Femme r vant de l Žvasion, 1945
Dona somiant l evasi
(fragment de l escala)
Joan Mir

UAB
Universitat Autònoma de Barcelona

B. Mothe Pujadas **Rational Design and Testing of Novel HIV T cell Immunogens** Doctoral Thesis 2012

Beatriz Mothe Pujadas

Rational Design and Testing of Novel HIV T cell Immunogens

Directors:

Christian Brander
Bonaventura Clotet i Sala

Doctoral Thesis
Departament de Medicina
Universitat Autònoma de Barcelona
2012

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DEPARTAMENT DE MEDICINA
UNIVERSITAT AUTÒNOMA DE BARCELONA

DOCTORAL THESIS, 2012

Rational Design and Testing of Novel HIV T Cell Immunogens

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'El teu perfil m'interessa. Truca'm quan tornis'
(Primer mail del Dr Bonaventura Clotet
Boston, maig 2007)

'I .... per què vols fer recerca?'
(Albert Tuldrà, entrevista a FLS
Badalona, estiu 2007)

'M'agrada fer-me preguntes'
(B. Mothe, Badalona estiu 2007)

'Tancat per vacances'
(First catalan words learned by Christian Brander after coming from Harvard
Barcelona, summer 2008)

'No saps com, però tú trobaràs la vacuna'
(Roger Paredes, declaració de fe setmanal des de 2008)

'Your charming smile is attracting everyone around you'
(First fortune cookie I ever opened -Boston 2008)

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A MIS PADRES
Por repetirme que no me olvide de sonreir

El Dr Bonaventura Clotet i Sala, i el Dr. Christian Brander (Unitat VIH i Institut de Recerca de la sida IrsiCaixa, de l'Hospital Universitari Germans Trias i Pujol i professors del departament de Medicina de la Unversitat Autònoma de Barcelona),

Certifiquen:

Que el treball experimental i la redacció de la memòria de la tesis doctoral: '**Rational design and testing of novel HIV T cell immunogens**' ha estat elaborada per na Beatriz Mothe Pujadas i consideren que és apta per al tràmit de lectura i defensa pública davant un tribunal per optar al grau de Doctor en Medicina per la Universitat Autònoma de Barcelona.

Badalona, a 3 de Març de 2012

Dr. Bonaventura Clotet i Sala　　　　Dr. Christian Brander

ABBREVIATIONS CODE

3TC	Lamivudine
ABC	Abacavir
Ad	Adenovirus
AEMPS	Agencia Española del Medicamento y Productos Sanitarios
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
ATV/r	Ritonavir-boosted atazanavir
CCR5	Chemokine receptor type 5
ChAd	Chimpanzee adenovirus
CTL	Cytotoxic T lymphocyte
CXCR4	Chemokine receptor type 4
CMV	Cytomegalovirus
COT-M	Group M Center-of-Tree
DMSB	Data Monitoring Safety Board
DRV/r	Ritonavir-boosted darunavir
EBV	Epstein-barr virus
EC	Elite controllers
EFV	Efavirenz
ELISPOT	Enzyme-linked immunospot
ETV	Etravirine
GALT	Gut-associated lymphoid tissue
GCP	Good clinical practice
GMP	Good manufacturing practice
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEPS	Highly exposed persitently seronegtives
HIV	Human Imunodeficiency Virus
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IMP	Investigational medicinal product
INSTI	Integrase strand-transfer inhibitors
IRB/IEC	Independent review board/Independent ethic comitte
LPS	Lipopolysaccharide
LPV/r	Ritonavir-boosted lopinavir

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LTNPs	Long-term non-progressors
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1-α, or CCL3
MIP-1β	Macrophage inflammatory protein-1- β, or CCL4
MRV	Maraviroc
MSM	Men who have sex with men
MVA	Modified vaccinia virus Ankara
nAb	Neutralizing antibodies
NK	Natural killer cells
NNRTI	Non-nucleoside analogue reverse transcriptase inhibitor
NRTI	Nucleoside analogue reverse transcriptase inhibitor
NVP	Nevirapine
OLP	Overlapping peptide
PBMCs	Peripheral blood mononuclear cells
PEP	Post-exposure prophylaxis
PI	Protease inhibitor
PMTCT	Preventing mother-to-child transmission
PR	Protease
PrEP	Pre-exposure prophylaxis
RAL	Raltegravir
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted; also known as CCL5 or chemokine ligand 5
RT	Reverse transcriptase
RTV	Ritonavir
SAE	Serious adverse event
SD50%	Half-maximal stimulatory antigen doses
SDF-1	Stromal cell derived factor-1, or CXCL4
SFC	Spot forming cell
SIV	Simian immunodeficiency virus
STD	Sexually transmitted diseases
STI	Structured treatment interruptions
TAM	Thymidine analogue-associated resistance mutations
TDF	Tenofovir disoproxil fumarate
TLR	Toll-like receptors
TCR	T cell receptor
pVL	Plasma viral load
VC	Viremic controllers
ZDV	Zidovudine

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PREFACE

From HIV-specific T cell-mediated immunity ...to a vaccine candidate

The AIDS pandemic is one of the greatest global health crises of our time. Since HIV was first identified 30 years ago, 23 million people have died and 34 million more are living with HIV. Despite advances in education, HIV prevention and improvements in access to antiretroviral drugs, the pandemic continues to outpace global efforts targeted at prevention and control. Every year, the same number of individuals are being infected with HIV than 20 years ago, and in our setting, 1 out of 4 individuals who is HIV positive does not know it.

We have the conviction that a preventive or prophylactic HIV vaccine is feasible and that a broadly applicable vaccine accessible to all humans is the only effective approach to halt the HIV pandemic and its devastating effects on global health and social structures. As with other pathogens, vaccines have demostrated over history to be the most cost-effective tools to contain several infectious diseases and reduce their mortality (smallpox, polio, measles, mumps, etc.)

It is well known however, that developing an HIV vaccine is one of the most important scientific challenges immunologists have ever faced. It is based on a) selecting targets of the virus, b) the construction of safe vaccines able to induce a neutralizing humoral response along with an effective cellular immunity and c) that are capable of acting on the possible sites of infection -the mucosa- before the virus can start spreading around the system in scarcely few hours. This is a really short window of opportunity. But there are mainly other roadblocks on its path: the high diversity of the virus worldwide. As the result of a very rapid replication capacity, mutation and recombination, the virus will evolve and diversify and thus escape from any immune pressure and suboptimal antiretroviral therapy.

Despite generalized skepticism about the feasibility of having effective vaccines after the announcement of the failure of the STEP trial in 2007, this past year the media has been fed with good news and hope. Sieve effects were demonstrated in volunteers who had been vaccinated but became infected, meaning that the vaccines 'did work' somehow, through avoiding infection by the few vi-

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ruses that matched the sequences contained in the vaccines. Obviously, that vaccine bottleneck was not enough to overcome the huge viral diversity.

On the other hand, the RV-144 study in Thailand was announced as the first Phase III trial to demonstrate modest efficacy in preventing HIV infection. With more than 16,000 volunteers recruited, the vaccinated group showed a 31% reduction in new infections (51 infections) compared with the placebo group (74), despite not observing a decrease in viral loads in the individuals who were infected. Not all the results were that positive: the reduction of infection was more important during the first year of vaccinations and declined over time, and was more remarkable in people with lower risk of acquiring HIV. In addition, significant levels of specific antibodies but without the expected neutralizing activity were observed in vaccinated volunteers and there was no induction of the desired specific cellular response. Many questions then arose on how the vaccines tested had really worked and whether their effect was lasting. It cannot be denied, however, that since it is the first trial to demonstrate a potential protective effect combined with the ad-hoc sieve analyses from the STEP trial, today the roadmap of where and how we should be moving forward in the vaccine field is now more clearly marked than ever.

We focus our research on the T cell immunity arm that an effective vaccine should modulate, mainly architectured by the HIV-specific cytotoxic T cell (CTL) immune response. The efficacy of the CTL component of a future vaccine will depend on the induction of responses with the most potent antiviral activity and broad HLA class I restriction. However, current HIV vaccine designs are largely based on viral sequence alignments only, without incorporating experimental data on T cell function and specificity, or have been overly guided by observations made in individuals with favourable genetics. In the following pages, we will try to adress how to overcome these past limitations in the work compiled in this thesis:

Firstly, in the introduction, which is divided into 3 sections, we will take a quick look at today's AIDS epidemics, the origins and basics of HIV pathogenesis and then attempt to give some immunobiological background on T cell mediated immunity and finally end by introducing the processes and difficulties involved in vaccine design, in addition to discussing some state-of-the-art candidates in development.

In the first chapter we review the role of viral factors, host genetic markers and specific immune responses in the control of HIV infection and their possible underlying mechanisms. In the second chapter, three large cohorts of HIV infected individuals from 3 different continents were screened for responses to the entire viral proteome to identify potential viral targets of protective HIV-1

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specific T cell responses. Responses to several sequences were identified as beneficial and were at least as predictive of individual viral loads as their HLA class I genotypes. In the study compiled in the third chapter, HIV Gag-p24 specific T cell responses were analyzed cross-sectionally in HIV controllers and non-controllers without beneficial genetics. Response rates using more sensitive peptide sets were compared and high avidity and broad variant cross-reactivity were identified as functional additional immune correlates of relative HIV control.

The results of these studies yielded 16 regions in HIV-1 Gag, Pol, Vif and Nef proteins that were i) preferentially targeted by individuals with low viral loads, ii) more conserved than the rest of the genome and iii) elicited responses of higher functional avidity and broader cross-reactivity than responses to other regions. The identified sequence candidates provided the backbone for a T cell immunogen design that aims to cover a broad HLA repertoire and break the immunodominance of responses to targets that do not emerge as particularly beneficial in the large cohorts screened. The complete approach of our T cell immunogen design is described in the fourth chapter, as well as the preliminary results of the first in-vivo immunogenicity experiments when vaccinating mice with a plasmid DNA expressing it.

Our findings will then be discussed in the context of other past and present vaccine approaches, trying to address potential limitations and benefits. The thesis ends by stating the conclusions and outlining our future research directions to better understand the mechanisms of HIV immune control, in order to improve vaccine trials read-outs based on better immune correlates and define a straightforward road map for ramping up promising vaccine candidates into clinical testing.

In summary, this work aims to provide a walk through the T cell immunology that plays a major role in HIV control. We have reviewed the most significant immune, virological and host genetic factors involved, identified potential viral targets, described new characteristics of CTL responses associated with better disease outcomes and ended by proposing a reductionist T cell vaccine candidate.

Many of the mechanisms of protection are still unclear and much time will pass before we have a commercially available vaccine effective for the entire population, but there is no doubt that each new small breakthrough is of great help in designing better vaccines that induce immune responses of improved quality and increase effectiveness in protection and disease control. We are all committed and hopeful that this can be achieved.

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PRÒLEG

Un recorregut pels limfòcits T citotòxics VIH específics ...fins al disseny d'un candidat a vacuna

La pandèmia del VIH/SIDA és indubtablement una de les majors crisis de salut global del nostre temps. D'ençà que es va identificar el VIH ara fa 30 anys, 23 milions de persones han mort i 34 milions més viuen amb el virus. Malgrat els avenços en educació i les millores en l'accés als medicaments antiretrovirals, la pandèmia continua sobrepassant tots els esforços mundials de prevenció i control. Cada any, contrauen el VIH el mateix nombre de persones que 20 anys endarrere i, encara en el nostre entorn, una de cada 4 persones que està infectada no ho sap.

Tenim la convicció que és possible desenvolupar una vacuna preventiva o terapèutica contra el VIH i que una vacuna d'abast global és l'únic mètode eficaç per aturar la pandèmia i els seus efectes devastadors en la salut mundial i les estructures socials. Igual que amb altres patògens, ha quedat demostrat al llarg de la història que les vacunes són les eines més cost-efectives per a la contenció de diverses malalties infeccioses (com ara la verola o malalties quasi eradicades com la poliomielitis i el xarampió, entre d'altres.)

No obstant això, és ben sabut que el desenvolupament d'una vacuna contra el VIH és un dels reptes científics més grans amb el qual els immunòlegs s'han hagut d'enfrontar mai. La dificultat rau en la selecció de les dianes virals i la construcció d'una vacuna en un vector segur que indueixi tant una resposta humoral neutralitzant com una immunitat cel·lular eficaç que pugui actuar sobre els possibles llocs d'infecció -les mucoses- i fer-ho en els pocs hores que triga el virus a començar a difondre's al llarg de tot el sistema. El principal obstacle és l'elevada diversitat dels virus en tot el món i el curt temps d'acció. El VIH té una capacitat de replicació, mutació i recombinació tan ràpida que és capaç d'evolucionar i diversificar-se per tal d'escapar a qualsevol pressió immunològica o teràpia antiretroviral subòptima.

Malgrat l'escepticisme sobre la viabilitat d'assolir vacunes eficaces que es va generalitzar després de l'anunci del fracàs de l'assaig STEP l'any 2007, durant tot el 2011 els mitjans de comunicació es van fer ressò de tot un seguit de notícies

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esperançadores. L'assaig STEP va demostrar que, en els voluntaris que es van vacunar però van adquirir la infecció, la vacuna havia produït un efecte 'tamís' o de cribratge de les soques virals. Només es produïen infeccions per virus diferents als inclosos en la vacuna. Això significava que les vacunes d'alguna manera sí que havien funcionat; tot i que, òbviament, el seu efecte havia estat insuficient per frenar l'entrada de virus més diversos.

Posteriorment s'anunciaren els resultats d'un estudi realitzat a Tailàndia anomenat RV-144, el primer assaig de fase III en demostrar una eficàcia molt modesta en la prevenció de la infecció per VIH. Amb més de 16.000 voluntaris reclutats, el grup de vacunats va presentar una reducció del 31% de noves infeccions (51 infeccions) en comparació amb el grup placebo (74), tot i no es va observar una disminució en la càrrega viral en les persones que van contraure el VIH. No tot van ser bones notícies: la reducció de les infeccions només va ser important durant el primer any després de les vacunacions i especialment en persones amb un menor risc de contraure el VIH; es van observar nivells elevats d'anticossos específics, però sense l'activitat neutralitzant esperada en els voluntaris vacunats, i tampoc no es va objectivar la inducció de la resposta cel·lular específica desitjada. Encara hi ha moltes preguntes obertes sobre com van funcionar realment les vacunes testades i fins a quin punt tenien un efecte durador. Tanmateix, és indubtable que ha representat la primera evidència que apunta a una vacuna amb un possible efecte protector i que, juntament amb els resultats dels anàlisis ad-hoc de l'efecte tamís viral dels anteriors candidats, avui tenim un full de ruta molt més ben perfilat que anys enrere de cap a on i com hem de dirigir-nos en el camp de la vacuna.

La nostra recerca es centra en el braç de la immunitat de cèl·lules T que una vacuna efectiva hauria de modular, principalment mitjançada pels limfòcits T citotòxics (CTL) VIH específics. L'eficàcia del component CTL d'una futura vacuna dependrà de la inducció de respostes amb l'activitat antiviral més potent en el context d'una àmplia restricció dels HLA de classe I. No obstant això, molts dels dissenys actuals de vacuna contra el VIH es basen principalment en alineacions de seqüències virals que no incorporen dades experimentals de la funció o l'especificitat de les cèl·lules T, o han estat excessivament guiats per estudis realitzats en individus amb característiques genètiques favorables. Al llarg dels treballs compilats en la tesi i dels projectes en curs, tractarem d'abordar algunes de les limitacions del passat:

Al llarg de la introducció, subdividida en 3 seccions, es dóna una ullada a l'estat actual de l'epidèmia de la sida, els seus orígens i els fonaments de la seva patogènesis, amb l'objectiu de presentar els coneixements immunobiològics més bàsics

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relacionats amb el nostre treball i de repassar els processos i les dificultats del disseny de vacunes pel VIH. A més, es presenten alguns dels resultats candidats desenvolupats fins el moment actual

En el primer capítol es revisa el paper dels factors virals, els marcadors genètics i de resposta immunitària específica en el control de la infecció per VIH i els seus possibles mecanismes subjacents. En el segon capítol s'analitzen les respostes immunitàries a tot el proteoma viral de tres grans cohorts de persones infectades pel VIH procedents de 3 continents diferents, per tal d'identificar possibles dianes virals de les respostes CTL associades amb un millor control de la infecció. Les respostes a diverses seqüències han estat identificades com a beneficioses i resulten ser tant o més predictives de les càrregues virals dels individus que el seu genotip d'HLA de classe I.

En l'estudi compilat en el tercer capítol, les respostes CTL a Gag-p24 del VIH s'analitzen de forma transversal en individus controladors i no controladors sense característiques genètiques favorables. Es comparen les taxes de resposta utilitzant sets de pèptids més sensibles dels emprats habitualment i s'identifica l'alta avidesa i l'àmplia reactivitat creuada a variants com a característiques funcionals de les respostes CTL correlacionades amb la capacitat relativa de controlar el VIH.

Els resultats d'aquests estudis apunten a 16 regions del VIH a les proteïnes de Gag, Pol, Vif i Nef que i) són les dianes a les quals es dirigeixen preferentment les respostes CTL dels individus amb càrregues virals baixes , ii) es troben en regions altament conservades del genoma viral, i iii) indueixen respostes de major avidesa funcional i àmplia reactivitat creuada que les respostes dirigides a altres regions. Les seqüències identificades són el punt de partida per al disseny d'un candidat reduccionista a vacuna del VIH per induir una resposta efectiva de cèl·lules T, que té com a objectiu cobrir un ampli repertori d'HLA i trencar amb la inmunodominància de respostes induïdes a altres regions que no apareixen com especialment beneficioses en les cohorts que hem testat.

El procés del disseny complert de l'esmentat immunogen es desenvolupa al quart capítol, així com els resultats preliminars dels primers experiments *in-vivo* d'immunogenicitat en ratolins emprant un plàsmid de DNA que expressa la nostra seqüència immunogènica.

Posteriorment, es discuteixen totes les troballes en el context d'altres enfocaments de candidats a vacuna anteriors i actuals, tractant d'abordar-ne les limitacions i potencials beneficis. La tesi conclou esmentant les conclusions i exposant les qüestions futures que cal abordar en els propers projectes de recerca, per tal de a) comprendre millor els mecanismes de control viral i immunològic del VIH, b) millorar els tests de mesura de resposta o *read-outs* emprats als as-

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saigs clínics de vacuna per aconseguir que es correlacionin millor amb els marcadors de resposta immunològica i c) definir millor el procés d'avençar gradualment els candidats a vacuna més prometedors de les fases pre-clíniques als assaigs clínics definitius en humans.

