incubated for 3 h at 37°C. Cells were then washed and resuspended in 3 mL of complete medium with IL-2 (15 ng/mL). Cells were plated in 24-well tissue culture plates and incubated at 37°C and 5% CO_2. After 5 days supernatants were collected for p24

antigen ELISA and MHC I analyses, while PBMCs and CD4$^+$ T cells were collected at 3 days and used for RNA extraction.

3.7 p24 ELISA

As a measure of HIV infection, p24 concentration was assayed using the Alliance HIV-1 p24 Antigen kit (Perkin Elmer, Boston, USA) following manufacturer's instructions. Analyses were performed on culture supernatants from both PBMCs and CD4$^+$ T cells collected 5 days post infection analyses. Briefly, cell culture samples are added directly to 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 resulting complex is detected by incubation with ortho- phenylenediamine-HCl (OPD) which produces a yellow color that is directly proportional to the amount of HIV-1 p24 captured. The absorbance of each microplate well is determined using a microplate reader and calibrated against the absorbance of an HIV-1 p24 antigen standard curve. Samples with absorbance values equal to or greater than the cutoff factor were considered initially reactive, but were retested in duplicate to determine whether the reactivity is reproducible.

3.8 RNA extraction

RNA was extracted from basal and cultured PBMCs and CD4$^+$ T lymphocytes by using the acid guanidium thiocyanate–phenol–chloroform method. RNAzol B reagent (TEL-TEST, Inc., Duotech, Milan, Italy), a monophase solution containing phenol and guanidine thiocyanate, was used.
Cells were lysed in RNAzol B and the lysate was separated into aqueous and organic phase by the addition of chloroform (20% of RNAzol B initial volume used).

Samples were centrifuged (at 12,000g 15 minutes at 4°C) to efficiently remove DNA and proteins from the aqueous phase containing RNA. The undegraded, pure RNA was obtained from the aqueous phase by isopropanol precipitation and washing with 75% ethanol. RNA quantity and purity was analyzed at Nanodrop 2000 Spectrophotometer (Thermo Scientific)

3.9 DNAse treatment and retrotranscription

The RNA was dissolved in RNase-free water, and purified from genomic DNA with TURBO DNAse (Applied Biosystems/Ambion, Austin, TX), a genetically engineered form of bovine DNase I with greater catalytic efficiency than conventional DNase I at higher salt concentrations and lower DNA concentrations. A reaction mixture, containing 1µg of RNA, Turbo DNasi 1U and TURBO DNasi Buffer, was incubated 30 minutes at 37 °C. Then DNase was inactivated by DNase inactivation reagent (Applied Biosystems/Ambion), that binds and removes the divalent cations from DNase.1 µg of RNA is reverse transcribed into first-strand cDNA in a 20-µl final volume. A reaction mixture, containing 1 µM random hexanucleotide primers, 1 µM oligo dT and the RNA, was heated at 70 °C for 5 minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent secondary structures from reforming. A dNTPs mix, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 U Recombinant RNasi inhibitor and M-MLV 5X reaction buffer were added (Promega). The reaction mix was incubated 60 minutes at 42 °C and then heated 5 minutes at 95 °C to inactivate the RT.

3.10 Real Time PCR

cDNA quantification for ERAP2-FL, ERAP2-AS, TAP1, IFNg, and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, MN). We also designed primers that do not discriminate ERAP2-

FL from ERAP2-AS, to quantify the total content of ERAP2 (ERAP2-TOT). Reactions were performed using a SYBR Green PCR mix (Finnzymes, Espoo, Finland). SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. Reactions were performed according with the following thermal profile: an initial denaturation (95 °C ,15 minutes) followed by 40 cycles of 15 sec at 95 °C (denaturation) and 1 min at 62 °C (annealing) and 20 seconds at 72 °C (extension). By recording the amount of fluorescence emission at each cycle, the PCR reaction was monitored during exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Melting point or dissociation curve analysis for amplicon identification, was performed.

The threshold line is the level of the detection or the point at which a reaction the reaction reaches a fluorescent intensity above background (the mean of fluorescence values detected from to third to tenth cycle, when target amplification is no appreciable yet). The threshold is set placed above baseline activity and in the exponential increase phase of the amplification for the most accurate reading. The parameter Ct (Threshold Cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct value. A Ct value of 40 or higher means no amplification and this value is not included in the calculations.

Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA. All the samples were analyzed in triplicate. All primers are summarize in Table 3.1.

GAPDH	For	CGGATTTGGTCGTATTGGG
	Rev	GCTTCCCGTTCTCAGCCTTG
TAP1	For	GCTGCCCACCAATGTAGAGGA
	Rev	GGCGAAGCCCAGAAGTTTAGG
IFNγ	For	GGCGACAGTTCAGCCATCAC
	Rev	TGTGGAGACCATCAAGGAAGACA
ERAP2tot	For	TCCAAACCAGCGGAAACCC
	Rev	CTGCTCCACAAGTCATCATTC
ERAP2FL	For	CCCAAGATGACAAGTAACATGCTC
	Rev	GGAAGCGCTCCTGTTGCAG
ERAP2SA	For	GAGATCTGTGGAATAGCCTGACC
	Rev	GGAAGCGCTCCTGTTGCAG

Table 3.1. *List of primers used to assess the expression of genes TAP1, IFNγ, ERAP-FL, ERAP-SA, ERAP-Tot and GAPDH, gene housekeeping used as a reference for assessing the increases.*

3.11 Antigen presentation pathway

Antigen presentation pathway was analyzed in a PCR array including a set of optimized real-time PCR primers on 96-well plates (LONZA, Basel, Switzerland). This approach permits to monitor mRNA expression of 84 genes involved in nearly all aspects of antigen processing and presentation following the procedures suggested by the manufacturer. The experiments were run on all of the subjects included in the study pooled into three distinct homo A, homo B and hetero AB groups. Thus, the results represents the mean value of the different targets analyzed in homo A, homo B and hetero AB groups. Furthermore, those targets showing marked differences between the three groups analyzed were retested by Real time PCR on each individual sample confirming the data obtained in the array.

3.12 Cloning plasmid construction and transfection

The cDNA of ERAP2 was PCR amplified using high fidelity polymerase (Pfu DNA Polymerase, Promega) from both hap A and hap B homozygous cell lines. To amplify the two isoforms of ERAP2, the following sequence of oligonucleotides were used:

Forward:5'ATACgcgatcgccATGcatcatcatcatcatcatATGTTCCATTCTTCTGCAATGG 3'

Reverse:5'CTTGgtttaaacTTAAGTATTAACCATTAGCCAAGTCCTCAGAGTC 3'

The two products were checked by direct sequencing and cloned into Flexi Vectors (Promega, Madison, WI, USA). After purification, PCR products were digested with the restriction enzymes SgfI and PmeI and ligated with T4 DNA Ligase. The translation reaction was performed with pF3WG (BYDV) Flexi Vector and the protein products were analyzed in SDS plyacrylamide gels (Promega, Madison, WI, USA).

3.13 Protein extraction

As ERAP2 is a transmembrane protein, the Kit Mem-Per Eukaryotic Membrane Protein Extraction Reagent (Thermo Scientific) was used to separate membrane protein from cytosolic protein so as to enrich the protein fraction of interest. 10×10^6 of PBMCs were centrifuged at 850 for 2 min. The pellet was washed with PBS and 150 µl of lysis solution was added for 10 min on ice. 450 µl of solubilizing membrane cell reagent plus a proteases inhibitor cocktail were then added for 30min on ice. Finally, supernatants were collected and stored in new tubes at -20 °C until use in a BCA assay (Pirce) for protein quantification.

3.14 Western Blot analyses

Protein extracts from approximately 10×10^6 PBMCs were separated on a 4–12% NuPage Bis-Tris gel (Invitrogen, Paisley, UK) at 120 V for 50 minutes in 16 NuPage MES SDS Running Buffer (Invitrogen, Paisley, UK). After transfer to a nitrocellulose membrane, proteins were detected using a 1:5,000 dilution of primary antibody [goat anti-ERAP2 polyclonal antibody (AF3830, R&D Systems, Minneapolis, MN, USA); anti-ß-actin monoclonal prepared in mouse (A5316, Sigma, Saint Louis, MO, USA)] and a 1:10,000 dilution of secondary antibody conjugated with alkaline phosphatase [goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology) and anti-goat IgG (A4187; Sigma, Saint Louis, MO, USA)]. Proteins were then visualized by enzymatic reaction after treatment with Sigma fast BCIP/NBT for 5 minutes (B5556; Sigma, Saint Louis, MO, USA).

3.15 Transfection

HeLa cells (uterine cervix carcinoma-derived cells) were cultured in DMEM high glucose supplemented with 10% FBS, 100 U/mL penicillin/ streptomycin, 2 mM glutamine. The cells were transfected with vectors pcDNA3.1/CT-GFP-MOUSE ERAP2-FL and ERAP2-SA using Lipofectamine 2000 (Invitrogen). The cells were spread in 6-well plates at a confluence of 70%. 3 µg of plasmid DNA and 15 µl of lipofectamine were incubated separately for 5 minutes in 250 µl of OptiMEM medium. Cells were then incubated 20 minutes at room temperature and added to the plates. Immunofluorescence analysis of transfected cells was performed 24 hours after transfection.

3.16 Immunofluorescence

The cells were incubated 2 h with the anti-HIS tag primary antibody (Chemicon International, Inc. Temecula, CA, USA) and anti-calreticulin as a

marker of the endoplasmic reticulum (Affinity BioReagents)(diluted 1:50 in PBS with BSA and saponin). Cells were subsequently washed in PBS and incubated with secondary antibodies Alexa Fluo 488 and 594 (Invitrogen Life Science, Carlsbad, CA, USA), diluted 1:500 in PBS with BSA and saponin. The nuclei were stained with 4 ', 6'-diamidino-2-phenylindole (DAPI, Sigma Aldrich) for 15 minutes at room temperature. The images were acquired using a Leica TCS SP2 AOBS confocal laser scanning with a 63x magnification and a resolution of 1024x1024 pixels.

3.17 HLA expression on CD45$^+$ cell surface

Flow cytometric analysis was performed 5 days post in vitro HIV-1 infection staining 0,5x106 PBMCs for 15 minutes with fluorescent directly labeled surface antibodies: CD45pecy5 and HLA-ABC FITC (Beckman Coulter, CA, USA). Cells stained with the appropriate isotype-matched Ig were used as negative controls. After staining, cells were fixed in 1% paraformaldehyde (PFA, Sigma-Aldrich). HLA-ABC expression was evaluated as mean intensity fluorescence (MFI) on leukocytes gated by CD45+ cells. Analyses were carried out blindly with respect to genotypes. Cytometric analysis was performed on 200,000 CD45 events using a FC500 flow cytometer (Beckman-Coulter CA, USA). Green florescence from FITC (FL1) is collected through a 525-nm band pass filter and deep-red fluorescence from Cy5PE (FL4) was collected through a 670-nm band pass filter using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1 and FL4. Flow data were analyzed by first gating on the leukocyte population as defined by forward and side light scatters and then on CD45 expression.