En resum, aquest treball pretén traçar un recorregut per la immunologia dels limfòcits T citotòxics implicada en el control del VIH. Es revisaran els factors genètics, virals i immunològics que més contribueixen a una contenció òptima del virus, s'identificaran possibles dianes virals i es descriuran noves característiques de les respostes CTL associades a una millor evolució de la malaltia. Tot plegat ens ha dut a proposar un candidat a vacuna del VIH per a cèl·lules T minimalista, actualment en fase pre-clínica.

Molts dels mecanismes de protecció segueixen sense estar gaire clars i haurà de passar força temps fins que tinguem una vacuna acceptablement eficaç i disponible al mercat per a tota la població, però no hi ha dubte que tots els petits nous avenços contribueixen a millorar el disseny de vacunes per induir una resposta immune de millor qualitat i millorar l'eficàcia en la protecció i el control de la malaltia.

Si més no, aquest és el nostre compromís.

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INTRODUCTION-I

Thirty years of HIV/AIDS

L.A., June 1981
A cluster of five cases of *Pneumocystis carinni* pneumonia in previously healthy gay men from Los Angeles reported in the Centers for Disease Control's *Mortality and Morbidity Weekly Report* (MMWR) in June 1981 hailed the official birth date of the AIDS epidemic. At that time, the standard treatment for *Pneumocystis* pneumonia was pentamidine. The people responsible for the distribution of pentamidine at the CDC in Atlanta found it quite striking to have received several requests for pentamidine within a short period of time from hospitals in California and New York. This was the first step in the process of identification of that new syndrome.

In 1983, a group of scientists headed by Luc Montagnier and François Barré-Sinoussi at the Pasteur Institute in Paris first isolated the causative retrovirus of a mysterious new immune syndrome from a lymph node biopsy without associating it to AIDS. It was named lymphadenopathy-associated virus, or LAV[1]. A year later, a team led by Robert Gallo from the American National Institute of Health, confirmed the discovery of the virus and that it caused AIDS, and renamed it human T-lymphotropic virus type III (HTLV-III)[2] In 1986, both the French and the US names (LAV and HTLV-III) were dropped in favor of the new term human immunodeficiency virus (HIV) In 2008, the Nobel Prize in Medicine was awarded to Montagnier together with his colleague Françoise Barre-Sinoussi for the discovery of HIV.

Three decades after the first descriptions of HIV outbreaks, 23 million people have died and 34 million more are currently living with HIV.

Despite being a worldwide pandemic, a global view of HIV prevalence shows enormous differences among continents and countries, with the biggest epidemics located in sub-Saharan Africa—Ethiopia, Nigeria, South Africa, Zambia, and Zimbabwe, with worrying adult HIV prevalence rates between 15 and 30%, reaching 60% in specific young age groups The estimated 1.3 million people who died of HIV-related illnesses in sub-Saharan Africa in 2009 comprised 72% of the global death toll attributable to the epidemic.

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Figure 1. Distribution of people living with HIV. Source: UNAIDS Report on the global AIDS epidemic 2010

Tremendous global efforts in education, HIV prevention and expansion of access to treatment have been implemented all over the world. Since 1999, the year in which it is thought that the epidemic peaked, globally, the number of new infections has fallen by 19% and AIDS-related deaths steadily decrease.[3] However, in seven countries from Eastern Europe and Central Asia, HIV incidence increased by more than 25% and the number of people living with HIV has almost tripled since 2000. A rapid rise in HIV infections among people who inject drugs at the turn of the century caused the epidemic in the Russian Federation and Ukraine to surge.

On the other hand, although the rates of annual new HIV infections have been stable for at least the past five years in Western, Central, and Eastern Europe, Central Asia, and North America, there is strong, evidence of a resurgence of HIV and outbreaks of STDs in several high income countries among men who have sex with men. Increases in higher-risk sexual behaviour are associated with this trend.[4,5] The most worrisome fact is that most HIV infections are not diagnosed. One out of every four individuals who is HIV positive does not know it, therefore, worsening their health status by not receiving appropiate treatment and triggering HIV transmission to other people.

The HIV epidemics are also disproportionately concentrated in racial and ethnic minorities in high-income countries, for instance, in the USA, where African-Americans constitute 12% of the population but accounted for 45% of the people newly infected with HIV in 2006.[6]

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Classification, HIV structure, and cell cycle.

The human immunodeficiency virus (HIV) is a member of the *Lentivirus* genus in the *Retroviridae* family. Lentiviral infections have been described in other species (B*ovine*IV, F*eline*IV, H*uman*IV or S*imian*IV), and commonly target cells of the immune system (macrophages or lymphocites) inducing different grades of immunodeficiency. Lentiviral infections are transmitted as single-stranded, positive-sense, enveloped RNA viruses. Upon entry into the target cell, the viral RNA genome is reverse transcribed into double-stranded DNA by a virally encoded reverse transcriptase (RT) that is transported along with the viral genome in the virus particle. The resulting viral DNA is then imported into the cell nucleus and integrated into the cellular DNA in infected cells, achieving latent or persistent cell infection. Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles that begin the replication cycle anew.

Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and termed both LAV and HTLV-III. It is more virulent, more infective, and is the cause of the majority of HIV infections globally. HIV-2 is largely confined to West Africa and it is thought to be less pathogenic.[7] Their genome differs very little; HIV-2 carries an extra gene not found in HIV-1 which encodes an extra viral protein X (vpx). Importantly, given the antigenic similarity between HIV-1 and HIV-2 occasionally it can be difficult to establish the proper diagnosis of HIV-2 infection using only serological tests, and some techniques do not detect viral infection by HIV-2 either. The interest in differentiating between both infections lies especially in the better prognosis of HIV-2 disease and also differences in the use of antiretrovirals needed.

The HIV-1 genome (about 10 Kb) contains the envelope, three structural Gag proteins (matrix –p17, capsid –p24 and nucleocapsid –p7), three enzymes (protease, reverse transcriptase and integrase), and six accessory genes that help regulate virus replication (tat, vif, vpu, vpr, nef and rev)

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Figure 2. Illustration of the genes/proteins of HIV-1. Open reading frames are shown as rectangles. Source: HIV Sequence Compendium 2010. Kuiken C, Foley B, Leitner T, Apetrei C, Hahn B, Mizrachi I, Mullins J, Rambaut A, Wolinsky S, and Korber B, Eds. Published by Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, NM, LA-UR 10-03684.

Figure 3. Schematic representation of the HIV-1 structure. Wikipedia Commons, Source: US National Institute of Health (redrawn by Carl Henderson) available at: http://en.wikipedia.org/wiki/File:HIV_Virion-en.png

The matrix forms the inner shell below the viral membrane; the capsid is a conical-shaped core that encloses two idendical RNA strands, and the nucleocapsid interacts with the viral RNA inside the capsid. These viral proteins are generated upon processing of the HIV p55 Gag precursor polyprotein by the viral protease (PR). The myristoylated p17 matrix is part of the viral structure and its interactions with the Env proteins are crucial for a proper assembly of mature virions.[8] The pol-encoded enzymes, PR, reverse transcriptase (RT), and integrase (IN) are initially synthesized as part of a large polyprotein precursor, p160GagPol, whose synthesis results from a rare frameshifting event during p55Gag translation and then cleaved by the viral PR (reviewed in [9]).

The envelope proteins derive from a gp160 precursor molecule, which is cleaved to gp120 and gp41 in the Golgi apparatus (reviewed in [10]). Gp41 is anchored to the viral membrane by its C-terminal transmembrane domain, whereas the central and N-terminal regions are expressed outside the virion and bind to the gp120 protein. Three of these units aggregate on the membrane surface to form the gp120/gp41 heterotrimer spikes in a –surprisingly low ratio of 7 to 14 Env spikes per virion released.[11]

Binding sites for CD4 cellular receptors of HIV, the seven transmembrane domain chemokine receptors that serve as co-receptors for HIV-1 as well as the majority of the antibody domains are located in gp120. Viral regulatory and ac-

cessory proteins, which include Tat, Tev, Rev, Nef, Vif, Vpr and Vpu do not need to undergo processing and they are the first to be translated to modulate the subsequent synthesis of the viral structural proteins. It is interesting to note that Nef is one of the first HIV proteins to be produced in infected cells, and it is –along with Gag- the most immunogenic of the HIV proteins, in terms of HIV specific T cell responses.[12] Nef is necessary for the maintenance of high virus loads, and viruses with defective Nef have been associated with reduced viral replication in some (elite) controllers.[13] It is noteworthy that Nef downregulates the CD4 and the HLA class I molecules, therefore undermining recognition by HIV-specific cytotoxic CD8+ T cells.[14]

HIV/SIV proteins

Name	Size	Function	Localization
Gag MA	p17	membrane anchoring; env interaction; nuclear transport of viral core (myristylated protein)	virion
CA	p24	core capsid	virion
NC	p7	nucleocapsid, binds RNA	virion
	p6	binds Vpr	virion
Protease (PR)	p15	gag/pol cleavage and maturation	virion
Reverse Transcriptase (RT)	p66, p51	reverse transcription	virion
RNase H	(heterodimer)	RNAse H activity	virion
Integrase (IN)		DNA provirus integration	virion
Env	gp120/gp41	external viral glycoproteins bind to CD4 and chemokine co-receptors	plasma membrane, virion envelope
Tat	p16/p14	viral transcriptional transactivator	primarily in nucleolus/nucleus
Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)	primarily in nucleolus/nucleus shuttling between nucleolus and cytoplasm
Vif	p23	viral infectivity factor, inhibits minus-strand viral DNA hypermutation	cytoplasm (cytosol, membranes), virion
Vpr	p10-15	promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	virion nucleus (nuclear membrane?)
Vpu	p16	promotes extracellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIVcpz)	integral membrane protein
Nef	p27-p25	CD4 and class I downregulation (myristylated protein)	plasma membrane, cytoplasm, (virion?)
Vpx	p12-16	Vpr homolog present in HIV-2 and some SIVs, absent in HIV-1	virion (nucleus?)
Tev	p28	tripartite tat-env-rev protein (also named Tnv)	primarily in nucleolus/nucleus

Table1. HIV proteins and their functions. Source: HIV Molecular Immunology 2011. Karina Yusim, Bette T. M. Korber, Christian Brander, Dan Barouch, Rob de Boer, Barton F. Haynes, Richard Koup, John P. Moore, Bruce D. Walker, and David I. Watkins, editors. Publisher: Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR-12-1007

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As an enveloped retrovirus, HIV-1 viral life cycle begins with the interaction between the viral envelope proteins and target proteins on the cell surface of susceptible cells. The first step is the binding of gp120 to its primary receptor on the cell surface, CD4, followed by binding to either the CCR5 or CXCR4 coreceptor. A conformational change of the gp41 structure then occurs, involving the ectodomain of gp41 -this region contains a highly hydrophobic N-terminus (the so-called "fusion peptide") and two heptad repeat motifs, referred to as the N-helix and the C-helix HR-1 and HR-2.[9] Structural analyses of these two helical sequences, in the context of a gp41 ectodomain trimer, indicate that they pack in an antiparallel fashion to generate a six-helix bundle to facilitate fusion between the viral and cellular membranes and to allow the release of the viral core into the cell.[15-18]Coreceptor recognition is defined by several structural elements of gp120 that include the first and second hypervariable regions (V1-V2) and most importantly, the V3 loop, which is the principal determinant of the CCR5 or CXCR4 viral tropism.

Specific regions of the membrane, known as lipid rafts, play a critical role in mediating the biological activity of the membrane. There is evidence that the ability of the receptors to move laterally within the membrane and accumulate in these specific membrane microdomains rich in cholesterol is relevant to their ability to mediate fusion with HIV-1 envelope.[19]

Following the deposition of the viral core in the host cell cytoplasm, the viral nucleoprotein complex traffics to the nucleus and the viral RNA genome is reverse transcribed into DNA, which is ultimately integrated into the host cell chromosome. This establishment of proviral infection leads to the expression of viral accessory and structural proteins, which must assemble within the target cell to form functional progeny virions capable of initiating a new round of infection.[20]

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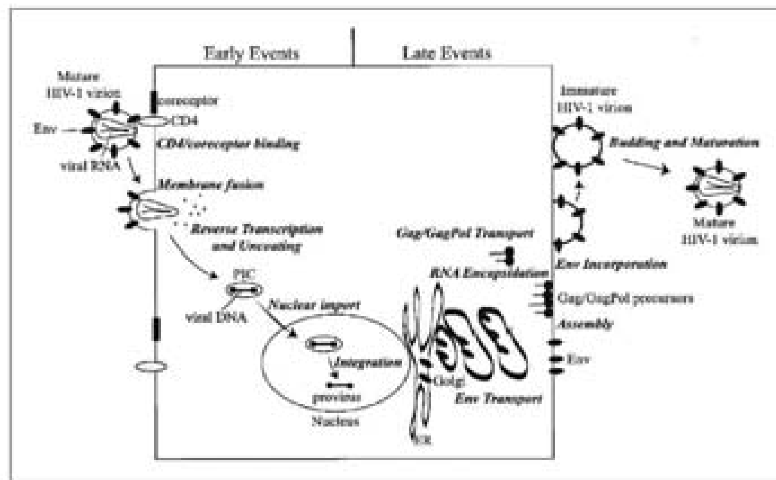

Figure 4. Schematic representation of the HIV-1 life cycle. The major steps in the early and late stages of the replication cycle are indicated. Source: Freed EO. HIV-1 replication. Somat Cell Mol Genet 2001 Nov; 26(1-6): 13-33.

Viral cell to cell transmission

Advances in fluorescence microcopy have helped tremendously in recent years in estimating that cell-to-cell transmission of HIV-1 virions is likely many times more efficient than infection by cell-free virus. Cell-to-cell transmission is facilitated by interactions between infected T cells and uninfected T cell targets by inducing the accumulation of HIV-1 Env and Gag and target cell CD4 and CXCR4 coreceptors at the point of the cell-to-cell contact (defined as the *virological synapse*).[21,22] A similar phenomenon has been described between T cells and dendritic cells. HIV-1 virions enter the endosomal compartment of dendritic cells and accumulate in the regions of the cell that come in contact with neighboring T cells, and are then transferable from these compartments to target T cells.[23,24] This accumulation of virions at the point of cell-to-cell contact provides a mechanistic understanding of how dendritic cells can enhance HIV-1 infection of T cells without becoming infected themselves, a process known as *trans*-infection. Lastly, cell-to-cell transfer of virus from antigen-presenting cells has also been observed in macrophages.[25]

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HIV diversity

HIV is highly heterogeneous within infected individuals owing to rapid turnover rates, high viral load, and an errorprone reverse transcriptase enzyme that lacks proofreading activity. High variability is also the consequence of recombination, which can shuttle mutations between viral genomes and lead to major antigenic shifts or alterations in virulence. On the basis of phylogenetic analysis HIV has been classified into the two mentioned types of HIV: HIV-1 and HIV-2.[7] The genetic sequence of HIV-2 is only partially homologous to HIV-1 and more closely resembles that of SIVsmm.

HIV-1 is divided into four 'groups': group M (main), which is responsible for the current pandemic and causes more than 99% of all HIV-1 infections in the world, group O (outlier), group N (non-M non-O) and group P, which did not spread outside central Africa. HIV-1 group M is further divided into nine 'subtypes' or clades: A, B, C, D, F, G, H, J and K, based on the whole genome, which are geographically distinct.[26] Circulating recombinant forms (CRF) come from the recombination of two different subtypes, which can then be transmitted forward. Forty-eight recombinants have been recognised to date.

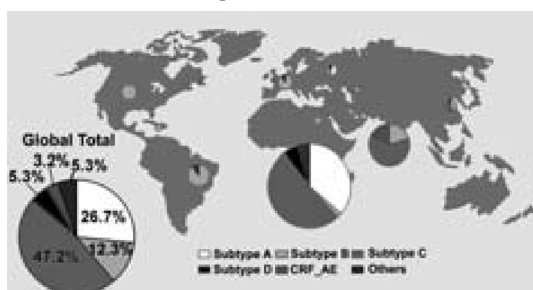

Figure 5. Estimated global distribution and regional spread of HIV-1 genetic subtypes. Source: Adapted from Wikipedia Commons, Based on Osmanov S, Pattou C, Walker N, Schwardlander B, Esparza J; WHO-UNAIDS Network for HIV Isolation and Characterization. Available at: http://en.wikipedia.org/wiki/File:HIV-1_subtype_prevalence_in_2002.png

HIV is among the most variable of human pathogens. In infected individuals, HIV exists as a swarm of highly related but non-identical viral genomes termed 'quasispecies.' As a reference, the diversity of viral variants infecting a single individual at any given moment is much higher than the variability of all influenza viruses generated around the globe every year.[27].

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The genetic diversity of HIV-1 in a given location is influenced not only by how long it has been there, but also by how efficiently it propagated. Conclusions from diversity studies pointed to the origins of the epidemics being in Central Africa. It was assumed it had had more time to diversify genetically and was the habitat of the simian source of the virus.

Origins: our closest relatives

'There are at least two good reasons for attempting to elucidate the factors behind the emergence of the HIV pandemic. First, we have a moral obligation to the millions of human beings who have died, or will die, from this infection. Secondly, this tragedy was facilitated by human interventions: colonisation, urbanisation and probably well-intentioned public health campaigns. Hopefully, we can gain collective wisdom and humility that might help avoid provoking another such disaster in the coming decades'

'The Origins of AIDS'. 2011. Jaques Pepin. Cambridge (Editors)

Thirty years after its discovery, HIV origins seem to be clearer than ever. Investigators have traced back into the early twentieth-century the first events of transmission from chimpanzees to man through cut hunting and butchery and how the existing urbanisation, massive use of reusable syringes and needles in large-scale colonial medical campaigns, plasma centers and later sexual and iv drug use transmission ended up spreading the virus from its origins in Léopoldville (Belgian Congo) to the rest of Africa, then the Caribbean, and ultimately the USA. [28]

Today, data from testing stored plasma samples worldwide suggest that HIV-1 was already present in 1960 and 1970, albeit at a low prevalence, in several locations in central Africa. The oldest HIV-1 isolates documented stem from a male adult recruited in Léopoldville (Belgian Congo) in 1959 (ZR_{59}) and an adult woman lymph node biopsy from 1960 (DRC_{60}) Both virus sequences differ by about 12%, and it has been suggested that they shared a common ancestor dating back to around 1921.