3.18 Statistical analysis

Statistical analyses were performed using SPSS Statistical Package for the Social Sciences (version 11; SPSS Inc., Chicago, Illinois, USA).

Differences between the groups were assessed using nonparametric analyses (Mann–Whitney U test). All P values are two-tailed. For meta-analysis a random-effects model as implemented in PLINK (Purcell et al., 2007) was applied.

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CHAPTER 4. RESULTS AND DISCUSSION

4.1 Genetic association with HIV-1 infection susceptibility

The haplotype A of ERAP2 was shown to have a higher frequency in a cohort of seronegative subjects who have been repeatedly exposed to HIV through unprotected heterosexual intercourses (Cagliani et al., 2010). To confirm this association we genotyped the rs2549782 variant that tags the two major ERAP2 haplotypes, with the G allele defining hap A (rs2549782G[392Lys]-rs2248374A [canonical splice site]) and the T allele defining hap B (rs2549782T[392Asn]rs2248374G[alternative splice site]) in a cohort of Spanish IDU-HESN and in age- and sex- matched HC. Thus, according to genotyping, participants were divided in Hap A/Hap A homozygotes (homo A), Hap B/Hap B homozygotes (homo B) or Hap A/Hap B heterozygotes (hetero AB). Results showed that the frequency of homo A was 26% and 14% in IDU-HESN and HC, respectively. The difference was statistically significant and the odds ratio for a recessive model with homo A being protected from HIV-1 infection is 0.49 (95% CI: 0.24- 0.99, Fisher's exact P= 0.033) (Table 1a). To perform a meta-analysis we used data from a previously published study. As estimation of the effect of heterogeneity is inaccurate when few data sets are included in the meta-analysis, we applied a random-effects model (Kavvoura 2008 10) and obtained a p value of 7.6 x10-5 (OR= 0.41) (Table 4.1). These results confirm that the rs2549782 ERAP2 variant is associated with protection against HIV infection and show how this effect is independent of the route of exposure.

	Association study in the Spanish cohort				Meta-analysis[a]			
Phenotype	Genotype counts (GG/GT/TT)	Genotype counts (recessive model, GG/GT+TT)	Fisher's p (recessive)	OR (95% CI)	Het. p-value[b]	I^{2} [c]	p_{meta} [d]	OR_{meta} [e]
IDU-HESN	27/46/31	27/77	0.033	0.49 (0.24- 0.99)	0.42	0	7.6 $\times 10^{5}$	0.41
HC	19/75/36	19/111						

[a] Meta-analysis was performed using data from a previous study of Italian sexually-exposed HESN subjects
[b] P value from Cochran Q heterogeneity test
[c] heterogeneity index
[d] random-effects meta-analysis p value
[e] Random-effects OR estimate

Table 4.1. *Association study and meta-analysis for rs2549782 in HESN*

4.2 Co-segregation of HLA-B*57 with rs2549782 genotypes

The protective effect of HLAB57, has been confirmed in different studies in which the authors highlighted the ability of HLAB57-restricted CTL to target multiple HIV peptides (Makadzange et al., 2006; Boutwell et al., 2009). To verify whether *ERAP2* haplotype status interacts with *HLA-B*57*, we genotyped rs2549782 and performed a co-segregation analysis on PBMCs isolated from 68 Italian sexually-exposed HESN and 187 HIV[+] patients. Results showed that 32% of homo A HESN are also HLA-B*57 positive, compared to only 6% of non-homo A subjects (*p*=0.010), indicating that the HESN phenotype is associated with the co-presence of the *HLA-B*57* allele and the homo A status at *ERAP2*, suggesting their synergy in protection from infection. The same situation is not observed among HIV[+]patients, as the percentage of homo A and non-homo A individuals carrying the *HLA-B*57* allele was similar (5% and 7%, respectively; *p*=0.73) (Tab. 4.2).

Phenotype	*HLA-B*57* status	rs2549782 genotype		*p* valuea
		GG (homoA)	GT or TT (non-homoA)	
HESN	*HLA-B*57*+	6	3	0.011
	HLA-*B*57*-	13	46	
HIV[+]	*HLA-B*57*+	5	9	0.14
	*HLA-B*57*-	29	144	

Table 4.2. *Co-segregation analysis of HLA-B*57 with rs2549782 genotypes in HESN and HIV*[+]*patients*

## 4.3 ERAP2-FL, ERAP2-AS and ERAP2-Tot mRNA basal expression

To further confirm these results, we enrolled 139 healthy controls and grouped them according to their ERAP2 genotype. The genotyping analysis of ERAP2 in these samples show that there were 35 homo A, 40 hetero AB and 64 homo B. Hap A and B were equally represented, reflecting the allele frequency reported for major human populations (www.hapmap.ncbi.nlm.nih.gov). On PBMCs isolated from subjects with different ERAP2 genotype we evaluated mRNA basal expression of ERAP2-FL, ERAP2-AS and ERAP2-TOT.

As expected basal mRNA transcription level for ERAP2-FL was significantly higher in homo A compared to hetero AB cells (p=0.04). Unexpectedly, marginal levels of ERAP2-FL mRNA are also detected in homo B PBMCs, these levels however, were significantly lower compared to those observed either in homo A (p=0.00031) or in hetero AB (p=0.006) cells (Fig. 4.1a).

Opposite results were obtained analysing ERAP2-AS mRNA. Thus, ERAP2-AS mRNA was found in homo B and, albeit in limited quantities, in hetero AB cells (p=0.0004), while it was completely absent in homo A PBMCs (Fig. 4.1b).

Finally, high, intermediate and low levels of ERAP2-Tot expression were seen in homo A, hetero AB and homo B samples respectively (Fig. 4.1c). These differences were statistically significant (P<0.05 in both cases), suggesting that subjects with different *ERAP2* genotype do express diverse quantities of ERAP2 that may qualitatively and or quantitatively affect the antigenic repertoire and consequently activating a diverse immunological response.
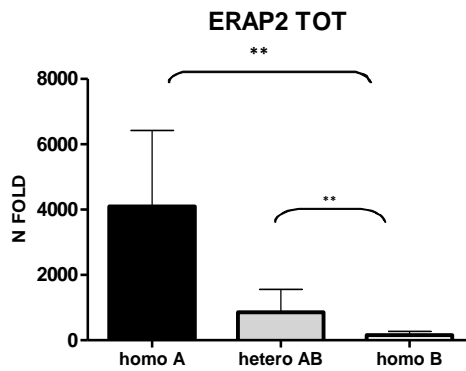
a

ERAP2-FL

b

ERAP2 AS

c
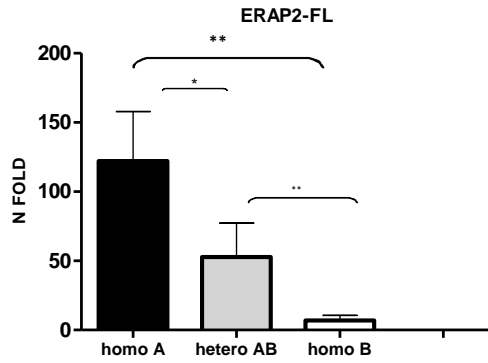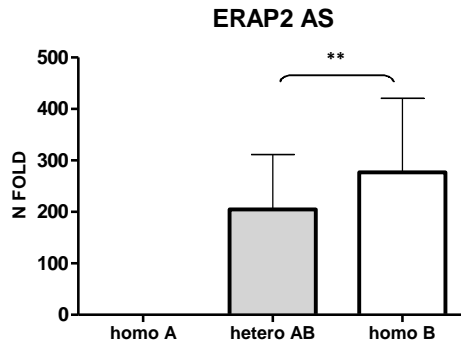
ERAP2 TOT

Figure 4.1. *Basal mRNA expression of ERAP2-FL (a) ERAP2-AS (b) and ERAP2-Tot (c) in cells of subjects with homo A (black bars); hetero AB (grey bars); and homo B (white bars) ERAP2 genotype*

4.4 mRNA expression of ERAP2-FL and ERAP2-AS in IFNγ-stimulated PBMCs

Since IFNγ is a potent stimulator of all genes directly involved in MHC class I antigen presentation, we investigated whether this cytokine affects the expression of ERAP2 variants.

We first analysed the kinetic of ERAP2-FL mRNA-expression on PBMCs isolated from 10 healthy controls stimulated by IFNγ. TAP1 was evaluated as positive control. Thus, PBMCs were stimulated with two doses of IFNγ (10 ng/ml and 100ng/mL) for 3h, 24h, 48h and 72h.

Results showed an increased expression of ERAP2-FL at 48h fold change (FC) 6 and a subsequent decrease at 72h after stimulation (FC 1) (Fig.4.2a). TAP1 mRNA expression mirrored ERAP2 trend (Fig 4.2b).In addition, since we did not observe any difference between the two doses of IFNγ, the lowest dose was used in the following experiments.

After 48h of IFNγ-stimulation, we evaluated mRNA expression of ERAP2-FL and ERAP2-AS on PBMCs isolated from 139 subjects divided according to their different ERAP2 genotype. As previously reported, following IFNγ stimulation we observed an increased ERAP2-FL-mRNA expression in homo A (FC 600) and hetero AB subjects (FC 400). These results were statistically significant, with a *p value* 0.05, in both subjects homo A and hetero AB compared with their unstimulated conditions (Fig.4.2c). Similarly, ERAP2-AS mRNA transcription level was augmented in homo B (FC 150) and hetero AB subjects (100) with a p-value < 0.05 (Fig 4.2d).

a

ERAP2-FL

b
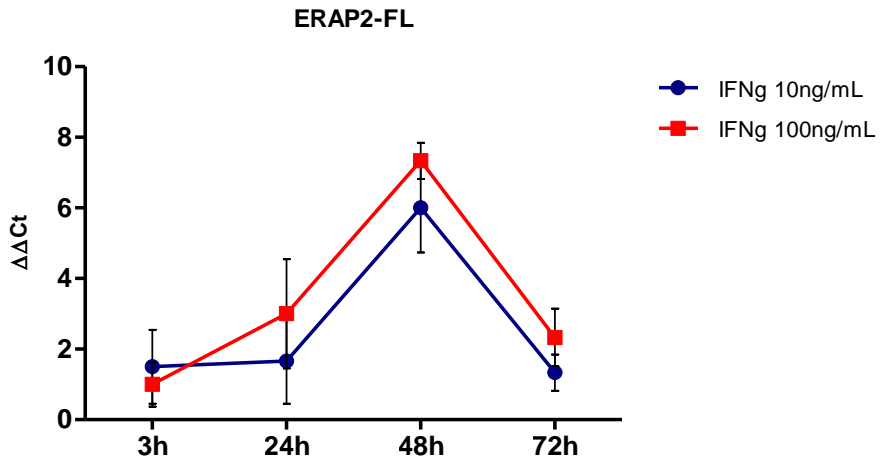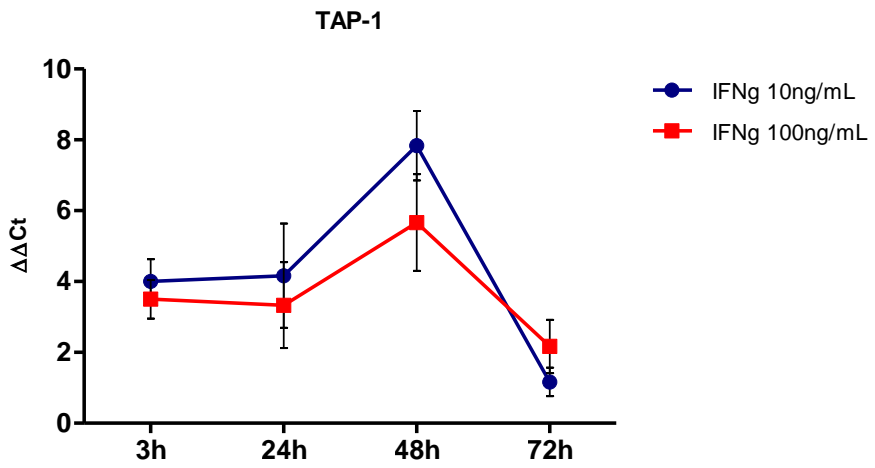
TAP-1

c

d

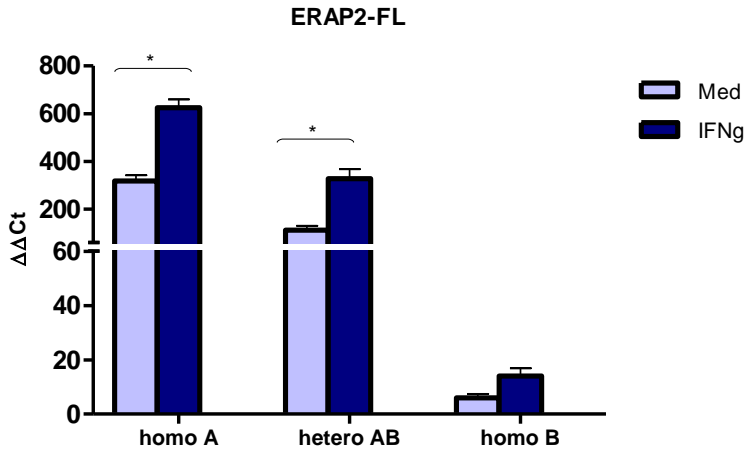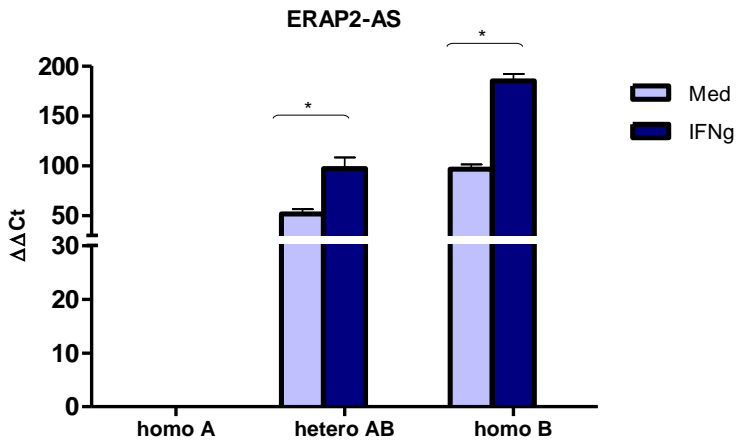**Figure4.2.** *mRNA expression of ERAP2-FL (a) and TAP 1 (b) in cells isolated from 10 HC and stimulated with 10 ng/ml (blue line); and with 100 ng/ml (red line) of IFNγ;at different time points. c & d mRNA expression of ERAP2-FL and ERAP2-AS in homo A hetero AB and homo B individuals in unstimulated (blue bars) and IFNγ-stimulated PBMCs (light blue bars). Results represents mean values ± SE.* p<0.05*
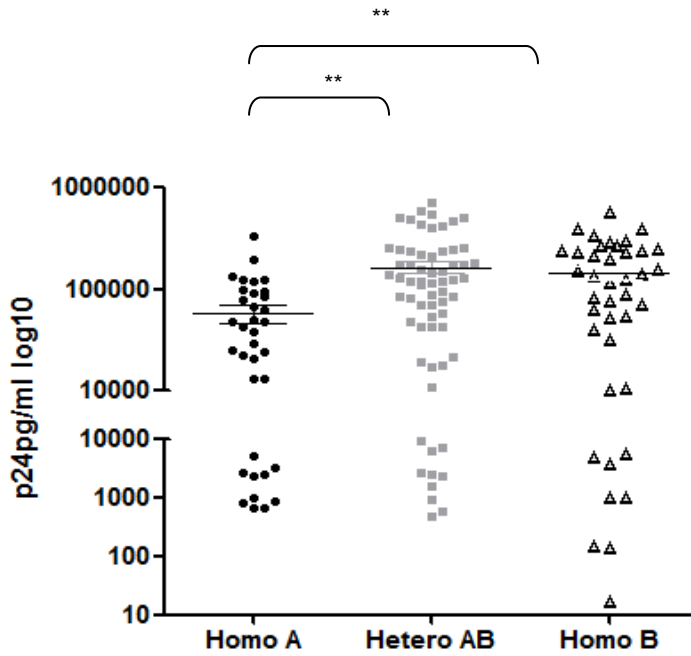
68

4.5 Susceptibility to HIV infection in subjects with different ERAP2 genotype

To verify if the expression of a diverse variant of ERAP2 influences susceptibility to HIV-1 infection, PBMCs isolated from the same 139 HC cohort described above were in vitro infected with a R5 tropic HIV-1 strain. Five days after infection p24 levels were significantly lower in homo A compared to both homo B (p=0.036) and hetero AB samples (p=0.003) (Fig. 4.3a). These results are consistent with the previously suggested recessive model of HIV-1 protection conferred by Hap A, and with the genetic association analysis in the IDU-HESN cohort (Table 4.1). Furthermore, it is interesting to observe that the heterozygotes have not an intermediate response compared to the other groups but become infected as much as homo B indicating a dominant negative effect of haplotype B on haplotype A.

Conversely, p24 levels in CD4^{+} T cell cultures isolated from 42 HC (7 homo A; 23 hetero AB; 12 homo B) were comparable in the three genotypic group, suggesting a role for CD8+ T cells in the regulation of this mechanism of resistance (Fig. 4.3b).
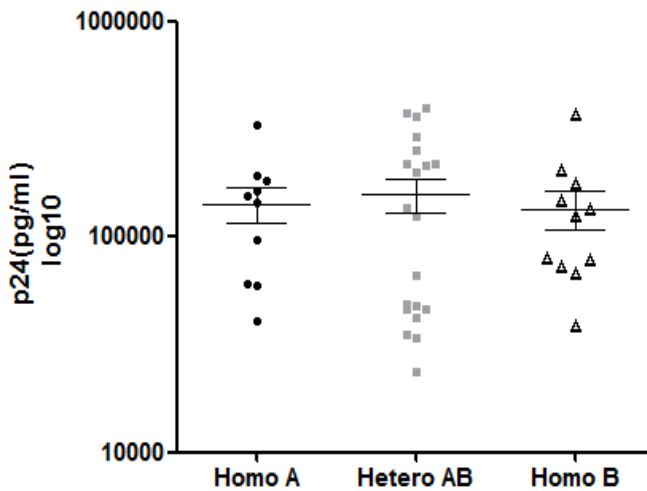
Figure 4.3. *p24 concentration in PBMCs (a) and (b) CD4^{+} T cells of subjects with different ERAP2 diplotype after 5 days of HIV infection. Mean values and S.E. are shown. ¼p <0.05, ¼p <0.005.*

4.6 Analyses of ERAP2-FL, IFNγ and TAP1 mRNA expression in PBMCs of individuals with different ERAP2 genotype

Following 3 days of *in vitro* HIV infection, analyses of mRNA specific for genes (ERAP2-FL, TAP1, IFNγ) involved in the presentation of peptides by MHC class I molecules to CD8^{+} T cells showed a completely diverse trend in subjects with different *ERAP2* genotype. Thus, a significantly higher transcription rate was observed for these three genes in homo A compared to both homo B and hetero AB cells ($p < 0.05$ for all comparisons) (Fig.4.4).

Notably, ERAP2-FL (Fig.4.4a), TAP1 (Fig.4.4b) and IFNγ (4.4c) mRNA expression was inversely correlated to p24 concentration, suggesting a possible association between the expression of these genes and susceptibility to HIV infection, possibly secondarily to a higher efficacy of cell mediated immunity.

a
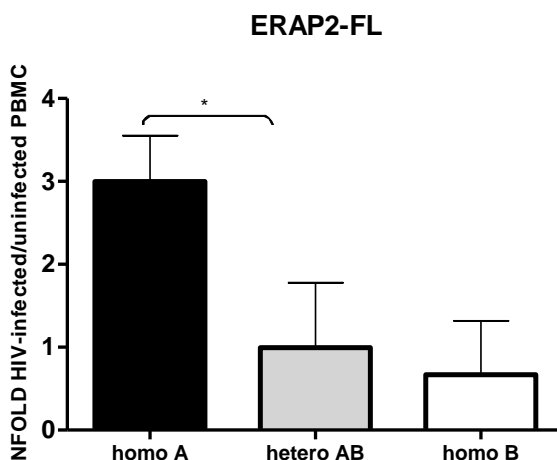
b
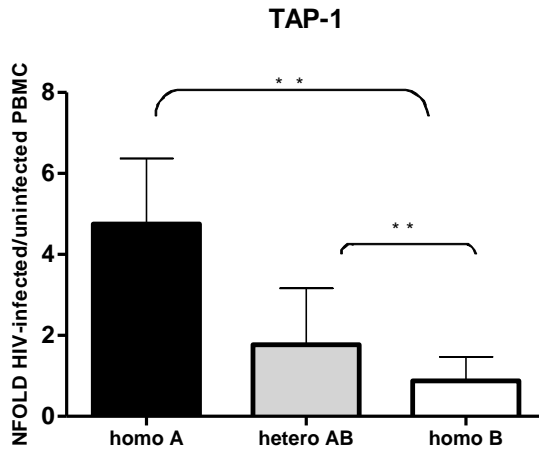
c
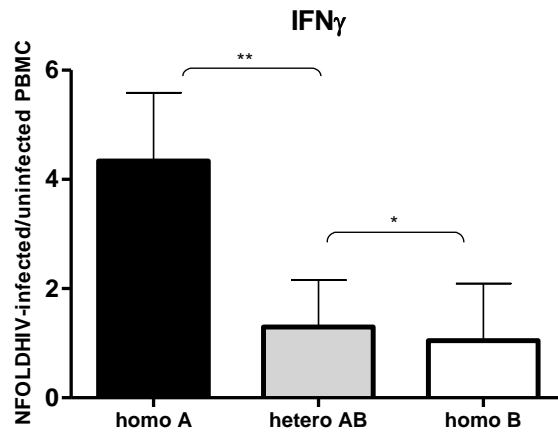
Figure 4.4. *mRNA expression of endoplasmic reticulum aminopeptidase type 2 (ERAP2)-full-length (a), transporter associated with antigen processing 1 (TAP1)(b) and interferon-g (c) after HIV-1 infection. ERAP2-FL, TAP1, and interferon-γ mRNA expression in PBMCs from homo A (black bars), hetero AB (grey bars) or homo B (white bars) ERAP2genotype participants after 3 days of HIV infection. Mean values and standard error are shown. * P<0.05, ** P<0.005.*

4.7 Antigen processing and presentation pathway

To determine whether the differences observed for ERAP2-FL, TAP1 and IFNγ expression in cells of individuals with different *ERAP2* genotypes could be extended to other factors involved in the antigen processing and presentation pathway, we used a Real-time PCR array, which screens for the expression of 84 genes implicated in that process.