Despite the fact that the exact moment of the cross-species transmission (within the first three decades of the twentieth century) is less clear, *Pan troglodytes troglodytes* (*Ptt*) chimpanzee is known to be the source of HIV-1, which lives in its natural state between the Sanaga and the Congo rivers. First reports of simian immunodeficiency viruses (SIV) isolat-

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ed from *Ptt* chimpanzees born in the wild were similar to HIV-1 strains from humans and through the use of molecular clocks, it has been suggested that several separate cross-species transmissions of SIV_{cpz} have occurred. The estimated dates of the most recent common ancestors of HIV-1 groups M, O, and N in central Africa are 1908 (range 1884–1924), 1920 (1890–1940), and 1963 (1948–77), respectively, and for HIV-2 groups A and B the dates are 1932 (1906–55) and 1935 (1907–61), respectively (reviewed in [28]).

SIVs have co-evolved with primate species since ancient times, becoming non-pathogenic in their natural hosts –despite being characterized by high viral loads, and a similar phenotype is seen with FIV in felines among other species. We –humans- were clearly not prepared in evolutionary terms for the irruption of HIV. The evolution of HIV-1 has been rapid, resulting in a complex classification, worldwide contagion, and intermixing of strains reaching the mentioned 9 clades and 48 circulating recombinant forms currently identified.

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Pathogenesis

Untreated HIV infection leads generally to a devastating erosion of the immune system, clinically characterized by a progressive decrease in the number of CD4+ T cells and a rise in the HIV viral load. In the absence of treatment, this decline in CD4+ T cells heralds the progression to the acquired immune deficiency syndrome (AIDS), associated with the development of opportunistic infections and cancers and ultimately death.

Within 1 to 4 weeks after initial HIV entry into the body, and usually before seroconversion, newly infected individuals may develop a virus-like illness (acute retroviral syndrome) characterized by headache, retro-orbital pain, muscle aches, sore throat, fever, swollen lymph nodes and a non-pruritic macular erythematous rash that can last from days to weeks. During acute HIV infection, a large and preferential depletion of mucosal CD4+ T cells is observed. Memory CCR5+ T cells are major targets for R5 HIV-1 replication and their frequency is relatively high in gut-associated lymphoid tissues (GALT), where they fuel HIV-1 replication. During the first weeks of infection, most individuals show peripheral lymphopenia and thrombocytopenia; whereas after the second week, the levels of CD8+ T cells start increasing and therefore progressively invert the CD4+/CD8+ T cell ratio. Also, during that period, plasma levels of cytokines associated with immune activation (IL-1β, soluble CD8, TNF-α, IFN-γ) are found at increased concentrations. This initial immune response, depending on the cytokine profile, may influence the extent of CD8+ T cell expansion and the severity of the retroviral syndrome. In parallel, within weeks after primary infection, the plasma viremia is reduced, reaching a virologic set point usually after 3 to 5 months. The magnitude of this virologic set point is considered to be a strong predictor for the rate of further disease progression.

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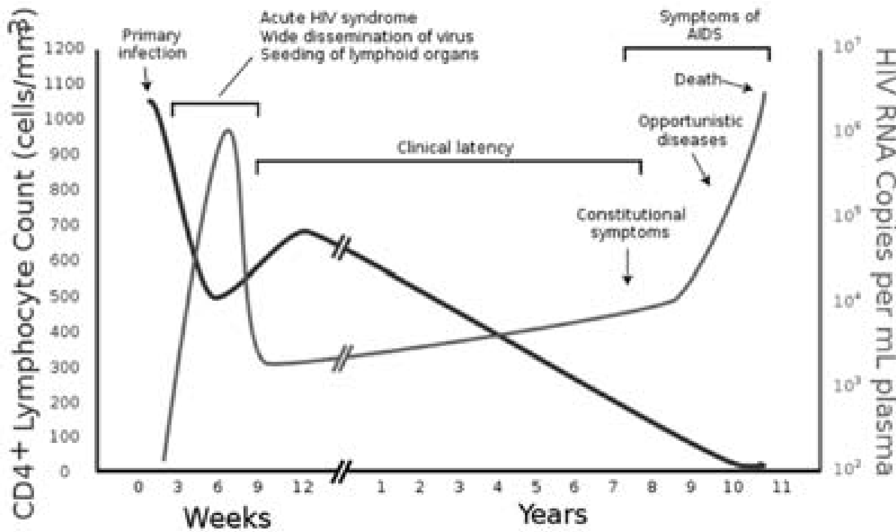

Figure 6. Schematic representation of evolution of viral load and CD4 cell counts over the average course of untreated HIV infection. Source: Wikipedia Commons. Original available at: http://en.wikipedia.org/wiki/File:Hiv-timecourse_copy.svg

Acute HIV infection is followed by a chronic and generally asymptomatic phase of the infection, which lasts for an average of 7 years and is characterized by a steady decrease of the CD4+ T cell counts and stable peripheral viral loads. Viral replication continues in the lymphoid tissues despite the presence of neutralizing antibodies and a strong HIV-specific CTL response during this stage. As a consecuence of viral evolution -partially driven by immune pressure- viral subpopulations or *quasispecies* become increasingly more heterogeneous. Progressively, CD4+ T cell counts reach levels below 200cells/ul, viral replication accelerates, HIV-specific CTL activity significantly decreases, lymphoid architecture is destroyed and AIDS-defining illnesses such as opportunistic infections start to appear. At these later stages, viral populations are significantly more homogeneous and are frequently preceded by a shift in cell tropism towards X4 viruses (using CXCR4 virus in 50% of patients).[29] CCR5 is expressed on a small fraction of memory CD4+ T cells, while the expression of CXCR4 is high on naive T-cells and decreases with differentiation and activation. Despite the higher frequency of CXCR4+ T cells, HIV-1 strains using CCR5 receptors remain dominant when early infection is established and only with X4 strains emerging usually at later stages of infection. Mechanisms of such a viral tropism switch remain elusive. Viral replication kinetics are faster at later phases and able to propagate to more and different cell types; for instance, neurologic tropism is fueled

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and viruses are less sensitive to neutralization or CTL activity.[18]

Overall, dynamics of CD4+ T cell depletion are mainly driven by [30,31] a) a direct cytophatic effect by the aggressive kinetics of viral replication in the cells –specially in acute infection, b) the recognition and further elimination by the HIV-specific cytotoxic T cells -suggested by the correlation between CD4+ T cell depletion and CD8+ antiviral clones expansion, c) apoptosis secondary to Env, Vpr and Tat viral proteins and d) a disturbance of T cell homeostasis caused by the combination of a central inhibition of lymphocyte proliferation, a relative sequestration of CD4+ T cells in lymph nodes and hyperactivation by continous antigen exposure partially responsible for progressive immune exhaustion.

AIDS is defined as the situation in which the CD4+ T cell counts have fallen below a threshold in the mucosa and other peripheral sites, such that a fast and efficient response to recall antigens can no longer be mounted and consequently, opportunistic infections, *de novo* malignancies or by reactivation of latent infections develop (such as EBV, SK, HPV, CMV, etc). This normally correlates with a level of peripheral CD4+ T cell counts below 200cells/μl and especially below 50-100 cells/μl[32] although some opportunistic diseases have also been described in individuals with higher cell counts under successful viral suppression.[33]

Among the HIV-infected population however, several groups of individuals have been identified that remain clinically stable and free from any AIDS-defining conditions for decades after infection in the absence of antiretroviral therapy.[34] These individuals (estimated to be 5–8% of the total HIV-infected population) have been referred to as **viremic controllers** (VC), **long term survivors** (LTS) or **long-term non-progressors** (LTNP) and are generally able to control viral replication to low levels – plasma RNA levels < 2,000 copies/ml (or < 5,000-10,000 copies/ml, depending on cut-offs) and to maintain normal CD4+ T cell counts over time with a reduced rate of CD4+ T cell loss (18 cells/μl/year) compared to that of normal progressors (around 60 cells/μl/year).[35] Few of such LTNP are able to maintain undetectable plasma viral loads for extensive periods of time and they are known as **elite controllers** (EC); they represent less than 1% of all HIV-infected individuals and are those who have the lowest probability of progressing to AIDS. There is considerable clinical and scientific interest in such individuals, as they may indeed hold the key to spontaneous control of HIV infection and could provide crucial help in developing an effective vaccine.

The other extreme of the spectrum of disease -**rapid progressors** or RP- are defined by a fast progressive immunosuppression soon after seroconversion and, in many cases, high levels of viremia.[36,37] As with the controllers, published data suggest that the concurrence of viral, immune and host factors contributes to the control or the severity of early disease. There are, however, few such RP individu-

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als in clinical cohorts as prospective recruitment is heavily limited by the need to identify patients with a known date of seroconversion, as well as the short window of clinical observation before antiretroviral treatment is initiated to have available biological material for study.

Also in very low percentages, individuals with high levels of HIV-1 replication during the chronic phase of infection that remain asymptomatic and maintain high CD4+ T cell counts for a very long time have been described (named VNP or **viremic non-progressors**) and are of great recent scientific interest.[38] This tolerant profile is poorly understood and reminiscent of the widely studied nonprogressive disease model of SIV infection in sooty mangabeys. Their more in-debth analysis may also prove to be highly informative for the comprehension of HIV immunopathogenesis, especially for understanding the role of chronic immune activation and viral control or diease progression.

Lastly, several studies have identified individuals who have been exposed to infectious viruses but did not establish a productive infection. These high-risk exposed people, generally referred to as HEPS, or **highly-exposed persistently seronegative individuals**, include sexual workers, partners of infected individuals, intravenous drug users, transfusion recipients, hemophiliacs and children born of infected mothers among others. The possible reasons for this resistance to infections will be further developed in chapter 1, as they may also provide important clues regarding the potential mechanisms of the prevention of HIV infection.[39,40]

Management of HIV/AIDS

There is no question that antiretrovirals and especially the combination of drugs (HAART, from Highly Active Antiretroviral Treatment) has changed the course of the AIDS epidemic and enabled HIV-infected people to live longer and survive to almost a normal life-time. Many drugs have been evaluated over the past 25 years, targeting different HIV cell cycle steps, and over 20 drugs can be used in combination.

Therapies for opportunistic infections among HIV-infected people as well as the management of diseases occurring in the context of the immune reconstitution inflamatory syndrome (IRIS) have also greatly improved since the onset of the epidemic. IRIS is a condition seen in some cases of AIDS upon treatment initiation. When viral suppresion is achieved and the immune system begins to recover, a response to a previously acquired opportunistic infection with an overwhelming inflammatory response that paradoxically causes nonspecific symptoms such as fever, and in some cases a worsening of infection is seen in IRIS.[41]

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Prevention and control of drug side effects, diagnosis and approach of drug resistances and an increasing incidence of non-AIDS related events dominates the current HIV management. Main drug toxicities (especially, but not exclusively, of earlier available drugs) include abnormalities in body fat distribution and in lipid and glucose metabolism, cardiac disease, and pancreatic, kidney, liver and bone disorders. In the present scenario, non-AIDS related events are globally more frequent than classic AIDS events[42,43] and are the main complications seen in our clinics. Non-AIDS related events comprised of cardiovascular disease, hepatic and renal impairment, non-AIDS neoplasias, osteoporosis, frailty and neuro-cognitive disorders have increased in frequency and are responsible for a higher mortality rate in the upper strata of CD4+ T cell counts. A subset of these events is expected in the elderly and could be due to a general phenomenon of premature aging of the infected population. A persistent immune activation - indirectably measurable through various inflammation markers- is seen to improve substantially upon HAART and successful viral suppression but always still remains above normal values compared to uninfected individuals. This 'hyperactivated status' could partially be driving the premature aging phenomena.[44,45]

The first insights regarding the importance of continuous viral suppression in containing non-AIDS events came from lessons learned from SMART and other substudiesassessing the potential benefits of treatment interruptions. An increase in global mortality and complications (especially cardiovascular, hepatic, renal and neoplasy) were detected with strategies of structured treatment interruptions (STI) guided by CD4+ T-cell counts in patients with sustained viral suppression under HAART. The risk increase was 160% ($p<0.001$) for the main variable (AIDS events and death) but also 70% ($p<0.009$) for the rest of non-AIDS events.[46,47]

Reviewing all the current antiretroviral drugs and possible combinations is beyond the scope of this introduction. Guidelines for the use of antiretroviral agents are available and updated every year. [48-50] They cover information about new drugs under development, new recommendations of drug combinations and regimen simplifications adapted to individual's comorbidities, updated drug labels for existing agents, new drug resistance tests and tropism assays available and specially, the new insights on the never-ending discussions of when to start treatment on recently diagnosed patients and establishing CD4+ cell counts thresholds (<250, <350, <500 cells/ul) .

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Just for classification purposes, antiretroviral drugs are divided into different classes according to their mode of action in the inhibition of the HIV cell cycle and include the following:

1) Entry inhibitors (or fusion inhibitors) interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. (enfuvirtide, T-20)

2) CCR5 receptor antagonists bind to the CCR5 receptor on the surface of the T-cell and block viral attachment to the cell, avoiding entry to replicate. (maraviroc)

3) Nucleoside reverse transcriptase inhibitors (NRTI) mimic nucleotides and inhibit reverse transcriptase directly by binding to the polymerase site and interfering with its function. (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, emtricitabine, tenofovir, abacavir)

4) Non-Nucleoside reverse transcriptase inhibitors (NNRTI) inhibit reverse transcription by being incorporated into the newly- synthesized viral DNA strand as a faulty nucleotide, causing DNA chain termination (delavirdine, efavirenz, nevirapine, etravirine and the newly-developed rilpivirine)

5) Integrase inhibitors inhibit the viral enzyme integrase (INSTI from integrase strand transfer inhibitors), which is responsible for integration of viral DNA into the genome of the infected cell. (Raltegravir has been the first approved but several more drugs from this class are currently undergoing clinical testing)

6) Protease inhibitors (PI) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for the final assembly of new virions (darunavir, atazanavir, amprenavir, lopinavir, fosamprenavir, tipranavir, nelfinavir, saquinavir, ritonavir)

7) Maturation inhibitors inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein (p24). Because these viral particles have a defective core, the virions released consist mainly of non-infectious particles. These drugs are still currently under development and include Alpha interferon, bevirimat and Vivecon.

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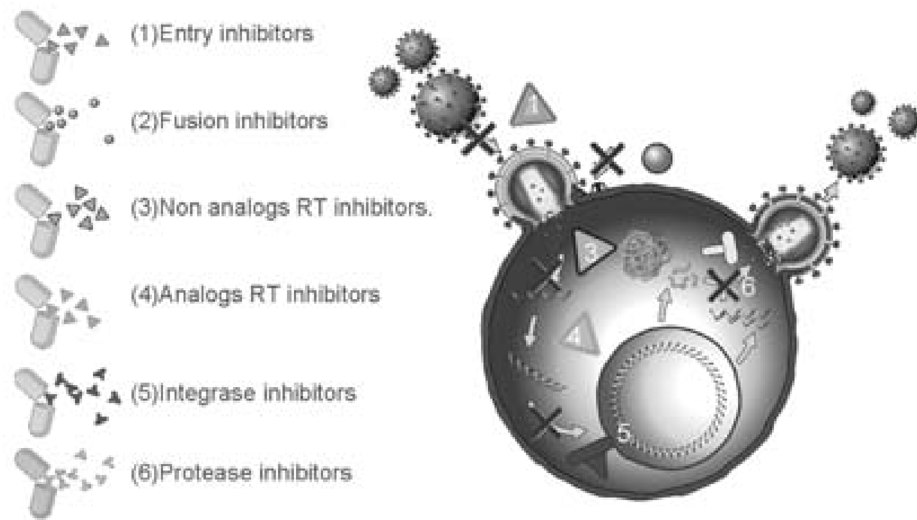

Figure 7. Schematic representation of targets of different antiretroviral drug classes. Source: IrsiCaixa outreach available at: http://www.irsicaixa.es/en/outreach

In addition to HAART, certain immune system-based treatments have received increased attention, but with somewhat disappointing results. They are sometimes included as components and/or adjuvants of 'therapeutic vaccine' approaches. Their main objective is attempting to restore immune function affected by HIV infection. Although a recovery of the CD4+ T cell counts is usually obtained with HAART, specific anti-HIV responses do not seem to improve substantially and eradication of the virus in the reservoirs is not achieved. These therapies include the use of cytokines (e.g., IL-2, GM-CSF, G-SCF, interferon-α, IL-10, IL-12, IL-15, IL-16 and rIL-7) and compounds helpful in decreasing immune activation. Some of the most relevant tested strategies that attempted to strengthen immune control of HIV infection included the use of IL-2 concomitantly with HAART treatment (SILCAAT and ESPRIT trials).[51] Despite demonstrating their capacity to stimulate specific immune responses and temporary increases in CD4+ T cell counts, they were unable to demonstrate reductions in opportunistic diseases or death over time.

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Potential immune-based therapies for HIV infection	Refs
IL-2 administration	52-54
IL-12 therapy	55-57
IL-15 therapy	58-60
rIL-7 administration in the context of intensified HAART	61
Antibodies to IL-4 and IL-10	57,62
IL-10 therapy	63
Type 1 interferons	64-66
Granulocyte-macrophage colony-stimulating factor	67,68
Anti-TNF-α drugs (Pentoxifylline, thalidomide, and antibodies)	69,70
Passive antibody administration	71-73
Immune modulators (Cyclosporine, hydroxy-urea and mychophenolic acid)	74-78
Thymopentin	79
Thymus replacement	80,81
CD8+ cell administration	82,83

Table 2. Potential immune-based therapies for HIV infection. Most of these approaches include the concomitant use of HAART. Source: adapted from Jay A. Levy. HIV and the Pathogenesis of AIDS. 3rd ed. ASM Press American Society for Microbiology, 2007.