Data obtained 3-days post *in vitro* HIV infection (Figure 4.5) showed a generalized and significant increase of a great number of the genes involved in the antigen processing and presentation pathway in homo A compared to both homo B and hetero AB cells. Notably, the 29 targets, which are expressed at a higher transcriptional rate in homo A, are involved in nearly all aspects of antigen processing and presentation, including: regulation and enhancement of class I and class II expression (*ABCB9, B2M, CALR, CD74, CTSE, CTSL2, HSPA5, HSP90AB1, HFE, IRF2, IRF8, LGMN, HLA-A, HLA-B, HLA-G, HLA-DQB2, HLA-DRA, PML, RFXANK, TAPBP*), the proteasome system (*PSME2*), costimulation of antigen presentation (*CD40, CD46, ICOS, PRKCD, STAT1*), lysosomal proteases (*LGMN*), antigen uptake by APC (*CD209, CDC42*), and aminopeptidases (*NPEPPS*).

Thus, it is tempting to speculate that the presence of a wider or more stable peptide repertoire generated by ERAP2 homo A cells is capable to boost the antigen presentation machine resulting in a more protective response to the virus.
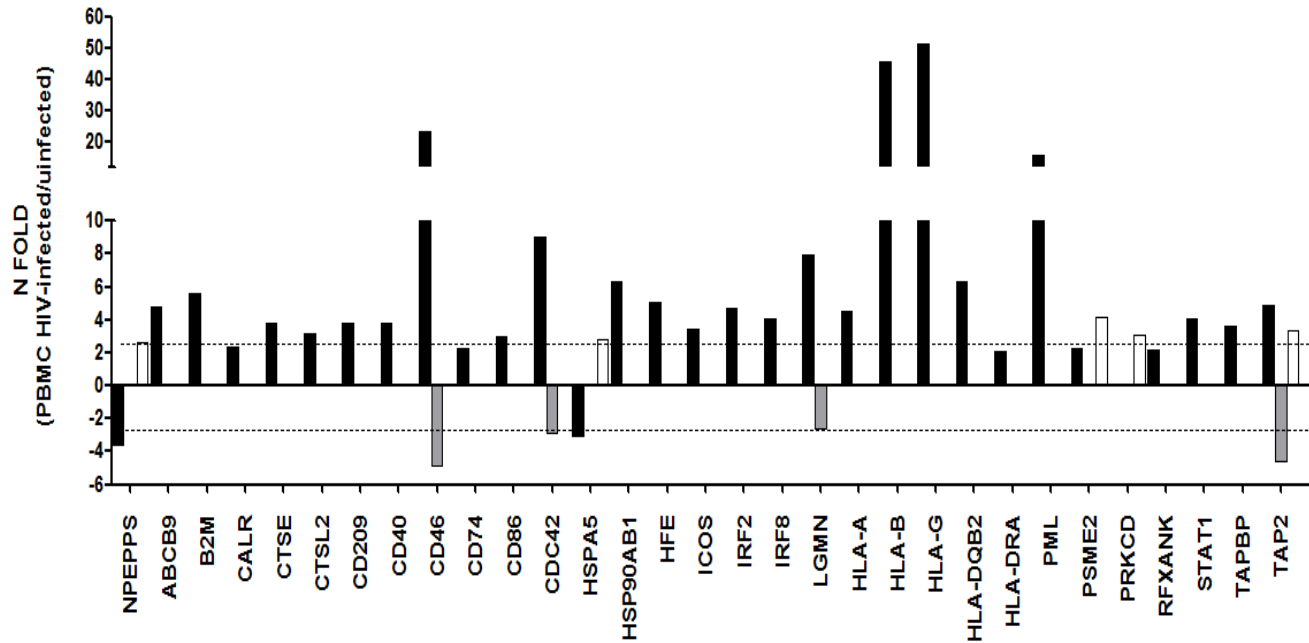
Figure 4.5. *Antigen processing and presentation pathway genes mRNA expression in cells of participants with homo A (black bars), hetero AB (grey bars) or homo B (white bars) ERAP2 genotype 3days after in-vitro HIV-infection. The expression of 84 genes involved in the antigen processing and presentation pathway was assessed by real-time quantitative RT-PCR and shown as fold-change expression from the uninfected sample. Only the targets showing different expression levels in the three groups are presented*

4.8 Effect of ERAP2 variants on MHC class I expression

Given that, experiments carried out by other research groups on cell lines showed that, silencing of ERAP2 causes a decreased expression of MHC class I molecules on the cellular surface and the transient knock-down of *ERAP1* and *ERAP2* was correlated to a reduced level of these molecules (Saveanu et al,.2005), we verified whether physiological down-regulation of endogenous ERAP2 had a similar outcome in haplotype B PBMCs. To this aim, we analysed HLA-A, B and C mean fluorescence intensities (MFI) on CD45$^+$ leukocytes. Results showed that MHC class I (HLA-A, B, C) MFIs are significantly reduced in hetero AB and homo B compared to homo A cells both in basal condition and upon HIV-1 infection (p< 0.05 in all conditions) (Figure 4.6). These results demonstrate that the presence of the *ERAP2* Hap B correlates with reduced levels of MHC class I expression on leukocyte surfaces, confirming a correlation between the presence of the homo A genotype and a stronger antigen presentation.
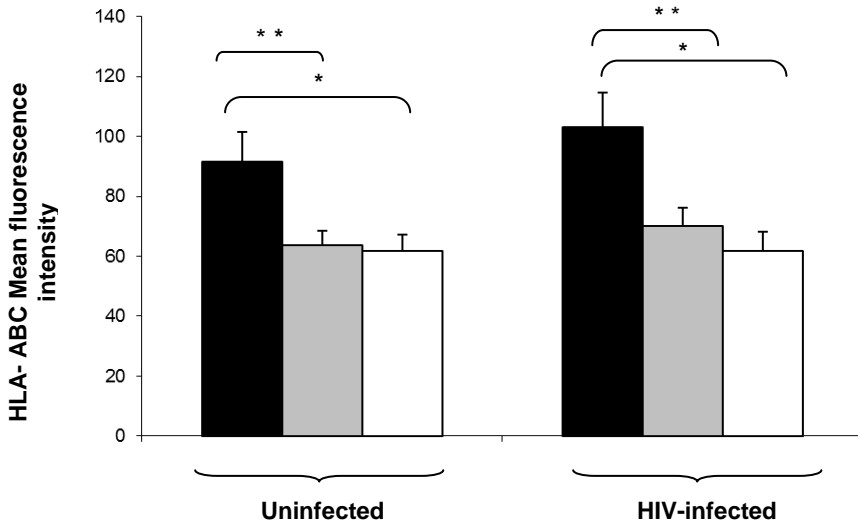
Figure 4.6. *Human leukocyte antigen (HLA)-ABC mean fluorescence intensities of CD45þ cells in homo A (black bars), hetero AB (grey bars) and homo B (white bars) participants. Mean values and standard error are shown. *P<0.05, **P<0.005.*

4.9 ERAP2 protein expression in PBMCs of individuals with different ERAP2 genotype

To verify if the mRNA derived from the full-length and alternative spliced form of *ERAP2* are both translated into functional proteins we performed a western blot analyses on PBMCs isolated from homo A, homo B and hetero AB individuals. Results showed that homo A as well as hetero AB PBMCs produce only full-length ERAP2 (120 kDa), while no detectable ERAP2 protein was found in cells isolated from homo B subjects. Notably, the intensity of the full-length ERAP2 band in hetero AB subjects was around half of the one detected in homo A subjects. Conversely, the truncated ERAP2 protein (62 kDa) could not be detected in any of the analysed subjects, independently of their *ERAP2* genotype. Recombinant ERAP2-AS and ERAP2-FL forms are used as positive controls (Figure 4.7). These data corroborate the assumption that ERAP2-AS mRNA undergoes a non-sense-mediated decay (Andres at al., 2010), but do not explain why hetero AB subjects display the same phenotype as homo B.
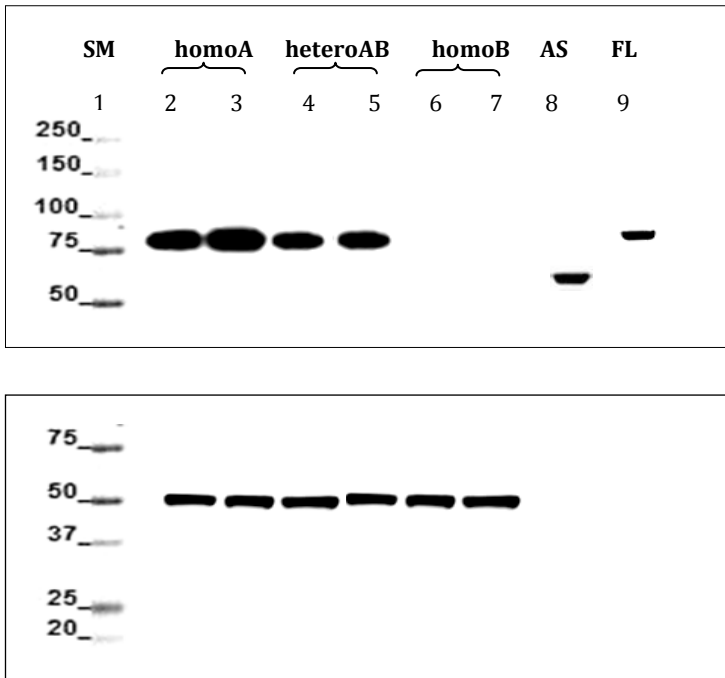
Figure 4.7. *PBMCs isolated from two participants representative of each ERAP2 diplotype (homo A, hetero AB and homo B) were tested for protein using primary antibodies specific to (a) ERAP2 (goat polyclonal) and (b) b-actin. Recombinant positive controls for the full-length positive (FLp) and alternative spliced positive (ASp) form of ERAP2 were also loaded.*