In developing countries, after the introduction of HAART in 1996, mortality rates were reduced by nearly 80-90% and were associated with an improvement in immune status, a sustained viral supression and a drastic reduction in opportunistic events. The effectiveness of HAART has been globally improving over the years (mostly because it is becoming easier to take and better tolerated) and has moved life expectancy at 20 and 35 years from 36 to 49 years and from 25 to 37 years respectively.[84]. However, the gap still exists; life expectancy in HIV+ individuals is still about two thirds of that expected in the non-infected general population.

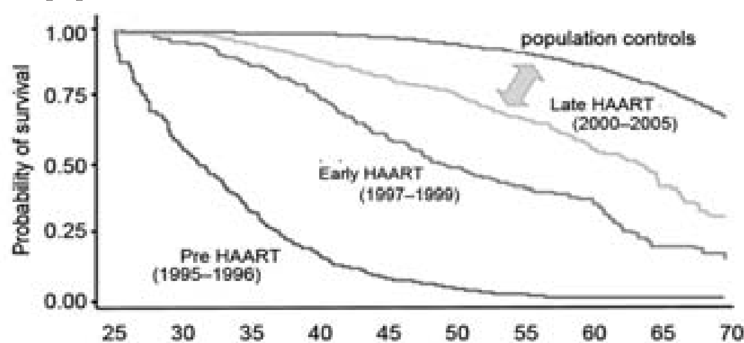

Figure 8. Estimated survival for HIV infected patients from age 25 years. Hepatitis C coinfected individuals excluded. Source: Adapted from Lohse N et al, 16th IAC 2006, Toronto, Canada, Abstract # MOPE 0310

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We all believe that the general population's life expectancy could be achieved but this requires virtually normalizing the immune system (for instance, to reach effective viral suppression and CD4+ counts above 500cells/µl persistently for more than 6 years). But signficant improvements in early diagnosis and expansion of treatment initation soon after infection are vital.[85]

Virological response to HAART is universal and fast, if an appropriate combination is selected in the absence of primary resistances and provided that the patients comply with the treatment regimen. In a few days or weeks there is a viral load drop of 1-2 log_{10} followed by a slower second phase of viral load decay over several months, so that 6-9 months after initiation of treatment, most of patients have reached undetectable plasma viral loads. At this time, the decrease is much slower, if it exists at all.[86,87]

Occasionally, transient increases in viral loads (called *blips*) are detected. In addition, current viral load assays show increased sensitivity and often provide measures of RNA levels <50 copies/mL, with unclear clinical significance.[88] However, measurement of two consecutive viral loads higher than 200 copies/mL –which eliminates most cases of viremia caused by isolated blips or assay variability- separated from one discrete time interval should be considered as an upcoming virological failure and a new salvage regimen should be designed and started as soon as possible.[89]

One of the main causes of the varying outcomes among patients on antiretroviral therapy is the emergence of resistant viruses. The causes of the selection of resistances appear to be a combination of increased transmission, evolution of HIV to avoid the effect of the drugs and a lack of adherence of subjects taking HAART.

Notwithstanding complete adherence, even in patients whose plasma viral RNA levels have been suppressed to below detectable levels, replication-competent virus can routinely be recovered from peripheral blood mononuclear cells (PBMCs) and biological fluids such as semen.[90] It is believed that the reservoir of latently-infected cells established early in infection may be involved in the maintenance of viral persistence despite HAART[91] and likely represents the major barrier to virus eradication.[92] Evidence for a rapid decay of the HIV-1 reservoir in patients has also been demonstrated in resting CD4+ T cells in the first 12–24 months after early initiation of antiretroviral therapy, and then its decay slows down.[93] Several attempts to achieve eradication by disrupting the viral reservoirs are of recent research interest, among them, treatment with more potent antiretroviral therapy in-

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cluding potentially antilatency therapies in individuals with recent HIV acquistion and/or use of immunomodulatory therapies to purge the viral reservoirs in the context of intensified regimens.[61,94]

Treatment for prevention

Antiretroviral drugs are also successfully used for post-exposure prophylaxis if prescribed early after a suspected high-risk contact for HIV acquisition. But more recently, increasing attention has been paid to pre-exposure prophylaxis (PrEP). PrEP has been tested in HIV-negative people who are at high risk of HIV acquisition by taking daily antiretroviral medication orally or by topical vaginal gels of antiretroviral drugs to try to lower their chances of becoming infected with HIV if they are exposed to it. The first two major successes of PrEP came with the Centre for the AIDS Programme of Research in South Africa CAPRISA004 and the Pre-Exposure Prophylaxis Initiative (iPrEx). The CAPRISA004 was a double-blind, randomized controlled trial conducted to compare 1% tenofovir gel (n = 445 women) with placebo gel (n = 444 women) in sexually-active, HIV-uninfected 18- to 40-year-old women in urban and rural KwaZulu-Natal, South Africa. HIV incidence in the tenofovir gel arm was 5.6 per 100 women-years compared with 9.1 per 100 women-years in the placebo gel arm. The use of Tenofovir gel reduced HIV acquisition by an estimated 39% overall, and by 54% in women with high gel adherence.[15] Soon afterwards, in November 2010, the National Institutes of Health (NIH) announced the results of the iPrEx clinical trial, a large, multi-country research study examining PrEP involving 2499 HIV negative individuals. The study found that daily oral use of tenofovir plus emtricitabine (TDF/FTC) provided an average of 47% additional protection for men who have sex with men (MSM) who also received a comprehensive package of prevention services that included monthly HIV testing, condom provision, and management of other sexually-transmitted infections. Importantly, when adjusted to adherence, protection rates were as high as 73%.[95,96]

These successes were buoyed by additional positive results from the CDC TDF2 and Partners PrEP trials, providing first evidence that a daily oral dose of antiretroviral drugs used to treat HIV infection could reduce HIV acquisition through heterosexual sex as well. However, disappointing results were obtained recently in the VOICE trial, evaluating three antiretroviral-based approaches for preventing the sexual transmis-

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sion of HIV in women (daily use of either TDF, TDF/FTC or a vaginal TDF gel). The monitoring board review determined that the trial would not be able to demonstrate that TDF tablets used orally were effective in preventing HIV acquisition in the women enrolled in the trial. The arms of the study that are testing oral TDF/FTC and the vaginal gel form of tenofovir are continuing. Future and ongoing trials aim to elucidate the potential protective effect of intermittent –instead of continuous- use of antiretrovirals (HPTN 067), other drug combinations (with maraviroc for instance) as well as evaluate the acceptability, uptake and adherence to daily PrEP when translating the strategy into non-research settings (San Francisco PrEP Demonstration Project).

In the meantime, national institutions are now leading the controversial demand to develop formal Public Health Service guidelines for PrEP in accordance with new available data, the impact of signature mutations selected by PrEP and its individual-level and public health consequences of ARV resistance[97] as well as sustainability, cost-effectiveness[98] and feasibility to scale up in the implementation of such strategies. The first example comes from CDC recommendations released in July 2011.

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CDC Interim Guidance on HIV Pre-Exposure Prophylaxis for Men Who Have Sex with Men

CDC

Below is CDC interim guidance for health-care providers electing to provide pre-exposure prophylaxis (PrEP) for the prevention of HIV infection in adult men who have sex with men and who are at high risk for sexual acquisition of HIV.

Before initiating PrEP

Determine eligibility

- Document negative HIV antibody test(s) immediately before starting PrEP medication.
- Test for acute HIV infection if patient has symptoms consistent with acute HIV infection.
- Confirm that patient is at substantial, ongoing, high risk for acquiring HIV infection.
- Confirm that calculated creatinine clearance is ≥60 mL per minute (via Cockcroft-Gault formula).

Other recommended actions

- Screen for hepatitis B infection; vaccinate against hepatitis B if susceptible, or treat if active infection exists, regardless of decision about prescribing PrEP.
- Screen and treat as needed for STIs.

Beginning PrEP medication regimen

- Prescribe 1 tablet of Truvada* (TDF [300 mg] plus FTC [200 mg]) daily.
- In general, prescribe no more than a 90-day supply, renewable only after HIV testing confirms that patient remains HIV-uninfected.
- If active hepatitis B infection is diagnosed, consider using TDF/FTC for both treatment of active hepatitis B infection and HIV prevention.
- Provide risk-reduction and PrEP medication adherence counseling and condoms.

Follow-up while PrEP medication is being taken

- Every 2–3 months, perform an HIV antibody test; document negative result.
- Evaluate and support PrEP medication adherence at each follow-up visit, more often if inconsistent adherence is identified.
- Every 2–3 months, assess risk behaviors and provide risk-reduction counseling and condoms. Assess STI symptoms and, if present, test and treat for STI as needed.
- Every 6 months, test for STI even if patient is asymptomatic, and treat as needed.
- 3 months after initiation, then yearly while on PrEP medication, check blood urea nitrogen and serum creatinine.

On discontinuing PrEP (at patient request, for safety concerns, or if HIV infection is acquired)

- Perform HIV test(s) to confirm whether HIV infection has occurred.
- If HIV positive, order and document results of resistance testing and establish linkage to HIV care.
- If HIV negative, establish linkage to risk-reduction support services as indicated.
- If active hepatitis B is diagnosed at initiation of PrEP, consider appropriate medication for continued treatment of hepatitis B.

Abbreviations: STI = sexually transmitted infection; TDF = tenofovir disoproxil fumarate; FTC = emtricitabine

* These recommendations do not reflect current Food and Drug Administration-approved labeling for TDF/FTC.

Source: "Interim Guidance: Pre-exposure prophylaxis for the prevention of HIV infection in men who have sex with men," CDC *Morbidity and Mortality Weekly Report*, January 28, 2011.

Figure 9. CDC's interim guidance for physicians on pre-exposure prophylaxis for HIV prevention for men who have sex with men who are at high risk for HIV infection. May 11, 2011. Available at: http://www.cdc.gov/nchhstp/newsroom/PrEPMSMGuidanceGraphic.html

Another and probably less controversial use of antiretroviral agents as prevention strategies has been reinforced following the HPTN Study 052 trial. [99] It just followed the principle '*The best way to prevent HIV with treatment is to treat those who have it*': 96% reduction in HIV transmission was achieved when antiretroviral treatment was initiated in HIV infected individuals to protect uninfected sexual partners. HIV treatment with three drugs is obviously more expensive than one or two for PrEP, but it is likely that the net double benefit (i.e., to the individual and the uninfected partner) easily justifies the incremental cost.

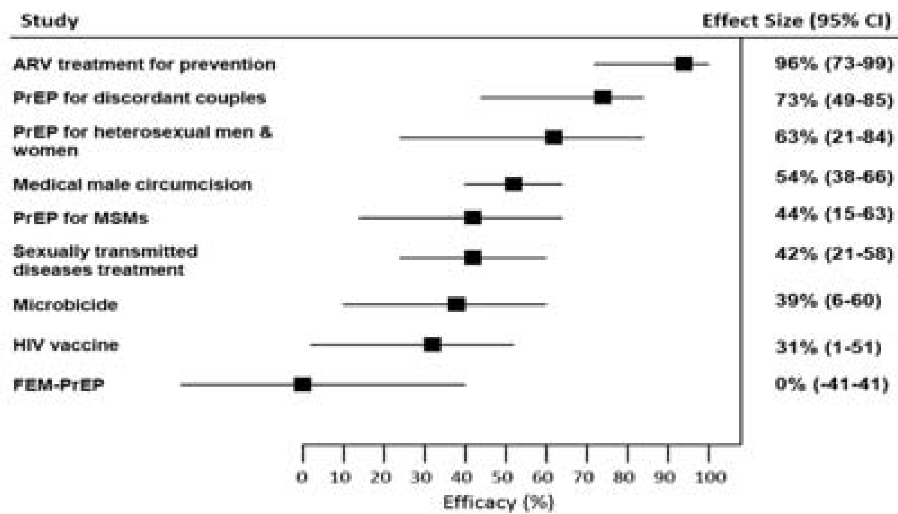

Figure 10. HIV incidence reduction by different prevention strategies. Source: Adapted from Dieffenbanch C. Combination HIV Prevention Research. Division of AIDS, NIAID, NIH. October 13, 2011.

Lastly, one of the most outstanding beneficial effects of antiretroviral drugs in prevention strategies: Virtual elimination of mother-to-child transmission (MTCT). South Africa is an example of the drastic reduction of transmission to infants after successful strategies, where presently, coverage to prevent mother-to-child transmission of HIV has been achieved by almost 90%. However, an estimated 370,000 children contracted HIV during the perinatal and breastfeeding period in 2009, which shows that access to services to halt mother-to-child transmission needs to be scaled up.

Despite all the evidence of the beneficial effects of HAART and despite having already more than 5 million people receiving treatment we cannot overlook the fact that it still represents only 35% of the people who need HIV therapy. Ten million people living with HIV who are eligible for treatment (based on WHO guidelines from early 2010) are still in need, so efforts to expand access to antiretrovirals cannot be restrained, especially in view of the dramatic effects of previous delays in treatment access programmes in the developing world.

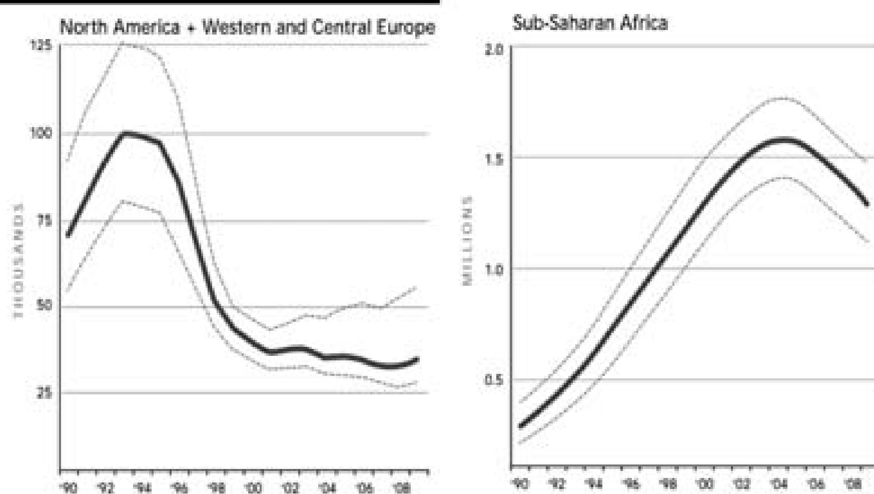

Figure 11. Comparison of annual AIDS-related deaths among regions, 1990-2009 reflecting the delayed introduction of HAART in Sub-Saharan Africa. Source: UNAIDS 2010.Report on the Global AIDS Epidemic. Available at: http://www.unaids.org

Test-and-treat

Increasing efforts to expand antiretroviral access undoubtedly go hand in hand with the implementation of generalized testing strategies, which are also failing in developed-world settings such as ours. One out of every four people infected with HIV has not been yet diagnosed and is estimated to be the main source of new transmissions. That is not only tremendously important to successfully put into practice test-and-treat strategies to prevent the epidemic from spreading, but also because 30% of diagnoses are unfortunately still made at advanced stages of the disease[100] when immune recovery is most difficult to attain (known as *discordant patients*)[101-103]. More efforts need to be implemented to pursue the diagnostic of acute infections, especially when a symptomatic acute infection occurs, as increasing evidence points towards the benefits of treatment initiation during these early phases.

Mathematical models report that if all persons were tested for HIV-1 once a year and all those with positive results immediately started and maintained the use of effective ART for life, the incidence could be reduced to <1 new infection per 1,000 persons within 10 years, and the prevalence would decrease to <1% within 50 years.[104,105]

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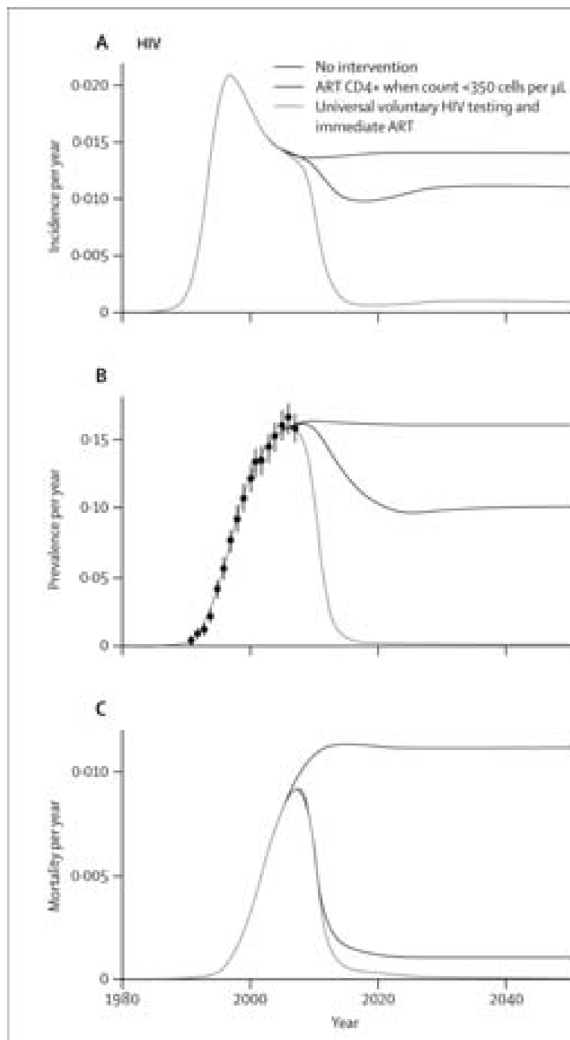

Figure 12: Modeled HIV incidence (A), prevalence (B), and mortality (C) of people placed on antiretroviraltherapy (ART). Source: Granich, R.M., Gilks, C.F., Dye, C., De Cock, K.M. & Williams, B.G. Universal voluntary HIV testing with immediate antiretroviral therapy as a strategy for elimination of HIV transmission: a mathematical model. Lancet 373, 48-57 (2009).

Such models reflect the effect of decreasing the community viral load. They rely, however, on many major assumptions such as a) heterosexual sex transmission, b) extremely high linkage to care and adherence rates, c) 100-fold ART reduction transmission, d) that treatment is coupled with ≥1 prevention intervention which collectively reduces incidence by 40%, and e) that significant drug resistance does not emerge, and that treatment failure rates are low (3% per year). Unfortunately, many of these assumptions are currently unattainable in real-world settings, as reviewed in[106].

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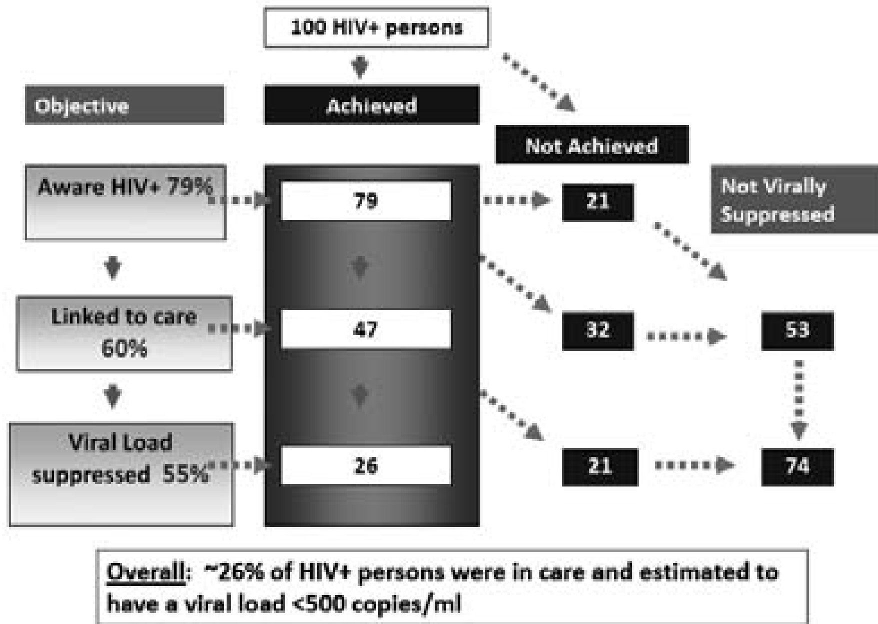

Figure 13. Representation of the cascade effect of progressive reduction of completely suppressed HIV infected patients showing the limited overall net effect of test-link to care-treat strategy. Source: Burns, D.N., Dieffenbach, C.W. & Vermund, S.H. Rethinking prevention of HIV type 1 infection. Clin Infect Dis 51, 725-731 (2010).

We therefore still need to be realistic: such a combination of HIV-1 prevention strategies will rarely be sufficient and cost-effective to fully implement an ideal strategy aimed at halting the pandemic. In conclusion, not only there is still considerable room for implementing test and treat strategies, and in particular through combining all the possible prevention programs, but especially for an available effective preventive vaccine.

INTRODUCTION-II

T Cell-Mediated Immunity

The body is protected from infectious agents by a variety of effector cells, molecules and antibodies that together make up the immune system. The innate immune response is always immediately available to combat a wide range of pathogens without any specificity but does not lead to lasting immunity. **Innate immunity** starts when foreign microorganisms breach the anatomical barriers and is mainly driven by an inflammatory response that implicates cells such as macrophages, phagocytic neutrophils, dendritic cells, and natural killer cells (NKs) and involves a release of several cytokines and activation of the complement.

However, most pathogens can overcome the innate immune defense mechanisms, and **adaptive immunity** is essential for defense against them. An adaptive immune response involves the selection and amplification of clones of lymphocytes bearing receptors which specifically recognize foreign antigens that will be able to eliminate the infectious agent. Many examples of immunodeficiency syndromes exist that are associated with failure of specific parts of the adaptive immune responses and are mostly characterized by recurrent infections (from general abnormalities such as thymic aplasia in DiGeorge's syndrome to more specific ones, such as defects in the WASP gene implicated in T cell activation responses responsible for the Wiskoot-Aldrich syndrome).[107]

Both the T cell-mediated immune response and the humoral antibody response produced by B cells have a crucial synergistic involvement in the control of HIV. More than ever, vaccinologists are again in favor of combinational strategies able to induce both a persistent and rapid T effector cell response to control the infection along with an effective humoral response to avoid HIV acquisition.[108] This introductory section will mainly focus on the T cell-mediated arm which is our major topic of interest in the work compiled in this thesis. The development of naive T cells in the thymus, their priming, their differentiation into effector T cells as well as their immunological memory, -their most important feature in vaccine development- will be covered. Lastly, some background on HLA to show the significant impact of host genetics in HIV specific response rates and its contribution to immune selection and viral evolution will be provided.

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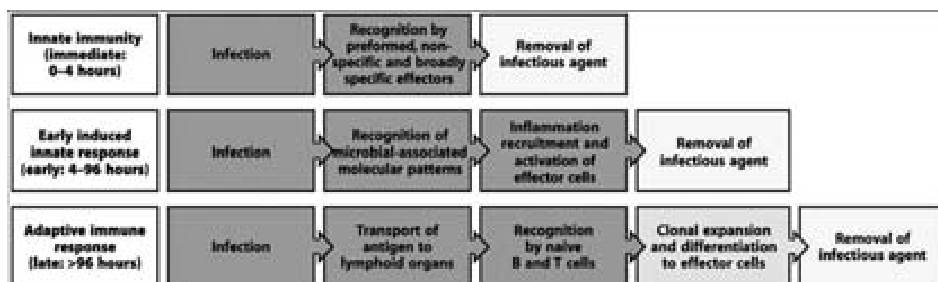

Figure 14. The response to an initial infecion occurs in three phases. The first two phases rely on the recognition of pathogens by germline-encoded receptors of the innate immune system, whereas adaptive immunity uses variable antigen-specfic receptors that are produced as a result of gene segment rearrangements. Adaptive immunity occurs later, because specific B and T cells must first undergo clonal expansion before they differentiate into effector clear cells. Source: Adapted from Janeway's Immunobiology, 7 ed. (Garland Science 2008)

T cells develop from progenitors that are derived from the pluripotent hematopoietic stem cells in the bone marrow and migrate through the blood to the thymus, where the generation of two distinct lineages of T cells takes place, the γ:δ and the α:β lineage, which express different antigen-receptor genes. α:β T cell receptors (TCR) genes are then rearranged: Immature T cells that recognize self MHC at an intermediate affinity receive signals to survive (positive selection) whereas those that interact strongly with self antigens are removed from the repertoire (negative selection). During this selection process, about 98% of the thymocytes die in the thymus, and just a very small part of the thymocytes leaves it as mature T cells. This apparent 'waste' of thymocytes emphasizes the intensive screening that each thymocyte undergoes for the ability to recognize self-peptide:self MHC complexes and for containing self tolerance.

The thymus is fully developed at birth in humans and the rate of T cell production by the thymus is greatest before puberty; thereafter, it begins to shrink and involute with subsequent progressive decrease of the production of new T cells over time. It seems, however, that once the T-cell repertoire is established, immunity can be sustained without the production of significant numbers of new T cells, as long as the pool of peripheral T cells is maintained by long-lived T cells and division of mature T cells.

The development of the thymocytes also includes passing through a series of phases mainly marked by the expression of the T cell receptor and cell-surface proteins such as the CD3 complex and the co-receptor proteins CD4 and CD8.

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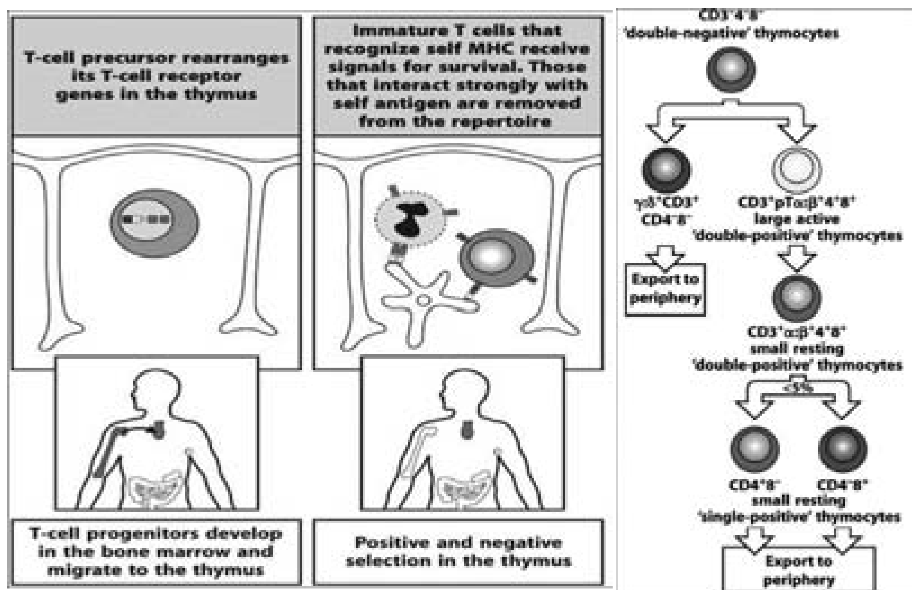

Figure 15. Schematic representation of the selection of T cells in the thymus and two distinct lineages of thymocites produced in the thymus: the minority population of $\gamma{:}\delta$ T cells (which lack CD4 or CD8) and the majority $\alpha{:}\beta$ T-cell lineage. Source: Adapted from Janeway's Immunobiology, 7 ed. (Garland Science 2008)

T cells that survive selection leave the thymus and enter the bloodstream. Circulating in the periphery, they repeatedly leave the blood to migrate through the peripheral lymphoid organs until they encounter foreign antigens and become activated. Mature recirculating T cells that have not yet encountered their specific antigens are known as **naive T cells**. Activation happens when a naive T cell meets its specific antigen, presented to it as a peptide:MHC complex on the surface of an **antigen-presenting cell** (APCs, generally dendritic cells)

All the adaptive primary immune response is initiated in the peripheral lymphoid organs, which includes lymph nodes, spleen and GALT (gut associated lymphoid tissue, such as the Peyer's patches). The adaptive immune response is initiated when **dendritic cells** (DC) capture the antigens at the site of infection and migrate to local lymphoid organs, where they will mature into cells that can both present antigens to naive T cells and trigger their activation. Maturation of DC occurs along with the expression of large amounts of peptide–MHC and co-stimulatory molecules on their surface to acquire the ability to prime naive T cells. There is great interest about the DC's modes of action in the HIV field for many reasons. Firstly, understanding the ability to induce functional and phenotypic maturation of peripheral blood monocyte-derived DCs by pulsing immature DCs with new antigens leading to the priming and expansion of Ag-spe-

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cific T cells ex vivo has potential applications for cellular vaccination and adoptive immunotherapy.[109-111] Second, because DCs can themselves enhance HIV infection of T cells through *trans*-infection processes[23,24] and could therefore be also alternative targets to inhibit HIV cell transmission. Lastly, in terms of the development of vaccine candidates, since the first step at local sites after vaccine inoculation will also depend on a DC efficient uptake and transportation of the foreign antigens into lymphoid tissues. In this regard, it has been experimentally shown that antigens placed in a superficial flap are not able to elicit a T cell response by themselves without proper DC-mediated transportation through their unique membrane transport pathways to lymphoid tissues.[112] Therefore, vaccine candidates need to account for such DC uptake, maturation and transportation phenomena.

Priming of naive CD8 T cells upon their encounter with pathogen-activated DCs in the T cell zones generates proliferation for several days leading to a clonal expansion and differentiation into **cytotoxic effector T cells** (CTL) capable of directly recognizing and killing pathogen-infected cells. CD4 T cells recognize pathogen peptides presented by APCs under MHC class II molecules and develop in a more diverse array of **effector types -T_H1, T_H2 and T_H17**, depending on the nature of the signals they receive during the priming. Their functions also include cytotoxic activity but more frequently involve the secretion of an array of cytokines to direct their different target cells, in order to give a particular response. The r**egulatory T cell subsets (T_{reg})** have an inhibitory activity that limits the extent of immune activation. Three APC cell types express MHC class II molecules, and thus have the potential to activate CD4 T cells: macrophages, B cells and DCs. Antigen presentation to naive CD4 T cells takes place within a complex microenvironment in which the movements of antigens, APCs and T cells are governed by the anatomical constraints of the lymph node architecture.[113]

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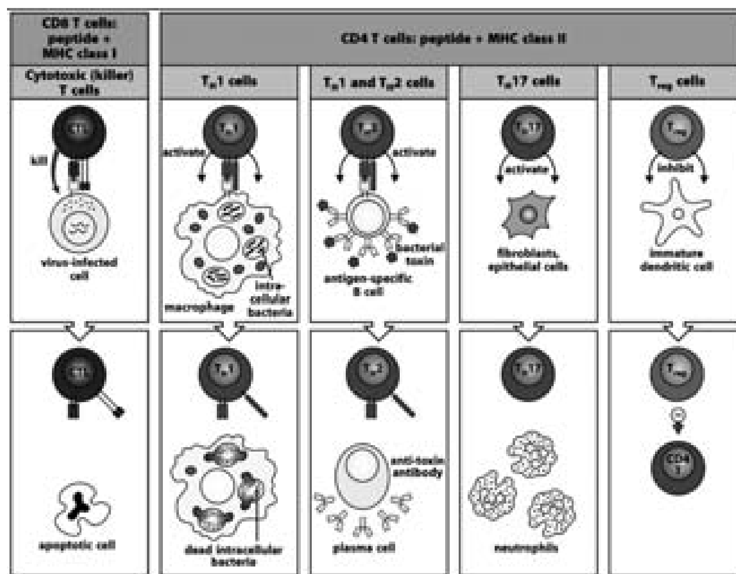

Figure 16. Roles of effector T cells in cell-mediated and humoral immune responses. Both cell-mediated cytotoxicity and humoral immunity are involved in many infections. CD4 T_H17 cells help to recruit neutrophils to sites of infection early in the adaptive immune response. T_{reg} cells, in supressing the T cell response, are important in preventing immune responses from becoming uncontrolled and in preventing autoimmunity. Source: Adapted from Janeway's Immunobiology, 7 ed. (Garland Science 2008)

Once an expanded clone of T cells achieves an effector function, its progeny can act on any target cell that displays an antigen on its surface. While progressive reduction in antigen stimulation occurs, a subset of effector T cells enter into a 'resting' state, long-lived cells that will give an accelerated response after reexposure to the antigen at subsequent challenges, by rapidly producing cytokines upon reestimulation.

Activated effector T cells emigrate from their site of activation and enter the blood via the thoracic duct at sites of infection. This migration processes are guided by changes in the endothelium adhesion molecules and by local chemotactical factors. When binding to their specific antigenic peptide:MHC complexes, the TCRs and co-receptors cluster at the site of cell-cell contact, forming what is called the immunological synapse. As in the virological synapses, clustering of TCRs triggers a reorientation of the cytoskeleton that polarizes the effector cell to orientate the release of the effector molecules at the site of contact with the target infected cell, and then cytotoxicity starts.

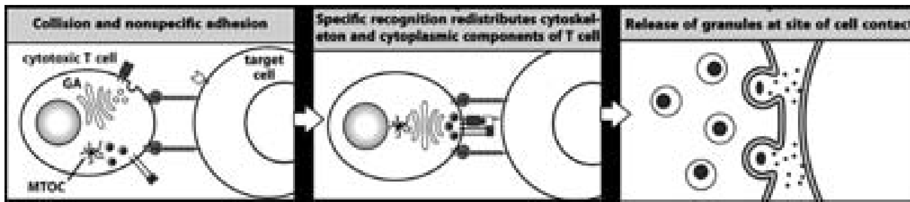

Figure 17. Cellular polarization of T cells during specific antigen recognition allows effector molecules to be focused on the infected target cell. Source: Adapted from Janeway's Immunobiology, 7 ed. (Garland Science 2008)

Once they enter the target cells, viruses such as HIV, although partially susceptible to antibody neutralization, can only be eliminated by the destruction of the infected cell. CD8 CTLs respond to the presentation of epitopes by APC in association with MHC class I. The antigens are recognized by the heterodimeric cell surface molecule of the TCR that interacts with the antigen presented as a short peptide:MHC molecule complex. Recognition through MHC class I molecules seems to be more restricted, not only because of limitations in the length of the peptides (8 to 10 residues) but also because the ends of the peptide (amino-terminal and carboxyl-terminal) must fit into defined pockets at opposite sides of the grooves in the MHC class I locus. Peptides presented in the MHC class II grooves to CD4 T cells can be longer (12 to 24 residues) and thus more heterogeneous.

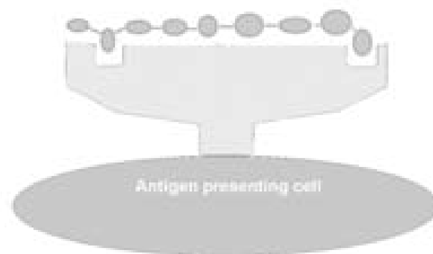

Figure 18: Schematic representation of presentation by the MHC class I molecules of the antigenic peptide

After recognition, CTLs kill their targets by cell lysis or inducing them to undergo apoptosis through the release of different specialized cytotoxic molecules (pore-forming perforin, granzyme, granulysin, Fas ligand) from modified lysosomes. Most CD8 CTLs also release cytokines such as IFNγ, TNFα, and LTα, also contributing to cytotoxicity. Of special interest, IFNγ induces an increased expression of MHC class I molecules in infected cells, and activates macrophages, recruiting them to the sites of infection and generally enhances recognition

by other effector cells and contains the spread of cytosolic pathogens. Measurement of IFNγ release has been widely used as an indirect marker of CTL activity, as will be discussed later on this section.

CTLs kill infected cells with high specificity, which is crucial in minimizing adjacent tissue damage while allowing the elimination of the infected target cells. However, the CTL lytic machinery can sometimes be directed against self-tissues in autoimmune disorders, transplanted cells during graft rejection and host tissues to cause graft-versus-host disease, which is one of the most serious diseases related to CTL function.[114]

Immunological Memory

Immunological memory is mainly determined by the small population of memory cells stablished during the primary adaptive immune response which can persist despite the reduction –and even absence- of the antigen that originally induced them. Many studies have attempted to determine the duration of immunological memory. By evaluating responses in people who for instance received smallpox vaccines in the past, strong vaccinia-specific CD4 and CD8 T cell memory responses were detected as far back as 75 years after the original immunization. Mechanisms with such a long maintenance are, however, quite unclear.