4.10 mRNA expression of ERAP2-FL and ERAP2-AS in gentamicin stimulated PBMCs

To verify whether the mRNA derived from the transcription of the non-synonym mutation Asp392Lys is degraded by intracellular mechanisms, as previously suggested by other authors (Andres et al., 2010), we analyzed the effect of pharmacological inhibition of non-sense-mediated decay (NMD). In this respect, PBMCs from 12 HC with different ERAP2 genotypes were isolated and treated with 300 µg of gentamicin for 20h, ERAP2-AS

and ERAP2 FL (positive control) mRNA expression are analyzed with real time PCR. Results showed that mRNA of both ERAP2-FL(Figure 4.8a) and ERAP2-AS (Figure 4.8b) is not increased after antibiotic treatment, thus confirming that ERAP2-AS is not degraded by the NDM pathway.

a
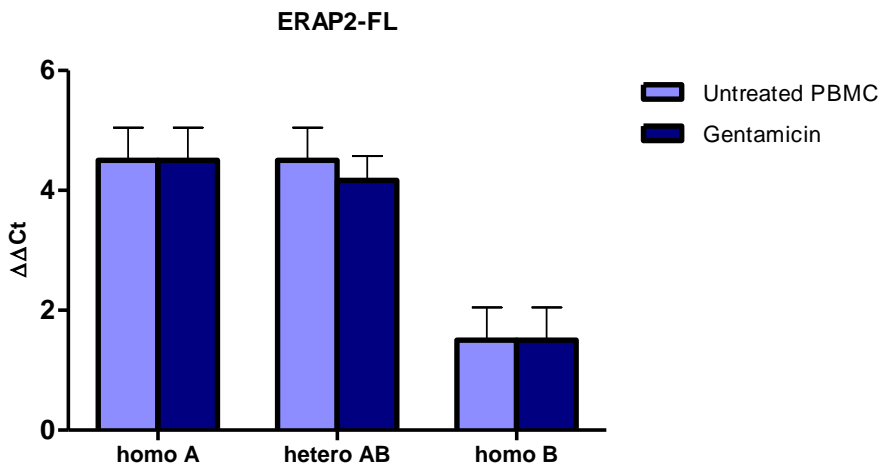
b
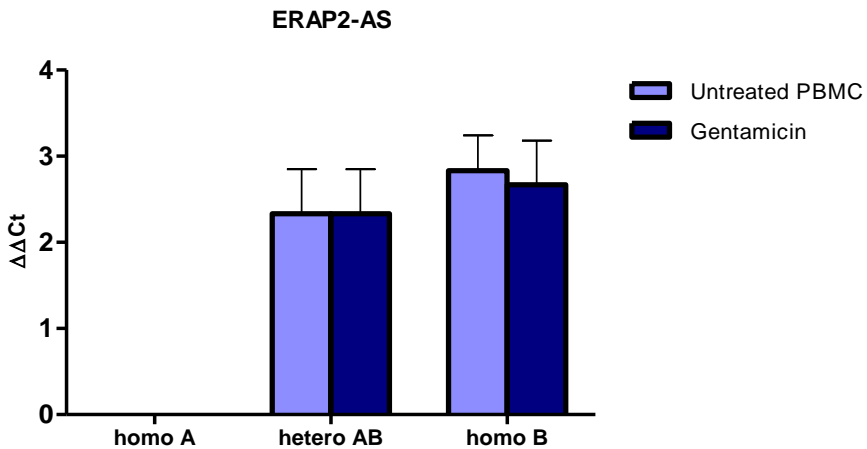
Figure 4.8. *Effect of NMD inhibitor on ERAP2-FL (a) and ERAP2-AS (b) mRNA transcript level. GAPDH was used as a reference gene.Mean values±standard errors are shown*

4.11 ERAP2-FL and ERAP-AS localization in endoplasmic reticulum

To identify the localization of ERAP2-FL and ERAP2-AS, an immunofluorescence assay is performed on HeLa cells transfected with the pcDNA3.1/CT-GFP-MOUSE vector, which encodes for both ERAP2 variants. Confocal microscopy showed that anti-histidine antibody, that tags both ERAP2 variants, co-localize with calreticulin antibody (Figure 4.10). Data suggest that whether ERAP2-AS mRNA might be translated in its functional product, it localizes at the level of the endoplasmic reticulum where it could exert its biological function.
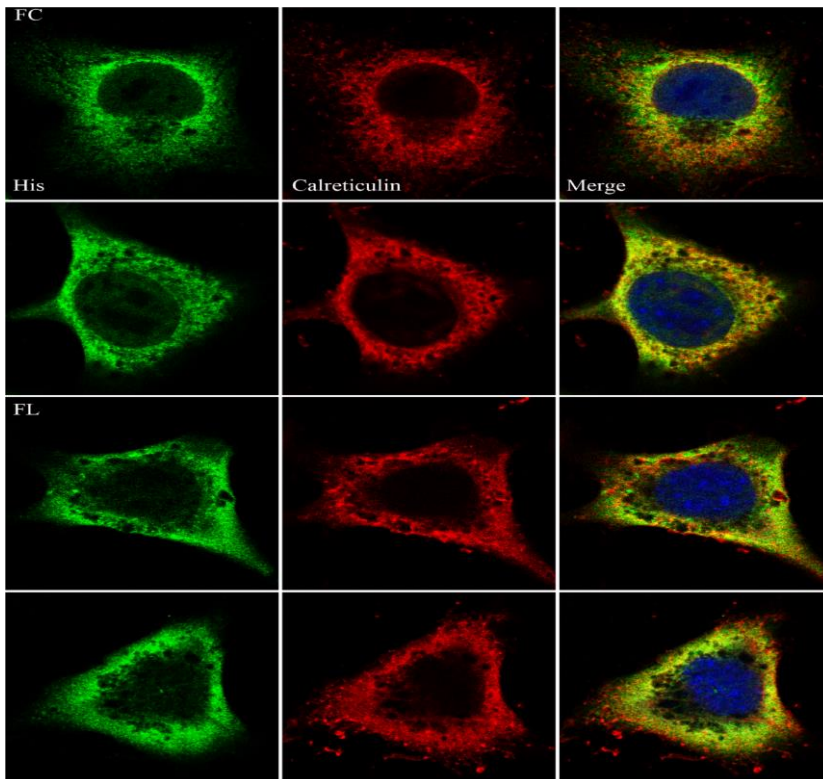
Figure 4.10. *Confocal microscopy of ERAP-FL and ERAP2-AS (green) in endoplasmic reticulum (red). Merge of ERAP2-AS and calreticulin (yellow)*

CHAPTER 5. CONCLUSIONS

The important biological functions of ERAP1 and ERAP2, in modulating the adaptive immune response are confirmed in recent studies. In particular, the variant that tags Hap A (rs2549782-G) in ERAP2 was shown to protect from sexually-transmitted HIV-1 infection (Cagliani et al., 2010). This initial observation is based on the analysis of a relatively small cohort of Italian HESN. Data herein replicate the association between the ERAP2 polymorphism and HIV-1 protection in an independent cohort of subjects with different geographic origin and a diverse infection exposure route. Meta-analysis of the results obtained in the two cohorts provided strong support to the association between diplotype status at ERAP2 and natural resistance to HIV-1 infection, suggesting that the protection from infection is independent on the route of exposure.

To confirm their role in susceptibility to infection, we analyzed the PBMCs from homo A subjects under HIV-1 infection, allow lower viral replication compared to cells derived from both hetero AB and homo B subjects. Conversely, p24 levels in CD4+ T cell cultures are comparable in the three genotypic groups, indicating an involvement of CD8^{+} T lymphocytes in the regulation of this mechanism of resistance.

This effect may at least partially be mediated by different ERAP2 expression levels in subjects with diverse ERAP2 genotype. In fact, results herein indicate that both in basal condition and following in vitro HIV-1 infection the transcript level for ERAP2-FL and ERAP2-Tot was significantly higher in homo A compared to hetero AB and barely detectable in homo B cells. These data are in line with previous observations and with western blot results, suggesting that ERAP2 diplotype status determines the abundance of the full-length protein product of ERAP2, which, in turn, might shape the antigen repertoire available for CD8^{+} T cell clone stimulation. Moreover, we were unable to detect any signal for the truncated protein. These data corroborate the assumption that ERAP2-AS mRNA undergoes

a non-sense-mediated decay, but we have shown that this mechanism does not affect the mRNA of ERAP2-AS. There are several possible explanations to understand why we were unable to detect the ERAP2-AS form. First, the amount of truncated protein might be under the sensitivity threshold of the technique, but could still exert a dominant negative effect. Second, the amount of full-length ERAP2 protein might be rate-limiting for efficient antigen presentation, so that a decrease in the overall available protein is sufficient to produce an immunologic response to HIV-1 infection that is scarcely protective. Third, alterations in ERAP2 availability might affect the stoichiometry of ERAP1/ERAP2 hetero-dimers formation. Indeed, the two aminopeptidases are known to physically interact, although the precise fraction of ERAP1 and ERAP2 which is engaged in complex formation is unknown (Saveanu,L. 2005). Lower levels of ERAP2 protein might thus alter the relative abundance of homo- and hetero-dimers and, as a consequence, the final repertoire of MHC I available cargos. This in turn might be relevant to the specificity of CD8$^+$ T cells, as suggested with *ERAAP*-deficient mice (Hammer 2007; Hammer 2006; Yan,J. 2006; York 2006; Firat 2007).

Confirming the assumptions and the results previously obtained we demonstrate that reduction of ERAP2-FL levels results in a quantitative diminution of MHC class I expression both in uninfected and HIV-1 infected cell cultures when homo A are compared both to homo B and hetero AB CD45$^+$ cells. Notably, results also demonstrated that, following HIV-1 infection, not only ERAP2-FL but also a number of genes directly involved in the antigen processing and presentation pathway are expressed at a higher levels in homo A compared to both homo B and hetero AB cells. The genes whose mRNA is upregulated in homo A cells are implicated in almost all phases of antigen processing and presentation, including the regulation and enhancement of class I and class II expression (TAP1,

ABCB9, B2M, CALR, CD74, CTSE, CTSL2, HSPA5, HSP90AB1, HFE, IRF2, IRF8, LGMN, HLA-A, HLA-B, HLA-G, HLA-DQB2, HLA-DRA, PML, RFXANK, TAPBP), the proteasome system (PSME2), antigen presentation costimulation (CD40, CD46, ICOS, PRKCD, STAT1), lysosomal proteases (LGMN), as well as antigen uptake by antigen presenting cells (CD209, CDC42) and aminopeptidases (NPEPPS). It is possible to speculate that in the presence of a wider or more stable peptide repertoire generated by ERAP2 homo A cells all the factors involved in the presentation of the antigen are boosted, contributing to the activation of an optimal immune response.

Indeed, the role of ERAP1 and ERAP2 in antigen processing and presentation suggests that variants in these genes might also interact with specific HLA class I alleles to modulate distinct phenotypes. Therefore, the role of ERAP1 and ERAP2 in antigen processing and presentation suggests that variants in these genes might also interact with specific HLA class I alleles to modulate distinct phenotypes.

Data herein indicate that, in HESN, the HLA-B*57 allele is much more common than expected among homo A subjects, suggesting synergy in protecting from infection. Whether additional HLA alleles interact with ERAP2 genotypes remains to be evaluated, but is a likely possibility as multiple alleles, as well as specific amino acid residues in HLA-B and HLA-C, are known to control HIV viral load.

To summarize, ERAP2 diplotype status is associated with resistance to HIV infection; this effect is probably secondary to the modulation of the antigen processing/presenting machinery, resulting in quantitative and possibly qualitative changes in MHC class I complexes on target cells.

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APPENDIX

Endoplasmic reticulum aminopeptidase 2 haplotypes play a role in modulating susceptibility to HIV infection

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Objective: Haplotype-specific alternative splicing of the endoplasmic reticulum (ER) aminopeptidase type 2 (*ERAP2*) gene results in either full-length (FL, haplotype A) or alternatively spliced (AS, haplotype B) mRNA. As ERAP2 trims peptides loaded on major histocompatibility complex (MHC) class I and CD8^{+} T lymphocytes protect against viral infections, we analysed its role in resistance to HIV-1 infection.

Methods: *ERAP2* polymorphisms were genotyped using a TaqMan probe, and human leukocyte antigen (HLA) typing of class-I *HLAB* locus was performed by single specific primers-polymerase chain reaction method. To verify whether *ERAP2* genotype influences susceptibility to HIV-1 infection *in vitro* we performed HIV-1 infection assay. We evaluated antigen presentation pathway with PCR array and the viral antigen p24 with ELISA.

Results: Genotype analysis in 104 HIV-1-exposed seronegative individuals (HESNs) exposed to HIV through IDU-HESN and 130 controls from Spain indicated that hapA protects from HIV infection. Meta-analysis with an Italian cohort of sexually exposed HESN yielded a *P* value of 7.6×10^{-5}. *HLAB* typing indicated that the *HLA-B*57* allele is significantly more common than expected among HESN homozygous for haplotype A (homoA). Data obtained in a cohort of 139 healthy Italian controls showed that following in-vitro HIV-1 infection the expression of ERAP2-FL and a number of genes involved in antigen presentation as well as of MHC class I on the surface of CD45^{+} cells was significantly increased in homoA cells; notably, homoA peripheral blood mononuclear cells, but not isolated CD4^{+} cells, were less susceptible to HIV-1 infection.

Conclusion: *ERAP2* hapA is correlated with resistance to HIV-1 infection, possibly secondarily to its effect on antigen processing and presentation.

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Introduction

CD8$^+$ T cells recognize targets through interaction between their T-cell receptor and a binary complex formed by a major histocompatibility complex (MHC) class I molecule associated with a peptide produced by the degradation of intracellular proteins [1,2]. This process allows CD8$^+$ T lymphocytes to identify and eliminate cells that are synthesizing abnormal or foreign proteins as may arise through mutations or viral infections. The composition of the peptide repertoire presented by the MHC I depends on the specificity of the MHC peptide-binding groove, but also on the availability of peptides generated by the antigen-processing pathway that can fit in such groove.

The processing of intracellular proteins is a multistep event beginning in the cytoplasm where the proteasome fragments intracellular proteins to cut them at the precise C-terminus of the final peptide and generates a mixture of N-terminally extended intermediates [3]. These peptides are then transported into the endoplasmic reticulum (ER) where the ER aminopeptidase 1 and 2 (ERAP1 and ERAP2) trim the N-terminal residues to generate the correct length peptides, which are subsequently bound by MHC class I molecules and presented on the cell surface [3].

Interestingly, studies in *Erap1*−/− mice show that this enzyme shapes the peptide repertoire presented by MHC I molecules in normal and virus-infected cells [4,5]. Recent data supporting this observation show that cytomegalovirus manages to avoid cytotoxic T lympho-cytes (CTL)-mediated immune responses by expressing a micro-RNA that specifically targets ERAP1, inhibiting the trimming of precursors to generate mature epitopes [6].

Both *ERAP1* and *ERAP2* have been targets of a long-standing balancing selection [7,8]. In particular, this process has maintained two highly differentiated *ERAP2* haplotypes at intermediate frequency in most human populations [7,8]. The two haplotypes, hereafter referred to HapA and HapB, differ at multiple variants in tight linkage disequilibrium. HapB harbours the T allele for rs2549782 (Asn392Lys) and the G allele for rs2248374; this latter has been shown to determine 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 [8,9]. Thus, the predicted protein product of HapB-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) trans-cribed from HapA.

Recently the G allele of rs2549782, which tags HapA, was found to be significantly overrepresented in HIV-1-exposed seronegative individuals (HESNs), indicating that association between rs2549782 and HIV-1 protection follows a recessive model [7].

Based on this observation we extended the analyses of the possible role of *ERAP2* in the modulation of resistance to HIV infection by verifying whether diverse *ERAP2* alleles differently modulate antigen processing and presentation.

Materials and methods

Study population

We recruited 104 white men exposed to HIV-1 infection by IDU from a Spanish cohort (Valme Hospital, Seville, Spain) whose epidemiological and clinical characteristics were described previously [10]. A group of 130 HIV-1 and HCV-seronegative healthy white men blood donors from the Jaen Hospital was used as healthy controls.

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

Similarly, informed consent was obtained from 139 HIV-1 and HCV-seronegative Italian healthy controls. Whole blood was collected from all participants, and DNA was extracted by phenol chloroform method (Promega, Madison, Wisconsin, USA). As for Italian sexually HESN, inclusion criteria are described in [7].

Genotyping

Genomic DNA was used as template for PCR amplifica-tion using TaqMan probes specifically designed to perform a SNP genotyping assay for rs2549782 (G/T) (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, California, USA) and using the allelic discrimination real-time PCR method. The polymorphic 32 bp deletion at the *CCR5* locus was typed by PCR using specific primers (forward: 5′-TGGTGGCTGTGTTTGCGTCT-3′; reverse: 5′-ATGACAAGCAGCGGCAGGAC-3′). The PCR products were electrophoretically separated on 3% agarose gels.

Human leukocyte antigen typing

Human leukocyte antigen (HLA) typing of class-I HLA-B locus was performed by single specific primers-polymerase chain reaction method using Histo-Type DNA plates (BAG Healthcare, Formedic s.r.l., Milan, Italy). Detection of the alleles recognized by the specific primers was possible after amplification in a GeneAmp PCR 9700 thermocycler (Applied Biosystems) and gel electrophoresis on 2% agarose gel.

HIV infections assay

Peripheral blood mononuclear cell (PBMC) were separated by whole blood on lymphocyte separation medium (Organon Teknica, West Chester, Pennsylvania, USA), and CD4+ cells isolated with the CD4+ Isolation Kit (Miltenyi Biotech Gladbach, Germany) were in-vitro infected as previously described [11]. After 5 days supernatants were collected for p24 antigen ELISA (PerkinElmer Waltham, Massachusetts, USA) and MHC I analyses, whereas PBMC collected at 3 days were used for RNA extraction.

Real-time PCR

cDNA quantification for ERAP2-FL, ERAP2-AS, interferon (IFN)-γ, transporter associated with antigen processing 1 (TAP1) and Glyceraldehyde 3-phosphate dehydrogenase was performed by real-time PCR as described elsewhere [11]. We also designed primers that do not discriminate ERAP2-FL from ERAP2-AS, to quantify the total content of ERAP2 (ERAP2-Tot). Primer sequences are reported in supplementary methods (Table S1, http://links.lww.com/QAD/A327).

Antigen presentation pathway

Antigen presentation pathway was analysed in a PCR array including a set of optimized real-time PCR primers on 96-well plates (LONZA, Basel, Switzerland) (Supplemental Material, http://links.lww.com/QAD/A327). The experiments have been run on 139 Italian healthy controls pooled into three distinct homoA, homoB and heteroAB groups. Thus, the results represent the mean value of the different targets analysed in homoA, homoB and heteroAB groups.

Human leukocyte antigen expression on CD45+ cell surface

Flow cytometric analysis was performed 5 days after in-vitro HIV-1 infection staining 5×10^5 PBMCs for 15 min with fluorescent directly labelled surface antibodies: CD45pecy5 and HLA-ABC FITC (Beckman Coulter, Brea, California, USA). Cells stained with the appropriate isotype-matched immunoglobulin (Ig) were used as negative controls. After staining, cells were fixed in 1% paraformaldehyde (PFA; Sigma-Aldrich). HLA-ABC expression was evaluated as mean fluorescence intensity (MFI) on leukocytes gated by CD45+ cells. Analyses were carried out blindly with respect to genotypes. Cytometric analysis was performed on 200 000 CD45 events using a FC500 flow cytometer (Beckman-Coulter). Green florescence from FITC (FL1) was collected through a 525-nm band pass filter and deep-red fluorescence from Cy5PE (FL4) was collected through a 670-nm band-pass filter using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1 and FL4. Flow data were analysed by first gating on the leukocyte population as defined by forward and side light scatters and then on CD45 expression.

Western blot analyses

Western blot analyses were performed as previously described [12]. Proteins were detected using a 1:5000 dilution of primary antibody [goat anti-ERAP2 polyclonal antibody (AF3830; R&D Systems, Minneapolis, Minnesota, USA), anti-β-actin monoclonal prepared in mouse (A5316; Sigma, Saint Louis, Missouri, USA)] and a 1:10 000 dilution of secondary antibody conjugated with alkaline phosphatase [goat antimouse Immunoglobulin G (IgG) (sc-2005; Santa Cruz Biotechnology Dallas, Texas, USA) and antigoat IgG (A4187; Sigma]. Proteins were then visualized by treatment with Sigmafast BCIP/NBT for 5 min (B5556; Sigma).

Cloning, plasmid construction and transfection

The cDNA of ERAP2 was PCR amplified using high-fidelity polymerase (Pfu DNA Polymerase; Promega) from both hapA and hapB homozygous cell lines. The two products were checked by direct sequencing and cloned in the cell-free pF3A WG(BYDV) expression vector (Promega).

Statistical analyses

Statistical analyses were performed using SPSS Statistical Package for the Social Sciences (version 11; SPSS Inc., Chicago, Illinois, USA). Differences between the groups were assessed using nonparametric analyses (Mann–Whitney U test). All P values are two-tailed. For meta-analysis a random-effects model as implemented in PLINK [13] was applied.

Results

Genetic association with HIV-1 infection susceptibility

The rs2549782 variant tags the two major *ERAP2* haplotypes, with the G-allele defining hapA (rs2549782G[392Lys]–rs2248374A [canonical splice site]) and the T-allele defining hapB (rs2549782T[392Asn]–rs2248374G [alternative splice site]). Thus, according to genotyping, participants were determined to be HapA/HapA homozygotes (homoA), HapB/HapB homozygotes (homoB) or HapA/HapB heterozygotes (heteroAB). Results showed that the frequency of homoA was 26 and 14% in IDU-HESN and healthy controls, respectively. Logistic regression indicated that the difference is statistically significant, and the odds ratio for a recessive model with homoA being protected from HIV-1 infection is 0.50 [95% confidence interval (95% CI) 0.26–0.97, $P = 0.0392$] (Table 1) [7]. The frequency of the *CCR5Δ32* allele is higher in IDU-HESN (0.102) than in healthy controls (0.052), but inclusion of genotype status at *CCR5* as a covariate in the logistic regression did not affect the association with rs2549782 ($P = 0.0391$).