Both CD4 and CD8 differentiate into two types of memory cells with distinct activation patterns which can be distinguished by their surface markers. As mentioned before, when naive T cells encounter an antigen, they can respond quickly by activation and become effector cells. With reduced antigen exposure, a subset of T cells turns into memory cells. Memory cells can be divided into two groups: one is referred to as **T_{CM} cells (central memory**, or resting), characterized by $CD45RO^+$ and $CCR7^+$ expression and traffic to lymph nodes as do naive CD8 cells. This subset also has a high proliferative potential; these cells, however, take longer to differentiate into effector T cells and do not secrete very large amounts of cytokines early after restimulation. The **effector memory cells (T_{EM})** express CD45RO but not CCR7 and mature into **terminal effector cells** containing perforin with a very rapid cytotoxic response driven by secretion of large amounts of IFNγ, IL-4 and IL-5 early after restimulation, but do not proliferate well. The factor that influences the formation of T_{CM} vesus T_{EM} cells upon antigen reduction is still not clearly defined. In general terms, CD8 T_{CM} cells predominate when an antigen is cleared or markedly reduced (e.g. with CMV infection), whereas T_{EM} cells predominate when high levels of antigen persist (as in HIV).

The precise mechanisms of the maintenaince of immunological memory seem to be mainly driven by the presence of certain cytokines and homeostatic

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interactions with self-MHC:self-peptide complexes. Vaccination-induced immunological memory however, is one of the major challenges in Immunology, as many pathogens –such as HIV- do not induce protective immunity that completely eliminates the pathogen.

Anti-HIV cytotoxic T cell responses

Among other common viral infections, HIV generates an incredibly strong cytotoxic T cell immune response. Although all HIV proteome is highly immunogenic, the density of the T cell response is mostly detected in Gag and Nef proteins, where immunology databases have the highest density of epitopes captured from the literature in these regions.[115] The immune response is often dominated by T cells specific for particular epitopes (named immunodominant) but subdominant responses can also play an important role.[116] A deeper dissertation on the role of HIV specific CTL immune response in the control of HIV infection will be provided along the articles compiled in this thesis and the discussion section, with special focus on defining the most protective specificities and functional characteristics.

An extensive series of data detected right after the peak of viremia in acute HIV infection suggests the potential role of the virus-specific CTL response in determining the viral set point in chronic stages of infection and HIV disease progression.[117-119] This is also supported by studies in the SIV macaque model, where transient depletion of CD8+ T-cells in controller animals resulted in 100- to 10,000-fold increases in viremia and where the re-establishment of the CD8+ T-cell populations restored the ability of these animals to control SIV replication.[120] More recently, a vaccine trial in the macaque model has provided a clear immunological CD8 CTL-mediated correlate of protection against disease. An innovative CMV vectored-based vaccine elicited a persistent and rapid T effector cell response to SIV antigens and resulted in control of the infection in 50% of the animals, not only reducing the initial infection but also controlling new viral rebounds of systemic infection at later time points. [121]

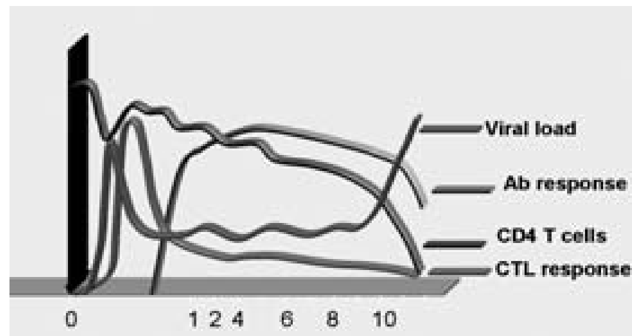

Figure 19 Schematic diagram of events occurring after HIV infection. Prior to seroconversion high levels of virus can be detected in the blood. After the first acute phase, viremia is reduced to low levels and maintained at the set-point level. Years later, viremia rises again throughout the development of AIDS period. CD4 cells counts decrease during acute infection with some recovery afterwards and then, a progressive decline occurs over time. CTL responses against HIV rise during primary infection and they are maintained over time and begin to decrease before or around the time of disease progression.

The relative importance of the CTL response in at least partially controlling viral replication is further supported by the identification of specific host genetic polymorphisms in the HLA region associated with relative slow disease progression (HLA-B27, 57, 58, 1516, 1517, emerging C alleles).[122] These association point directly towards a contribution of the HLA class I restricted CD8+ T cell immunity in virus control, also revealed by the demonstration of differences in HIV specific T cell response patterns among different ethnicities.[123,124] Elegantly, diversity in HLA class I alleles was shown to have a significant impact on HIV evolution through the discovery of CTLs targeting subdominant epitopes in association with HLA frequencies[116]. More recenlty, Kawashima et al described HLA footprints driving immune selection pressure in an exhaustive analysis of viral sequences and HLA alleles in >2,800 subjects from 5 different continents.[125] This process of viral adaptation to host genetics highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.

The investigation of the CTL response to HIV-1 has led to the most extensive characterization of class I restricted CTL epitopes in any viral infection studied this far. Optimally-defined CTL epitopes are listed and updated every year at the Los Alamos HIV Immunology database and include more than 200 well-characterized HIV CTL epitopes located across all different HIV proteins. These epitopes are restricted by a wide array of different HLA class I alleles with a few highly promiscuous epitopes being targeted in the context of as many as 8 different HLA class I alleles.[126]

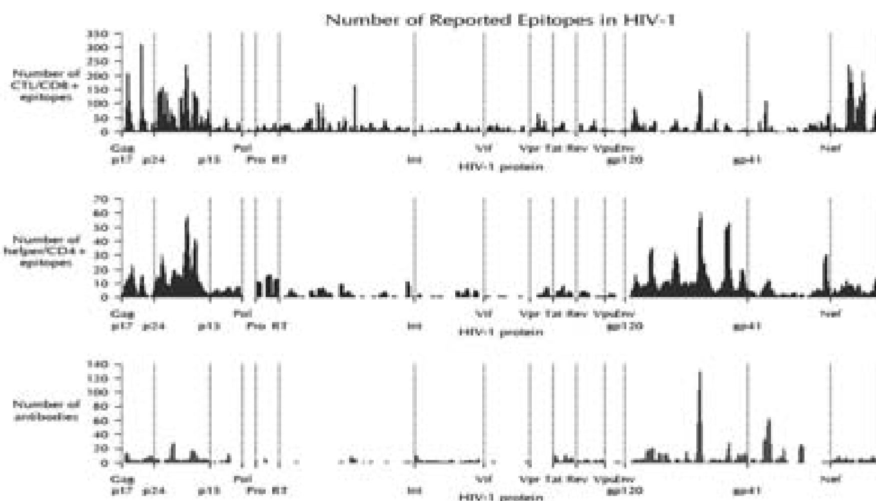

Figure 20. The number of unique epitopes included in the database that span each position in the HIV proteome. Source: HIV Molecular Immunology 2011. Karina Yusim, Bette T. M. Korber, Christian Brander, Dan Barouch, Rob de Boer, Barton F. Haynes, Richard Koup, John P. Moore, Bruce D. Walker, and David I. Watkins, editors. Publisher: Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR-12-1007

Optimal epitopes have usually been defined as the shortest peptide truncation that results in the highest stimulation of epitope-specific T cells at the lowest peptide concentration. Based on the anchor residues described for various HLA class I alleles, viral protein sequences can be screened for the presence of such anchor residues to identify potential CTL epitopes.[127] These peptides can then be synthesized and tested either in cellular or cell-free assays for their capacity to bind to the selected class I molecule. With subsequent assays that test the ability of peptides thus defined to serve as a target for CTL in natural infection, epitopes can be rapidly identified and characterized.[128] Identification and optimal definition of HIV-derived cytotoxic T lymphocyte epitopes is crucial to identify precise targets of the host cellular immune response to HIV and its associations with better disease outcomes, which is the main goal of the work discussed in the Chapter 2. It also allows the identification of viral sequence changes in epitopes that may mediate CTL escape through different mechanisms such as abrogated HLA binding, missing TCR interaction or impaired antigen processing. In addition, the precise definition of epitopes and their HLA restriction also enables the identification of epitopes that can be presented by multiple HLA alleles and which could thus be employed in the context of different host genetic backgrounds.

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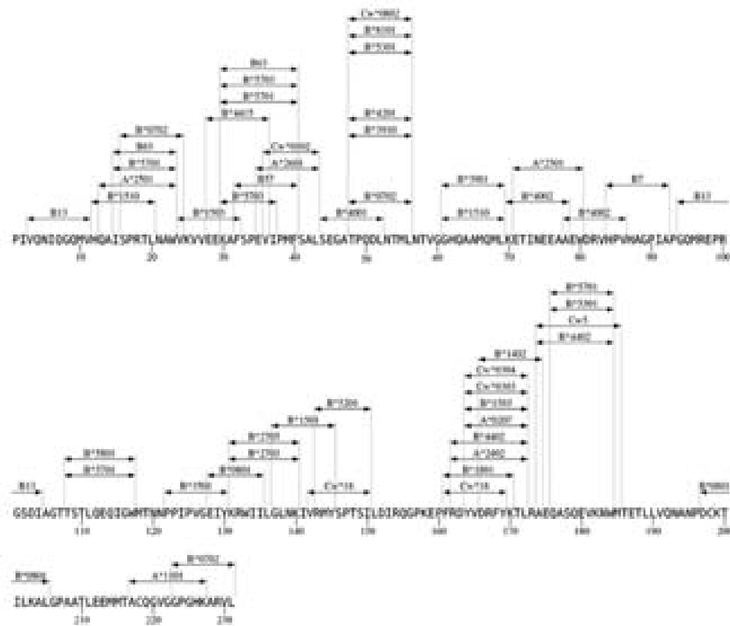

Figure 21. Example of a map of optimal HIV-1 CTL epitopes (shown for HIV Gag p24). The location and HLA restriction elements of CTL epitopes are indicated on protein sequences of HXB2. Source: HIV Molecular Immunology 2009. Karina Yusim, Bette T. M. Korber, Christian Brander, Barton F. Haynes, Richard Koup, John P. Moore, Bruce D. Walker, and David I. Watkins, editors. Publisher: Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR 09-05941

Aside from CTL epitopes located in proteins encoded by HIV open reading frames (ORFs), recent data also points out the relevance of responses to alternative reading frames (ARF)-encoded HIV epitopes (cryptic epitopes), as described during natural infection. It has been suggested that they may also contribute to viral control and drive viral evolution on a population level.[129,130]

Understanding HLA : 'Our cellular Id'

The human leukocyte antigen (HLA) system is the name of the major histocompatibility complex (MHC) in humans. The genomic map of the HLA (3.6 Mb, 224 loci in HLA class I, II and III) resides on chromosome 6, and encodes cell-surface antigen-presenting proteins, with 40% of expressed genes with a role in immunity. HLAs corresponding to MHC class I (A, B, and C) present peptides from inside the cell (for instance viral peptides produced in the proteasomes,

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generally about 8-10 amino acids in length) CTLs cells require presentation via MHC molecules to recognize the antigens, what is known in the field as 'HLA restriction'. HLAs corresponding to MHC class II (DP,DM, DOA,DOB,DQ, and DR) present antigens from outside the cell to T lymphocytes, particularly T helper cells, which in turn stimulate antibody-producing B cells. HLAs corresponding to MHC class III encode components of the complement system. Other than its role in infectious diseases, HLA is also determinant in organ transplant rejections and may mediate autoimmune diseases (i.e. type I diabetes, ankylosing spondylitis, celiac disease, SLE, myasthenia gravis, inclusion body myositis and Sjögren's syndrome among others)

MHC class I proteins form a functional receptor that is comprised of the binding of β2-microglobulin with major and minor gene subunits to produce a heterodimer structure such as the following:

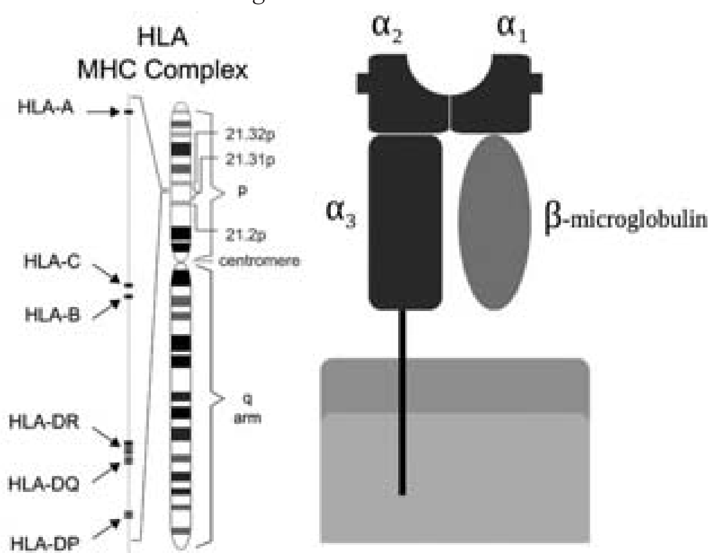

Figure 22: Schematic representation of cromosome 6 and an MHC class I molecule. Source: Wikipedia commons, available at: http://en.wikipedia.org/wiki/Human_leukocyte_antigen.

HLA alleles nomenclature has varied over time. The IMGT/HLA Database is part of the international ImMunoGeneTics project and contains at present 7,269 allele sequences (4,946 Class I alleles/1,457 Class II alleles up to May 2011) and is freely available at: http://www.ebi.ac.uk/imgt/hla. They provide the official sequences for the WHO Nomenclature Committee.

The last nomenclature update (Dec 2010) established the following: Each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons. The length of the allele designation is

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dependant on the sequence of the allele and that of its nearest relative. All alleles receive at least a four digit name, which corresponds to the first two sets of digits and longer names are only assigned when necessary. The digits before the first colon describe the type, which often corresponds to the serological antigen carried by an allotype, The next set of digits are used to list the subtypes, the numbers being assigned in the order in which the DNA sequences have been determined. Alleles whose numbers differ in the two sets of digits must differ in one or more nucleotide substitutions that change the amino acid sequence of the encoded protein. Alleles that differ only with respect to synonymous nucleotide substitutions (also called silent or non-coding substitutions) within the coding sequence are distinguished by the use of the third set of digits. Alleles that only differ with respect to sequence polymorphisms in the introns or in the 5' or 3' untranslated regions that flank the exons and introns are distinguished by the use of the fourth set of digits.

In addition to the unique allele number, there are additional optional suffixes that may be added to an allele to indicate its expression status. Alleles that have been shown not to be expressed, 'Null' alleles have been given the suffix 'N'. Those alleles which have been shown to be alternatively expressed may have the suffix 'L', 'S', 'C', 'A' or 'Q'.

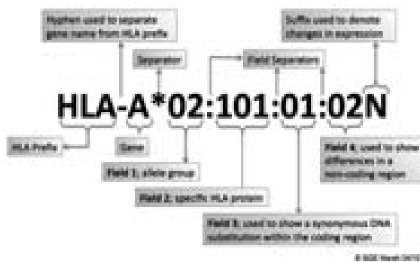

Figure 23. Example of Nomenclature for Factors of the HLA System. Source: IMGT/HLA Database, available at: http://hla.alleles.org/announcement.html

Through the three papers compiled in this thesis, the old pre-2010 nomenclature has still been used. Different associations of HLA polymorphisms with disease outcomes will be developed further in Chapter 1.

As mentioned before, the diversity of HLA in the human population is one aspect of disease defense and, as a result, HLA diversity will have a crucial impact in influencing response patterns[116,124] and driving viral evolution.[125]

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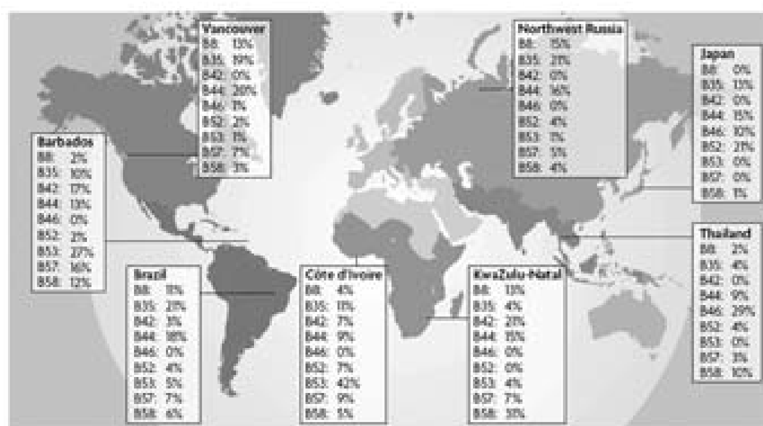

Figure 24. Representation of the variety of HLA class I allele frequencies in global terms. Source: Kawashima, Y., et al. Adaptation of HIV-1 to human leukocyte antigen class I. Nature 458, 641-645 (2009).

Lastly, to understand the impact of HLA in the adaptation of the virus in the context of MHC restriction and the promiscuity [126] of HIV specific CTL responses, it is important to bring in the concept of HLA supertypes. Based on HLA footprints, we can identify novel CTL epitopes [131] and even track the portion of sequence evolution thought to be driven by HLA-mediated immune-escape mutations.[132]. But, when assessing the extent of promiscuous CTL epitope presentation, MHC restriction can be understood in several –non-exclusive- ways:

1) the original HLA allele described for that given epitope, or
2) a "super-type-matched" class I allele, or
3) an "alternative" allele which may share binding motif similarities.

Sidney et al gave a comprehensive review on the classification of this "super-type-matched" class I allele, named HLA supertypes[133] which helps in understanding such CTL promiscuity. Despite the polymorphisms of MHC receptors, class I molecules can be clustered into sets of molecules that bind largely overlapping peptide repertoires. Different groups or supertypes for HLA-A (6) and B (6) class I alleles have been described on the basis of their main anchor specificity. The utility of the supertype knowledge is critical for epitope identification studies and especially interesting for us, in promoting the rational design of vaccines based on immunogenicity data that aim to provide a broad HLA coverage

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HLA A-supertypes

A01

A01 A03

A01 A24

A02

A03

A24

Figure 25. Supertype classification of HLA-A alleles. In each supertype, the alleles are grouped on the basis of the stringency of selection (from left to right):experimentally established motif (i.e., reference panel), exact match(es) in the B and F pockets, one exact and one key residue pocket match, key residue match(es) at B and F pockets (grey). Alleles with no match at one or both pockets (unclassified) are not listed. Source: Adapted from Sidney, J., Peters, B., Frahm, N., Brander, C. & Sette, A. HLA class I supertypes: a revised and updated classification. BMC Immunol 9, 1 (2008).

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HLA B-supertypes

B07

[illegible]

B08

[illegible]

B27

[illegible]

B44

[illegible]

B58

[illegible]

B62

[illegible]

Figure 26: Supertype classification of HLA-B alleles. The alleles associated with each HLA-B supertype, multiple supertypes are shown. In each supertype, the alleles are grouped (from left to right) on the basis of the stringency of selection as described above. Source: Adapted from Sidney, J., Peters, B., Frahm, N., Brander, C. & Sette, A. HLA class I supertypes: a revised and updated classification. BMC Immunol 9, 1 (2008).

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Assessment of HIV-specific T cell responses

The analysis of direct ex vivo T cell activity can be detected and quantified by many assays. Initially-developed **lymphoproliferative assays** (using 3H Thymidine or expansion with subsequent Cr51 release assays) have progressively been substituted by the **IFNγ based ELISpot** assay (from enzyme-linked immunospot). ELISpot has proven to be a sensitive and specific tool to determine the frequency of antigen-specific T cells. It enables the direct visualization of single IFNγ reactive cells with high sensitivity and despite its mostly monofunctional design, is still widely used in HIV immunology and in vaccinology for the quantification of HIV-specific T cells. In particular, it is relatively easy to perform, requires no expensive instrumentation and has the potential for high-throughput screening.[123], reviewed in [134]

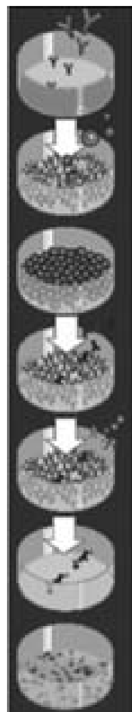

Figure27 Schematic representation of the ELISpot assay. Cytokine-specific antibodies are bound to the membrane. T cells are added to the wells. Cytokines secreted by some activated T cells are captured by the bound antibody and then revealed by a secondary cytokine-specific antibody, which is coupled to an enzyme, giving rise to a spot of insoluble colored precipitate.

Quantification of antigen-specific T cells is given in terms of breadth and magnitude. Breadth provides the total number of different responses, which represents how many regions/proteins an individual's T cells recognize (and gives detectable responses based on pre-specified criteria for positive/negative responses). Information on the breadth of the responses allows further assessments on dominance patterns to inquire which viral targets predominate within the specificities of the total CTL response. As well, most novel concept of 'depth' of the responses can also be assessed by ELISPOT through testing different epitope variants (also named cross-reactivity potential) On the other hand, the magnitude of an immune response defines how strong the responses are by determining the exact number of antigen-specific T cells within an aliquot of input cells (for instance, peripheral blood mononuclear cells #SFC/10^6 PBMC). Using ELISpot assays, the functional avidity of responses can also be assessed by performing limiting peptide dilutions and determining the peptide concentration required to induce half-maximal responses. Half-maximal stimulatory antigen doses (SD50%) are calculated as the peptide concentration needed to achieve a half-maximal number of spots in the ELISpot assay.

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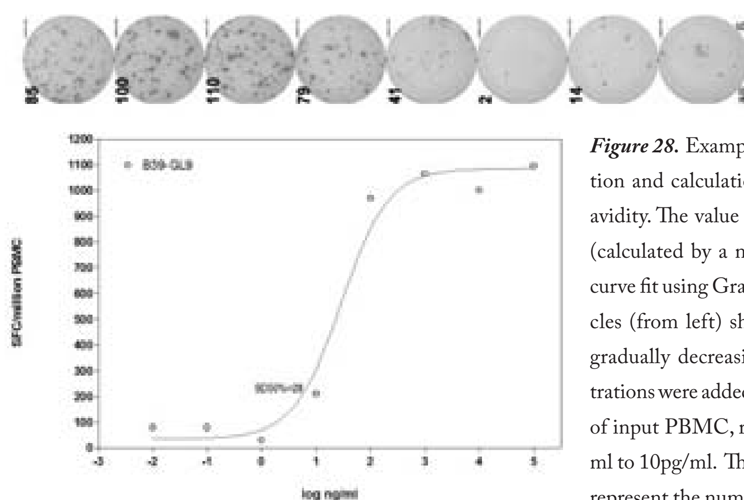

Figure 28. Example of a peptide titration and calculation of the functional avidity. The value of SD50% is shown (calculated by a non linear regression curve fit using GraphPad Prism4). Circles (from left) show the wells where gradually decreasing peptide concentrations were added to the same number of input PBMC, ranging from 100ug/ml to 10pg/ml. The numbers indicated represent the number of spots per well.

Apart from the quantitative monofunctional ELISpot assay, the measurement of additional *in vivo* antiviral properties of HIV-specific CTLs provides an extraordinary complementary information, specially after the insights from T cell vaccine trials where immunogenicity measured by ELISpot failed to predict efficacy outcomes.[135] **Flow ctyometry** using TCR labeling (peptide-MHC tetramers) or intracellular cytokine staining enables for instance the definition of T cell <u>phenotypes</u>. Of special interest is the fact that the HLA-DR+, CD38- activated CD8 T cell phenotype represents a T cell population with a superior ability to expand upon exposure to antigen and increased capacity to exert effector functions. In addition, increasing attention has been focussed on the <u>polyfunctionality</u> of the $CD8^{+}$ T cell response to HIV measured by complex flow panels to discriminate effective from not-effective CTL responses. The assessment of <u>proliferative capacity</u> with high perforin expression and the secretion of multiple cytokines such as IFNγ, IL2, TNFα, MIP-1b and/or CD107a surface expression after antigen contact are among those most commonly performed.[136-138]

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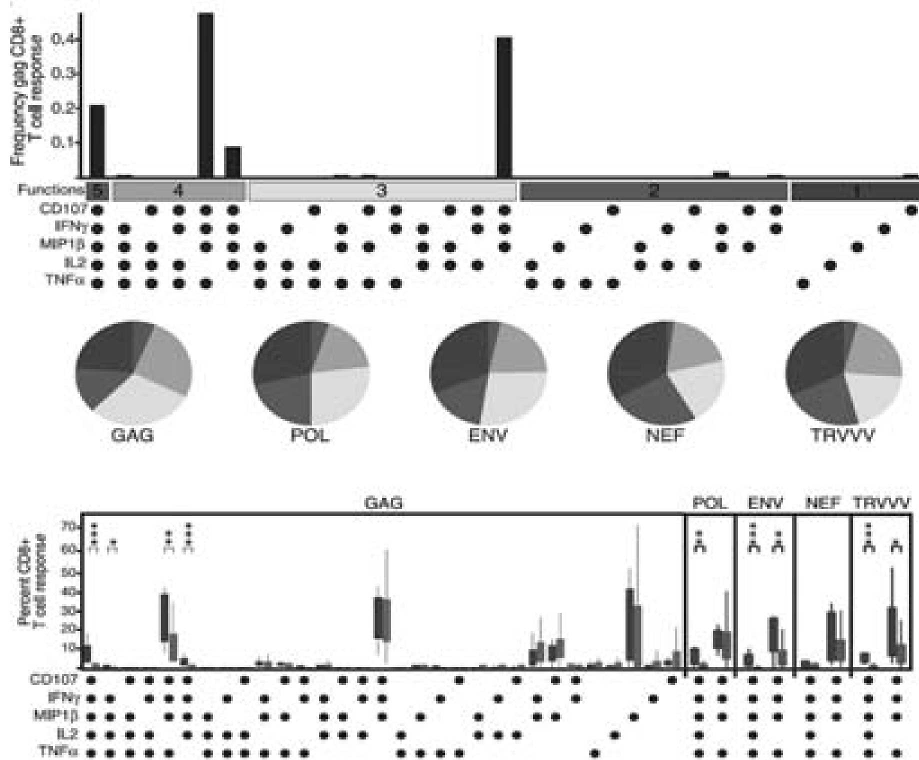

Figure 29. Example of a flow cytometry panel for HIV-specific CD8 T cell polyfunctionality assessment. The first panel shows a particular combination of functions. Each dot denotes CD107a, IFNγ, MIP-1β, IL-2, and/or TNFα positivity. The panel also contains horizontal bars showing those combinations of 5, 4, 3, 2, or 1 function for reference. Below, each pie chart represents the mean response across the individuals to the 5 different HIVantigen stimulations (Gag, Pol, Env, Nef, TRVVV). For simplicity, responses are grouped by number of functions. The box plots in the lower panel represent the 10th, 25th, 75th, and 90th percentiles of the proportion of the respective functional response to the total CD8 T cell response against HIV Gag (left) or other HIV antigens (right panels) in the 2 cohorts assessed (Controller and non-controller individuals) Source: Adapted from Betts, M.R., et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 107, 4781-4789 (2006).

Increased attention has been given to assays for assessing direct *ex vivo* T cell activity for the purpose of evaluating the ability of CTL to inhibit HIV replication. Through these **in-vitro viral inhibition assays** (adapted from the first developed by Yang et al.[139,140]), co-culture of HIV infected target cells and virus-specific CTL or unstimulated CD8 T cells as effectors aim to measure direct viral replication inhibition, either by p24 release reduction measurement by ELISA or flow cytometry.[141,142] Increasing data

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indicate that quantification of direct *ex vivo* CD8 T cell inhibitory capacity may provide a more accurate indication of immune control *in vivo* than ELISpot measurements as it relies on the recognition of endogenously-generated viral peptides within CD4 T cells (targets), in contrast to stimulations with synthethic peptides at high/ non-physiological/saturating concentrations.

However, differences between HIV test sequences from laboratory isolates, the need for HLA class I matched target cell lines, inefficient outgrowth and/ or variable infectivity of autologous CD4 T cells plus the difficulty in screening large number of CTL specificities are some of the limitations that have prevented these assays from being used in previous vaccination trials.[143] Significant improvements on the standardization of these assays are being achieved in order to a) require less input cells, b) shorten incubation periods and c) use flow cytometry to the endpoint p24+ cell reduction measurements, and may in general foster their use as a benchmark of protective immunogenicity in earlier vaccine phase I/II trials in the future, as well as for predicting CD4 T cell loss over time in early HIV-1 infection.[144]

Lastly, significant importance must be given to the <u>use of suitable antigen sets</u> (proteins, peptides, virus). The sensitivity of some of the mentioned assays relies enourmously on the chosen peptide sets, for instance. Some results showing these effects will be developed in the work compiled in Chapter 3.

In summary, HIV infection induces a strong, deep and broad T cell response to the majority of viral proteins with different patterns of dominance. The assays available today for quantifying CTL responses are very sensitive and can detect epitope-specific cells at a frequency of less than 1/2,000 CD8 cells. On the other side, these high-throughput assays may be of limited use because of their single cytokine measurement and complementary, more comprehensive functional read-outs are desirable, especially after the failure of previous assays used in predicting protection in some T cell vaccine trials. However, more efforts on the standardization and evaluation of samples, time, expertise and cost requirements are needed to establish a consensus on its feasible use in large-scale vaccination trials.

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INTRODUCTION-III

Vaccine development

More than 300,000 scientific articles have been published on HIV/AIDS, and we still do not have an effective vaccine

Preventive vaccines have historically been shown to be the most cost-effective tools in controlling the spread of some infectious diseases. including: smallpox, diphtheria, tetanus, yellow fever, pertussis, *Haemophilus influenza* type b disease, poliomyelitis, measles, mumps, rubella, typhoid and rabies. In the case of smallpox, eradication was achieved in 1979 and cases of poliomyelitis have been reduced by 99% thanks to vaccination [145]. In terms of public health, vaccines are effective strategies for various reasons including a) reducing the morbidity and mortality from the disease on a population level, b) granting protection at an individual level, c) limiting transmission to others and ultimately, protecting the community as a whole.

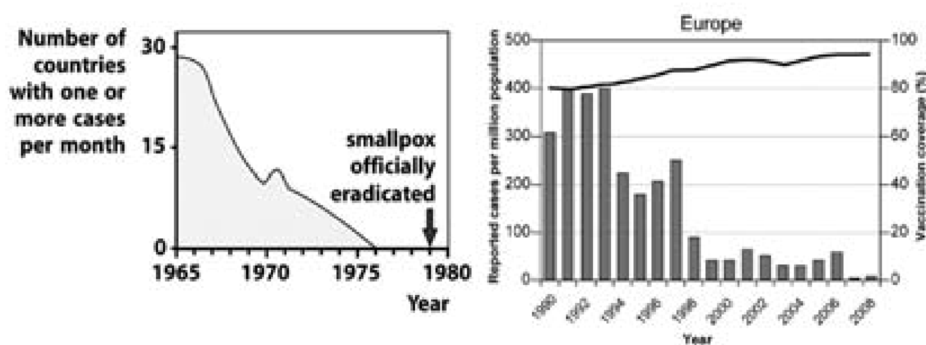

Figure 30. Two examples of the impact of vaccination on the incidence of smallpox and measles. Source: WHO report on measles eradication program 2009

Despite these enormous successes, the generation of vaccine-mediated protection remains a complex challege. Importantly, currently available vaccines have largely been developed empirically, with little or no understanding of how they activate the immune system and what components of the vaccine-induced immunity mediates control from infection. The HIV vaccine field is no exception: In addition to the already-mentioned obstacles posed by the extensive global diversity of HIV-1, there is no data from spontaneuosly cleared infections that could define immune correlates of sterilizing immunity against HIV. In this light, it is not surprising that other clinical endpoints apart from sterilizing immunity would be considered a partial breakthrough. In particular, vaccines may not prevent primary infection, but may decrease the possibility of HIV transmission from an infected person who has been vaccinated to another person and slow down the course of infection when it occurs (mimicking the LTNP or EC outcomes). Thus, even if a vaccinated person became HIV infected, the vaccine would help that person to remain healthier longer and without or with only limited drugs toxicity. A lower transmission rate as well as reductions in treatment and social costs would be still remarkable and desirable.

Sterilizing immunity – Block infection
complete protection from HIV infection
no detectable HIV at any time
no transmission of HIV to others
Transient infection - Clearance
infection occurs, but the immune system is able to detect and kill off infected cells
disease does not advance (the immune system is able to control the infection)
no detectable HIV at a later stage (6-12 months after infection)
seroconversion (becoming HIV+) may or may not occur
transmission to others might occur within a brief window of time and might be completely prevented some time later
Long-term controlled infection (mimicking EC/LTNPs phenotype)
undetectable or very low viral load throughout life
no immunodeficiency disease progression (HIV does not advance to AIDS)
seroconversion (becoming HIV+) likely
transmission to others prevented or greatly diminished

Table 3. List of possible ways a preventive HIV vaccine might work. Adapted from HIV Vaccine Trial Network (HTVN) Vaccine outcomes. Available at: http://www.hvtn.org/science/outcomes.html

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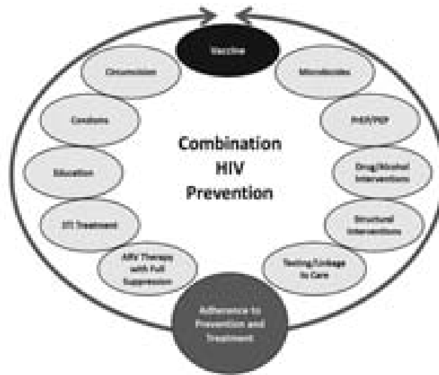

As mentioned in the first part of the introduction, HIV vaccine development is more than ever part of a larger prevention portfolio that also includes behavioral modification, circumcision, pre-exposure prophylaxis, test-and-treat strategies and the use of microbicides. The vaccine field however, still believes that having a successful vaccine candidate is attainable and would represent the most effective tool to contain the HIV burden.

Yet vaccine development is difficult, complex, extremely risky, and costly, especially for HIV. The risk is high since most vaccine candidates fail in preclinical or early clinical development. Such clinical development involves studies to assess vaccine safety, immunogenicity and efficacy through a staged process of serial human clinical trials. Briefly, process development involves making preparations of test vaccine that satisfy regulatory requirements for clinical testing such as clinical lot preparation, preclinical toxicology testing and analytical assessment, and finally, scale-up methods that need to be robust enough to ensure a consistent manufacturing process. Process development can be as costly as clinical development and as the development proceeds toward licensure, costs escalate as clinical studies become larger. Finally, postlicensure studies of safety and vaccine efficacy will also represent a large additional cost.

All clinical, process development tasks as well as future manufacturing must be closely integrated, and in most cases, require potent industrial partners engaged in the process and manufacturing steps. The requirements for the manufacture of vaccines are among the most rigorously designed and monitored, as the final products are administered to millions of perfectly healthy individuals. Every new vaccine will thus be subjected to a well-defined regulatory process for approval, which consists of four main stages:

1) Preparation of pre-clinical materials for proof of concept testing in animal models, manufacture of material according to current Good Manufacturing Practices (cGMP) and toxicology and stability analysis in an appropiate animal system.

2) Submission of investigational new drug application (IND, or PEI in Spanish) for submission to the national regulatory drug agency (FDA, EMEA, AEMPs) for review. In cases where vaccines are generated by genetically-modified organisms, authorisation for voluntary release to the environment is also re-

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quired by most environmental ministries.

3) Testing for safety, immunogenicity and effectiveness through clinical studies (Phase I to III clinical trials)

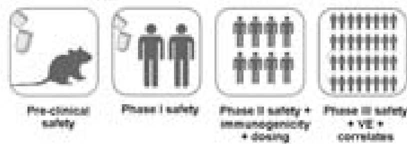
4) Submission of all clinical, non-clinical, and manufacturing data to the drug agency in the form of a Biologics License Application for final review and licensure.

In summary, HIV vaccine development does not only face an enormous scientific challenge, but has encountered and will continue to encounter considerable difficulties in delivering final products that are held to a strict standard of safety while providing sufficient doses at reasonable costs that can improve success rates of combination of behavioral+treatment prevention strategies.

All these steps result in a lengthy process, from the concept to licensure, as illustrated by past timelines for some of the currently licensed vaccines.

Vaccines	Years to Approval
Varicella	25-30
HPV	14-16
Rotavirus	14-16
Pediatric combination vaccines	10-12
HIV	>30?

Table 4. Vaccine development timelines for some of the currently licensed vaccines. Source: Plotkin, Stanley. Vaccines.5th Edition. 2011. Elsevier Press.