Table 1. Association study for rs2549782 in HIV-1-exposed seronegative individuals.

| Phenotype | Association study in the Spanish cohort | | | | Combined[a] | | | | |
	Genotype counts (GG/GT/TT)	Genotype counts (recessive model) GG/GT+TT)	P (recessive model)	OR (95% CI)	Het P-value[b]	I[c]	P$_{meta}$[d]	OR$_{meta}$[e]
IDU-HESN	27/46/31	27/77	0.0392	0.50 (0.26–0.97)	0.42	0	9.6 × 10⁻⁵	0.41
HC	19/75/36	19/111						

CI, confidence interval; HC, healthy controls; HESN, HIV-1-exposed seronegative individual; OR, odds ratio.
[a]Meta-analysis was performed using data from a previous study of Italian sexually exposed HESN subjects [7].
[b]P value from Cochran Q heterogeneity test.
[c]Heterogeneity index.
[d]Random-effects meta-analysis P value.
[e]Random-effects OR estimate.

To perform a meta-analysis we used data from the previously published study [7]. We applied a random-effects model [14] and obtained a P value of 9.6×10^{-5} [odds ratio (OR) = 0.41] (Table 1). These results confirm that the rs2549782 ERAP2 variant is associated with protection against HIV infection and show how this effect is independent on the route of exposure.

Human leukocyte antigen-B typing and co-occurrence analysis

The HLA-B* 57 allele has previously been associated with protection from HIV-1 infection [15,16]. To verify whether ERAP2 haplotype status interacts with HLA-B* 57, we genotyped rs2549782 and performed a co-occurrence analysis in Italian sexually exposed HESN and HIV-positive participants. Results showed that 32% of homoA HESN are also HLA-B*57 positive, compared with only 6% of nonhomoA participants (P = 0.011), indicating that the HESN phenotype is associated with the co-presence of the HLA-B*57 allele and the homoA status at ERAP2. The same situation is not observed among HIV-positive individuals, as the percentage of homoA and nonhomoA individuals carrying the HLA-B* 57 is not significantly different (P = 0.14) (Table 2).

Endoplasmic reticulum aminopeptidase 2 mRNA basal expression

PBMC of 139 Italian healthy controls were genotyped for rs2549782; results showed that HapA and B were equally represented, mirroring the allele frequency reported for major human populations [7,8].

ERAP2-FL mRNA levels were significantly higher in homoA compared to heteroAB cells (P = 0.04). Unexpectedly, marginal levels of ERAP2-FL mRNA were also detected in homoB PBMC, although were significantly lower compared with those observed either in homoA (P = 0.00031) or in heteroAB (P = 0.006) cells (Fig. 1a).

ERAP2-AS mRNA was found in homoB and, even though in limited quantities, in heteroAB cells (P = 0.0004), whereas it was completely absent in homoA PBMC (Fig. 1b).

Finally, high, intermediate and low levels of ERAP2-Tot expressions were seen in homoA, heteroAB and homoB samples, respectively (Fig. 1c). These differences were statistically significant (P < 0.05 in both cases), suggesting that participants with different ERAP2 genotype do express diverse quantities of ERAP2 to shape their antigen repertoire.

Susceptibility to HIV infection

To verify whether ERAP2 genotype influences susceptibility to HIV infection in vitro, PBMCs isolated from the 139 Italian healthy controls (35 homoA, 64 heteroAB and 40 homoB) were in-vitro infected with HIV-1. Five days after infection p24 levels in PBMC cultures were significantly lower in homoA compared with both homoB (P = 7 × 10⁻⁴) and heteroAB samples (P = 2.94 × 10⁻⁵) (Fig. 1d). These results are consistent with the previously suggested recessive model of HIV-1

Table 2. Co-segregation analysis of HLA-B*57 with rs2549782 genotypes in HESN and HIV positive.

| Phenotype | HLA-B*57 status | rs2549782 genotype | | P value[a] |
		GG (homoA)	GT or TT (nonhomoA)	
HESN	HLA-B*57+	6	3	0.011
	HLA-B*57⁻	13	46	
HIV⁺	HLA-B*57+	5	9	0.14
	HLA-B*57⁻	29	144	

HESN, HIV-1-exposed seronegative individual.
[a]Fisher's exact test P value.

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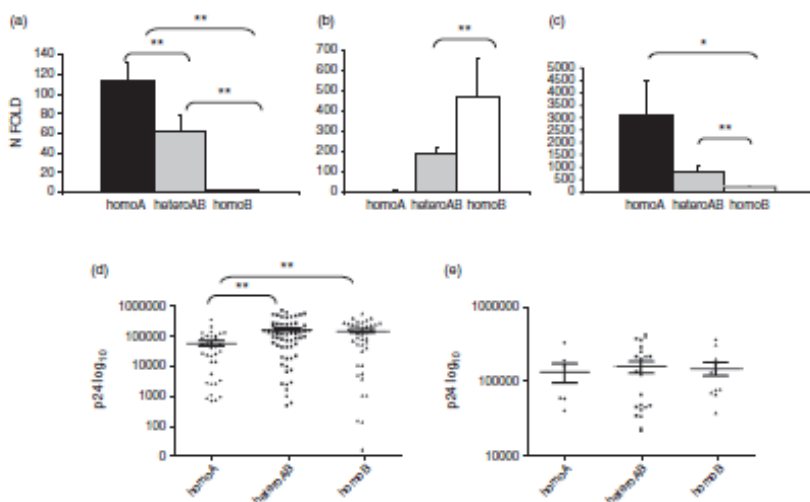
Fig. 1. Basal mRNA expression of endoplasmic reticulum aminopeptidase 2 (ERAP2)-full-length. (a) ERAP2-AS (b) and ERAP2-Tot (c) in cell of participants with homoA (black bars), heteroAB (grey bars) and homoB (white bars) *ERAP2* genotype. (d) p24 concentration in peripheral blood mononuclear cell and (e) CD4$^+$ cells of participants with different *ERAP2* diplotype after 5 days of HIV infection. Mean values and standard error are shown. *$P < 0.05$, **$P < 0.005$.

protection conferred by HapA [7], and with the genetic association analysis in the IDU-HESN cohort (Table 1).

Conversely, p24 levels in CD4$^+$ cell cultures isolated from 42 healthy controls (7 homoA, 23 heteroAB, and 12 homoB) were comparable in the three genotypic group, suggesting a role for CD8$^+$ cells in resistance to HIV-1 infection in homoA participants (Fig. 1e).

Analyses of endoplasmic reticulum aminopeptidase 2-full-length, interferon-γ and Transporter associated with antigen processing 1 mRNA expression

Following 3 days of in-vitro HIV infection, a significantly higher transcription rate was observed for ERAP2-FL, IFN-γ and TAP1 in homoA compared with both homoB and heteroAB cells ($P < 0.05$ for all comparisons) (Fig. 2a).

Notably, ERAP2-FL, TAP1 and IFN-γ mRNA expression were inversely correlated to p24 concentration, suggesting a possible association between the expression of these genes and susceptibility to HIV-1 infection.

### Antigen processing and presentation pathway genes mRNA expression

To determine whether the differences observed for ERAP2-FL, TAP1 and IFN-γ expression in cells of

individuals with different *ERAP2* genotypes could be extended to other factors involved in the antigen processing and presentation pathway, we used a real-time PCR array, which screens for the expression of 96 genes implicated in that process.

Data obtained by real-time PCR array 3 days after in-vitro HIV infection (Fig. 2b) show a generalized and significant increase of a great number of the genes involved in nearly all aspects of antigen processing and presentation pathway in homoA compared with both homoB and heteroAB cells.

Effect of endoplasmic reticulum aminopeptidase 2 variants on major histocompatibility complex class I expression

As the transient knockdown of *ERAP1* and *ERAP2* was correlated to a reduced level of MHC class I molecules expression [17], we analysed HLA-A, B and C MFIs on CD45$^+$ leukocytes from 139 healthy controls with different genotype. Results showed that MHC class I (HLA-A, B, C) MFIs were significantly reduced in heteroAB and homoB compared with homoA cells both in basal conditions and upon HIV-1 infection ($P < 0.05$ in all conditions) (Fig. 3). Notably, such a difference was not seen with CD45, a marker constitutively expressed by leukocytes (data not shown). These results demonstrate that the presence of the *ERAP2* HapB correlates with

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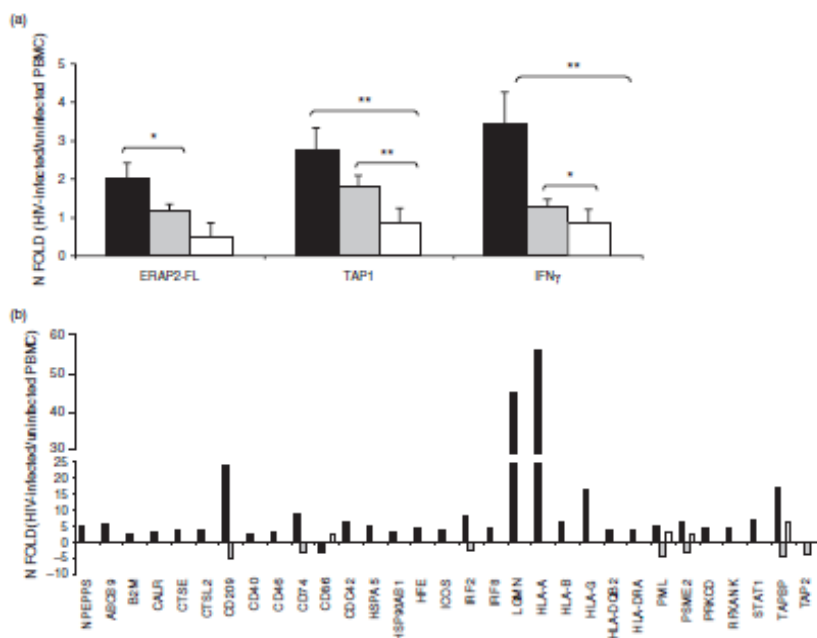
Fig. 2. mRNA expression of endoplasmic reticulum aminopeptidase type 2 (ERAP2)-full-length, transporter associated with antigen processing 1 (TAP1) and interferon-γ after HIV-1 infection. (a) ERAP2-FL, TAP1, and interferon-γ mRNA expression in peripheral blood mononuclear cell isolated from homoA (black bars), heteroAB (grey bars) or homoB (white bars) ERAP2 genotype participants after 3 days of HIV infection. Mean values and standard error are shown. *$P < 0.05$, **$P < 0.005$. (b) Antigen processing and presentation pathway genes mRNA expression in cells of participants with homoA (black bars), heteroAB (grey bars) or homoB (white bars) ERAP2 genotype 3 days after in-vitro HIV-infection. The expression of 96 genes involved in the antigen processing and presentation pathway was assessed by real-time quantitative RT-PCR and shown as fold-change expression from the uninfected sample. Only the targets showing different expression levels in the three groups are presented.

reduced levels of MHC class I expression on leukocyte surfaces.

Endoplasmic reticulum aminopeptidase 2 protein expression

To verify whether the mRNA derived from the full-length and alternative spliced form of ERAP2 are both translated into functional proteins, we performed a western blot analyses on PBMCs isolated from 15 Italian healthy controls (five homoA, five heteroAB and five homoB). The recombinant full-length and truncated proteins were also loaded. Results showed that homoA and heteroAB PBMCs produce only full-length ERAP2 (120 kDa), whereas no detectable ERAP2 protein was found in cells isolated from homoB participants. Notably,

the intensity of the full-length ERAP2 band in heteroAB participants was lower of the one detected in homoA participants. Conversely, the truncated ERAP2 protein (62 kDa) could not be detected in any of the analysed participants, independently of their ERAP2 genotype (Fig. 4).

Discussion

The repertoire of peptides presented by MHC I molecules on cell membranes is shaped by the aminopeptidases located in the ER, also known as ERAPs. In line with their central role in antigen

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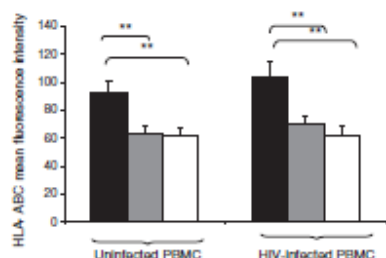
Fig. 3. Human leukocyte antigen-ABC mean fluorescence intensities of CD45^{+} cells in participants with different endoplasmic reticulum aminopeptidase type 2 (ERAP2) genotypes. (a) Human leukocyte antigen (HLA)-ABC mean fluorescence intensities of CD45^{+} cells in homoA (black bars), heteroAB (grey bars) and homoB (white bars) participants. Mean values and standard error are shown. *P < 0.05, **P < 0.005.
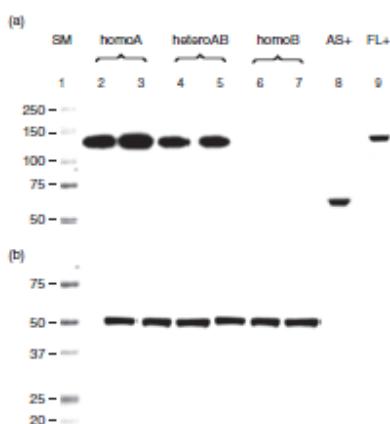
Fig. 4. Peripheral blood mononuclear cell (PBMC) isolated from two participants representative of each endoplasmic reticulum aminopeptidase 2 (ERAP2) diplotype (homoA, heteroAB and homoB). PBMC isolated from two participants representative of each ERAP2 diplotype (homoA, heteroAB and homoB) were tested for protein using primary antibodies specific to (a) ERAP2 (goat polyclonal) and (b) β-actin. Recombinant positive controls for the full-length positive (FL[+]) and alternative spliced positive (AS[+]) form of ERAP2 were also loaded.

processing, variants in *ERAP1* and *ERAP2* have been associated with ankylosing spondylitis [18,19], Crohn's disease [20], and multiple sclerosis [21], and a variant that tags HapA (rs2549782–G) in *ERAP2* was shown to protect from sexually transmitted HIV-1 infection [7]. Data herein replicate the association between the *ERAP2* polymorphism and HIV-1 protection in an independent cohort of participants with different geographic origin and a diverse infection exposure route. Meta-analysis of the results obtained in the two cohorts provided strong support to the association between diplotype status at *ERAP2* and natural resistance to HIV-1 infection.

In the attempt to explain this effect we performed analyses on cells of healthy individuals who were genotyped for their *ERAP2* gene. Results of infection assays with HIV-1$_{IIIB-L}$ showed that PBMC from homoA participants allow lower viral replication compared with cells derived from both heteroAB and homoB participants. This effect may at least partially be mediated by different *ERAP2* expression levels in participants with diverse *ERAP2* genotype. Supporting this possibility, results herein indicate that both in basal condition and following in-vitro HIV-1 infection, the transcript level for ERAP2-FL and ERAP2-Tot was significantly higher in homoA compared with heteroAB and barely detectable in homoB cells. These data are in line with previous observations [8] and with western blot results, suggesting that *ERAP2* diplotype status determines the abundance of the full-length protein product of *ERAP2*, which, in turn, might shape the antigen repertoire available for CD8^{+} T-cell clone stimulation. The relationship correlating ERAP2 genotype to ERAP2 quantity, antigen presentation and CD8^{+} cells activation is strengthened by the observation that HIV-1 infection assay performed in the absence of CD8^{+} cells did not show differences in susceptibility among the three genotype groups.

In accordance with these observations, different groups independently generated endoplasmic reticulum aminopeptidase associated with antigen processing (*ERAAP*)-deficient mice and established that trimming of antigenic peptides by ERAAP in the ER is critical for generating a standard peptide–MHC class I repertoire [4,22–25] as, in the absence of *ERAAP*, the overall expression of cell surface classical and nonclassical MHC I molecules is reduced. In agreement with these results, we demonstrate that reduction of ERAP2-FL levels results in a quantitative diminution of MHC class I expression on CD45^{+} cells both in uninfected and HIV-1 infected cell cultures.

Following HIV-infection, not only ERAP2-FL but also a number of genes directly involved in the antigen processing and presentation pathway are expressed at higher levels in homoA cells. The genes whose mRNA

was upregulated in homoA cells are implicated in almost all phases of antigen processing and presentation, including the regulation and enhancement of class I and class II expression (*TAP1, ABCB9, B2M, CALR, CD74, CTSE, CTSL2, HSP45, HSP90AB1, HFE, IRF2, IRF8, LGMN, HLA-A, HLA-B, HLA-G, HLA-DQB2, HLA-DRA, PML, RFXANK, TAPBP*), the proteasome system (*PSME2*), antigen presentation costimulation (*CD40, CD46, ICOS, PRKCD, STAT1*), lysosomal proteases (*LGMN*), as well as antigen uptake by antigen presenting cells (*CD209, CDC42*) and aminopeptidases (*NPEPPS*). It is possible to speculate that in the presence of a wider or more stable peptide repertoire generated by *ERAP2* homoA cells all the factors involved in the presentation of the antigen are boosted, contributing to the activation of an optimal immune response.

Data herein show that heteroAB individuals display a phenotype that was very similar to that of homoB individuals, supporting the previously suggested recessive model for HIV-1 protection conferred by HapA [7], and suggesting that chromosomes carrying HapB could encode a product with dominant negative effect. However, accordingly with the very low expression of ERAP2-Tot seen in homoB individuals, we were unable to detect any signal for the truncated protein. These data corroborate the assumption that ERAP2-AS mRNA undergoes a nonsense-mediated decay [8], but do not explain why heteroAB participants display the same phenotype as homoB. There are several possible explanations for this observation. First, the amount of truncated protein might be under the sensitivity threshold of the technique, but could still exert a dominant negative effect. In fact, recently, Evnouchidou *et al.* [26] established that ERAP2 SNP rs2549782, associated to HapB leads to a substrate-specific changes in enzymatic activity, allowing the enzyme to alter its specificity profile for peptidic substrate. Second, the amount of full-length ERAP2 protein might be rate-limiting for efficient antigen presentation, so that a decrease in the overall available protein is sufficient to produce an immunological response to HIV-1 infection that is scarcely protective. Third, alterations in ERAP2 availability might affect the stoichiometry of ERAP1/ERAP2 heterodimers formation. Indeed, the two aminopeptidases are known to physically interact, although the precise fraction of ERAP1 and ERAP2, which is engaged in complex formation, is unknown [17]. The trimming specificity of heterodimers and homodimers remains to be evaluated, but can reasonably be thought to differ, allowing the trimming of a wider range of antigenic peptides. Lower levels of ERAP2 protein might thus alter the relative abundance of homodimers and heterodimers and, as a consequence, the final repertoire of MHC I available cargos. This in turn might be relevant to the specificity of CD8$^+$ T cells, as suggested with *ERAAP*-deficient mice [4,22–25].

It was previously reported that, in the presence of the same MHC class I alleles, CD8$^+$ T cells from HESN recognize unusual peptides that may confer resistance to HIV-1 infection [27]. It is thus tempting to speculate that the homoA status at *ERAP2*, which is prevalent in HESN, is capable to shape a peptide repertoire that activates a biologically more advantageous pool of CD8$^+$ T cells. Indeed, the role of *ERAP1* and *ERAP2* in antigen processing and presentation suggests that variants in these genes might also interact with specific *HLA* class I alleles to modulate distinct phenotypes. For example, polymorphisms in *ERAP1* predispose to ankylosing spondylitis and psoriasis depending on the allelic status at *HLA-B* and *HLA-C*, respectively [28,29]. A systematic analysis of genetic interaction with *HLA* alleles requires extremely large sample sizes, due to the high allelic diversity at these loci. Thus, we focused on *HLA-B*57*, which has been associated with both delayed progression to AIDS and decreased HIV infection susceptibility [15,16]. Data herein indicate that, in HESN, the *HLA-B*57* alleles is much more common than expected among homoA participants, suggesting synergy in protecting from infection. Whether additional *HLA* alleles interact with *ERAP2* genotypes remains to be evaluated, but is a likely possibility as multiple alleles, as well as specific aminoacid residues in HLA-B and HLA-C, are known to control HIV viral load [30].

Finally, it will be interesting to clarify whether the HapA-associated protective effect is specific for HIV-1 infection or if it is applicable to other pathogens. Remarkably Draenert *et al.* [31] demonstrated that HIV-1 evolved a strategy to avoid ERAP1 trimming, underling the key role played by aminopeptidases in the shaping of HIV-1 antigen repertoire and suggesting that, despite the high redundancy of peptides offered by MHC class I, epitope specificity controls immune response. Further, cytomegaloviruses are endowed with a miRNA-based CTL-evasion mechanism that specifically targets ERAP1, inhibiting the trimming of precursors to HLA class I-presented mature epitopes [6]. Different alleles in ERAP proteins are thus likely to determine the differential processing of peptides originated from intracellular pathogens, resulting in a unique repertoire of antigens presented to CD8$^+$ T cells and in a diverse vulnerability to infections.

To summarize, *ERAP2* diplotype status is associated with resistance to HIV-infection; this effect is probably secondary to the modulation of the antigen processing/presenting machinery, resulting in quantitative and possibly qualitative changes in MHC class I complexes on target cells. Further studies will be necessary to verify whether genetic variations in *ERAP2* influence the epitope repertoire and to establish whether these variations play a role in determining susceptibility to other intracellular pathogen infections.

Acknowledgements

Conflicts of interest

The authors declare no conflict of interest.

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