Phases of Testing and Clinical Trials

The pre-clinical phase of vaccine development includes in vivo animal testing. These studies are often conducted in mice, rabbits and rhesus macaques for toxicity and vaccine stability studies. Particularly, immunogenicity and efficacy preclinical data generated in non-human primates have served for many years as a gatekeeper for the progression of candidate vaccines to evaluation in a human clinical setting. International vaccine initiatives seem to be changing that view, especially also since the non-human primate model is an expensive approach for comparative immune analyses and of limited value in assessing the protective effect of human-designed vaccine candidates.[146]

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Clinical phase I studies involve a small number of healthy, uninfected participants at low risk of HIV infection, and primarily test the safety of the candidate vaccine. They usually last 12-18 months and generally include less than 100 subjects. .After an initial safety assessment in phase I trials, phase II trials must be adequately powered to define immunogenicity of new constructs. Initial phase II clinical trials must assess laboratory and clinical efficacy and also attempt to define correlates of protection with validated assays. Phase II trials often involve between 300 and 600 persons with varying degrees of risk to acquire HIV infection. Evaluations to define optimal dose and schedules are also conducted at this stage. Phase II trials can last 2 or more years.

Phase III studies have averaged from 2,500 to 10,000 persons per trial and generally involve high-risk volunteers to further assess whether the vaccine works in preventing HIV infection. Phase III trials can last 3-5 or even more years. Multiple phase III trials are needed to assess the protective efficacy of different vaccine concepts against different HIV-1 clades and in populations that may differ on the route of HIV-1 transmission or genetic background.

Implications of PrEP in the design of VE trials

In the coming years, PrEP might be also proposed as part of baseline combination interventions for AIDS vaccine trials, raising some of the following unavoidable challenges in the field [147,148] (and reviewed in [149]):

-higher prevention standard will impact HIV incidence rates, therefore limiting the window for a vaccine effect (VE) on HIV acquisition. It will increase the number of events required and number of subjects enrolled and expand the expected trial duration and with this, increase dropout rates. Partially efficacious vaccine will not have a viable option to be evaluated and the criterion for moving a candidate into phase III testing will in general be even higher. An intrinsic value on high incidence cohorts will develop when it comes to locating the trial sites.

-additional assumptions will have to be made in order to estimate vaccine efficacy in an unbiased fashion within the context of ARVs (control for adherence for instance)

-possibility of complicating endpoint measurements other than VE and immune readouts (lower VL setpoint after HIV acquisitions, delay in identification of acute infections, emergence of fast drug resistances and impact on having valid immune correlates)

-increased complexity in trial implementation: number of visits required to monitor ARVs adherence and HIV testing, safety assessments to control for

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drug adverse events with overall major increases in the cost of the trials.
-interventions such as TDF gels may not be licensed or available in the countries selected to develop the clinical trial, thereby posing potential ethical dilemmas for those who must pay for the intervention.

Considering this scenario within which future HIV vaccine trials will likely be conducted (larger, longer and costlier trials), increased emphasis on adaptive designs[150] is urgently needed. Adaptive clinical trial designs can accelerate vaccine development by rapidly screening out poor vaccines while extending the evaluation of efficacious ones, improving the characterization of promising vaccine candidates and the identification of correlates of immune protection. The main goal is thus to significantly shorten the timeline of HIV vaccine efficacy trials and accelerate vaccine development.

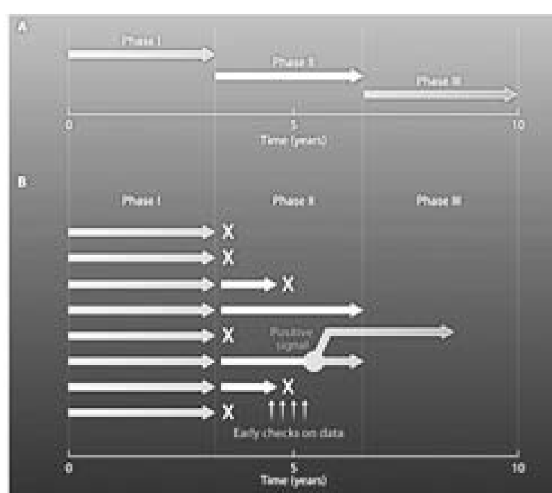

Figure 31. Adaptive trial designs accelerate vaccine development. (A) The traditional approach to testing vaccine efficacy is iterative testing in phase I, II, and III clinical trials. (B) In contrast, adaptive trial designs enable real-time analysis of immunogenicity and vaccine efficacy. This information can be used to make a decision to proceed to phase III efficacy trials much sooner. The ability to run multiple trials in parallel and focus on an optimal vaccine candidate could save considerable time by avoiding multiple iterations of the phase I, II, and III testing cycle. Source: Corey, L., G. J. Nabel, C. Dieffenbach, P. Gilbert, B. F. Haynes, M. Johnston, J. Kublin, H. C. Lane, G. Pantaleo, L. J. Picker, and A. S. Fauci. 2011. HIV-1 vaccines and adaptive trial designs. Sci Transl Med 3:79ps13.

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Candidate HIV vaccines

Having an effective HV vaccine still remains one of the most important scientific challenges immunologists have ever faced. Its major hurdles stem from the high diversity of the virus, its fast ability to evade immune pressure and its capacity of early integration into the host's genome, thus establishing a life-long latent infection.

Based on today's knowledge, HIV vaccine development can be divided into a) the selection of appropiate viral targets (immunogens) able to induce a neutralizing humoral response along with an effective cellular immunity, b) the insertion of the immunogens in safe vectors as delivery systems and c) the use of (different) vaccine candidates in the most effective combinations to induce long-lasting immunity at the possible sites of initial infection -genital and rectal mucosa surfaces . In the following section we will discuss the major vaccine candidates that have been developed for HIV to date:

A) Classical vaccine strategies based on attenuated live virus or whole inactivated virus have severe safety limitations. Firstly, due to the concern that inactivation of virion preparations may not be able to completely inactivate all virions, particularly when viral aggregates occur. Secondly, HIV production would need to occur in human transformed cell lines not considered acceptable from a regulatory prespective.[151] The first live, attenuated vaccines were developed in macaques, where SIVmac239Δ-nef efficiently protected rhesus macaques against infection with wild-type SIVmac.[152] Despite some success, some macaques presented CD4 T-cell depletion and progression to simian AIDS, most likely due to the fact that SIV with a deletion in the nef gene was able to revert to its initial virulence.

Surprisingly, this last December 2011, a Korean biotech company in partnership with Canadian researchers, announced FDA approval for the clinical testing of a genetically-modified killed whole virus, but no other technical details were released about the inactivation of the biologicals.

B) An alternative to whole inactivated virus is to use pseudovirions (virus-like particles, VLPs). VLPs are composed of an outer shell that does not contain a viral genome while closely mimicking a native configuration of authentic virions. Such VLPs are thus not self-replicating but maintain the potential to elicit both humoral and cell-mediated immune responses. VLPs have been employed in the production of several licensed human vaccines,

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such as vaccines for hepatitis B and human papillomavirus. The ones in development for HIV usually include Gag and Env structural proteins and generally expose subunit gp120 molecules on their surface. Some constraints of VLPs include the limited size of the insert they can hold and their inability to mimic complex conformations. A way of overcoming such limitations is to try to induce antibodies to shorter conserved envelope regions, such as the MPER gp41 region. Recently, some investigators have designed modified VLPs exhibiting reactivity to monoclonal antibodies 2F5 and 4E10 and showed HIV neutralizing activity in the serum of immunized guinea pigs.[153]

C) Recombinant proteins were the first HIV vaccine candidates that scaled up into clinical testing in 1987. Examples of recombinant subunit proteins are gp120, gp140, or gp160 produced by genetic engineering. Recombinant soluble HIV envelope glycoproteins have been the target of intense research aiming to elicit broadly reactive neutralizing antibodies (Nab) by active immunization with an alternating interest over the 'short' history of HIV vaccinology. The failure of the first antibody-based vaccines[154] led to an almost exclusive focus on T cell–based vaccines, and afterwards the STEP T cell–based vaccine failure swang back again to nAb activities.[155] In addition, the modest protection reported in the Thai trial[156] apparently correlated with the induction of binding antibodies to scaffolded gp70-V1V2 antibodies further reinforced this view. In addition, recent discoveries of broadly neutralizing antibodies detected in few infected humans[157,158] have been a greatly attractive finding, again promoting scientific interest in humoral immunity, although how to induce such responses with a vaccine is not yet clear.[159] Vaccines that a) present the epitopes described capable of neutralizing a wide array of virus strains to the immune system in b) a conformationally-precise manner, may elicit such antibodies and provide a high level of protection from HIV infection. To produce candidates that mimic the mature envelope trimer on the virion surface seems to be one of its major hurdles. Also, eliciting nAb with enough high avidity to the highly conserved regions of the HIV envelope is more complex than simply presenting the desired envelope epitope to the immune system.

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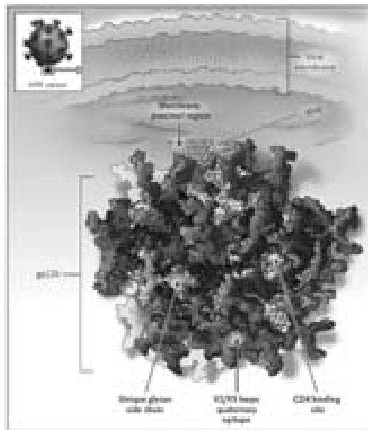

Figure 32 **.** Epitopes Targeted by Broadly Neutralizing Human Monoclonal Antibodies.
The HIV envelope proteins consists of a gp41 transmembrane portion and a gp120 protein spike. Below the virion is an enlarged depiction of the viral membrane and the HIV envelope protein. The arrows indicate four epitopes of the envelope that are the targets of human monoclonal antibodies. Source: Adapted from a figure provided by William Schief, University of Washington. Johnston, M.I. & Fauci, A.S. HIV vaccine development--improving on natural immunity. N Engl J Med 365, 873-875 (2011).

Some strategies try to present the selected epitopes more effectively than wild type virus, by the deletion of variable loops and the elimination of carbohydrate attachment sites or hyperglycosylation to mask unwarranted epitopes.[160,161] Others try to produce epitope-mimics of the binding sites of broad Nab determined by structural studies of the antigen-antibody complexes.[162-164]

D) One of the most widely-used types of experimental HIV vaccines include those generally classified as live recombinant vector vaccines (reviewed in [165]) These include a broad array of non-HIV live attenuated viruses (or bacteria) engineered to carry genes encoding HIV antigens (full proteins or synthetic polypeptide immunogens). One of their major attractive features is the ability to stimulate both humoral and cell-mediated immunity following the general vaccinology principle that 'infection' with a vector induces more robust cellular responses than immunization with proteins or particles.

Live Recombinant Vaccines	Examples
Vaccinia virus	MVA (modified Ankara virus), NYVAC (Copenhagen vaccine strain)
Canarypox	ALVAC
Adenovirus	Ad5, Ad26, ChAd
Paramyxoviruses	Sendai, Measles
Rhabdoviruses	VSV (vesicular stomatitis virus), rabies
Herpesvirus	CMV (Cytomegalovirus)
Mycobacterium bovis	BCG (bacillus Calmette Guerin)
Bacteria	Salmonella
Alphavirus	VEE (Venezuelan equine encephalitis virus)
Parvovirus	Adeno-associated virus

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Vaccinia virus vectors expresing a variety of HIV and SIV genes have been tested in animals and humans. Safety concerns surrounding the use of replication competent vaccinia drew attention to poxviruses that replicate poorly in mammalian cells, such as MVAs -Modified Vaccinia virus Ankara- and the modified Copenhagen NYVAC strain. Avian poxviruses, which are themselves replication-incompetent in mammalian cells, are also an alternative, but are therefore less immunogenic. In fact, canarypox are the only live-vectored vaccines that have reached phase III clinical testing for HIV. Although in previous preventive and therapeutical phase II vaccine trials ALVAC-HIV-recombinant canarypox vaccines did not show any promising results in either preventing infection or controlling viral loads upon treatment interruptions[166], the ALVAC vCP1521 (Env/Gag/Pro) was used as a prime along with a gp120 AIDSVAX boost in the phase III RV144 Thai trial. [156]

MVA is a vaccinia virus strain attenuated by serial passage in chick embryo fibroblasts (CEF). It has lost 15% of its parental genome, including cytokine receptor genes. It replicates well in CEF and baby hamster cells but poorly in most mammalian cells.[167] It was successfully used as a smallpox vaccine in the early 1970s towards the end of the eradication campaign in nearly 120,000 people without any serious adverse events. MVA has been shown to be effective as a vaccine vector for HIV antigens by inducing potent CD8+ T cell responses to passenger proteins, especially when used as a booster element in the vaccine regimen. A further derivative, the NYVAC strain, was originally specifically produced by deleting 16 open reading frames from vaccinia virus to achieve an extreme attenuation in mammalian cells. Recently, investigators have further modified it to increase its immunogenicity while maintaining its highly attenuated phenotype by the re-incorporation into the virus genome of two host range genes, K1L and C7L, in conjunction with the removal of the immunomodulatory viral molecule B19, an antagonist with a type I interferon effect. These novel vectors are referred to as NYVAC-C-KC and NYVAC-C-KC-ΔB19R)[168,169]

Adenoviruses are medium-sized (90–100 nm), nonenveloped viruses that offer great advantages as vaccine vectors: They can be administered orally releasing the virus in the intestine, or intranasally inducing both systemic and mucosal immunity. Attenuated adenoviruses have repeateadly been shown to be highly immunogenic vaccine vectors[170] A drawback with human Ad5 recombinant vaccines is the prevalence of pre-existing anti-Ad5 neutralizing antibodies in the general population (with seroprevalences as high as 90% in several regions of Africa). This may not only dampen the response to the vaccine, as shown in the STEP trial[171] (see below) but also limit repeated boosting. In that context, human adenoviruses from other serotypes with less seroprevalence of Ab (such

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as Ad26, Ad35) or chimpanzee adenovirus are currently being explored as improved vectors for human use, as these would be less affected by pre-existing antibodies (www.clinicaltrials.org NCT01215149 and NCT01151319 respectively)

Replication defective alphavirus 'replicons' (i.e. VEEV, from Venezuelan equine encephalitis virus) [172,173] are attractive vectors because of the enormous amplification of the viral message that occurs after infection. Vector virion 'replicons' are completely defective for replication and are produced by packaging the vaccine gene inside the vector virion coat. By deleting most of the viral genes from the packaged DNA the coding capacity of the replicon for vaccine antigens is significantly increased. Similar to the defective poxviruses, the genes delivered by these systems are still remarkably immunogenic, despite the lack of a replication cycle. Importantly, these viruses have the potential to target DCs, resulting in efficacious antigen presentation. As with whole inactivation virus approaches, a major limitation is that they require transformed cells for the packaging system which complicates their regulatory approval for human use.

Promising data were reported last year by investigators at the Californian Institute of Technology showing the secretion of broadly-neutralizing antibodies through vector-mediated gene transfer, called 'vectored immunoprohylaxis' or VIP using humanized mice.[174,175] VIP uses the modified adeno-associated virus (AAV) with alternative capsides (from serotype 8) that express Nab such as 4E19, b12, 2G12 or 2F5 driven by cytomegalovirus (CMV) promoters. AAVs are small (20 nm), replication-defective, nonenveloped viruses that infect humans and some other primate species without any known pathogenicity, making them attractive candidates for gene therapy strategies. Vectors using AAV can infect both dividing and non-dividing cells and persist in an extrachromosomal state without integrating into the genome of the host cell. Apparently AAVs present very low immunogenicity, seemingly restricted to the generation of neutralizing antibodies, while they induce no clearly defined cytotoxic response. At the present time there are several clinical trials using AAV-based vectors in a diverse range of diseases such as Cystic Fibrosis, Alzheimer, Muscular Distrophy, Parkinson, Hemophilia B, etc.

Lastly, live recombinant bacterial vaccines have been developed as vectors using baccilus Calmette-Guérin (BCG)[176,177] and *Salmonella* strains that have been attenuated by mutagenesis of genes involved in virulence and invasiveness.[178]

E) Naked DNA vaccines. Injection of purified plasmid DNA that carry a gene encoding an antigen under the control of an appropiate mammalian transcription promoter into the muscle or the epidermis leads to the expression of the antigen in situ and triggers an immune response, mostly the Th1 type. Such DNA

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vaccination has great potential due to its safety, versatility, and feasible scalability. DNA vaccines can be administered repeatedly without generating immunity against the vector itself. For many years HIV DNA vaccines have been considered as having relatively low immunogenicity in comparison to viral vectored-vaccines. Significant advances have been achieved by including expression-optimized antigens as well as improving their delivery methods by intramuscular electroporation.[179] RNA/codon optimization aims to enhance antigen expression and is based on introducing multiple nucleotide changes to destroy identified RNA processing, inhibitory and instability sequences in the mRNA without affecting the encoded protein sequence.[180] This process can also include the elimination of predicted splice sites from coding sequences by the appropriate codon changes, thereby minimizing the possibility of adverse immunogen splicing. DNA delivery by in vivo electroporation significantly increases the uptake and immunogenicity of DNA vaccines[181,182]; it has already been shown in the macaque model and it is currently being tested in human trials[183].

One of the few ongoing phase IIb clinical trials at present time is the HVTN 505, which tests a regimen including a DNA prime. HTVN 505 was delayed and redesigned after the announcement of STEP failure from its initial PAVE 100 design for evaluating the protective efficacy of a DNAprime/rAd5 boost vaccine expressing Gag, Pol, and three Env antigens. The trial was required to exclude individuals with Ad5 nAb titer above 1:18 and only circumcised men and male-to-female transgender persons could be included. A second amendment was implemented in September 2011. Initially designed to only determine differences in viral load setpoints among placebo recipients who become HIV infected versus those who received vaccination, it was planned to recruit 1,350 volunteers. The goal of the second amendment was to add protection against infection as an additional primary endpoint (VE à Phase IIb). As a consequence, the trial now requires the recruiting of a total of 2,200 individuals. (www.clinicaltrials.org NCT00865566)

Lastly, DNA plasmids are now being also used to express cytokines, as in the IAVI sponsored Phase I B004 trial, where a multiantigen plasmid DNA is co-administered with recombinant human IL-12 pDNA followed by recombinant Ad35 in HIV uninfected, low-risk, healthy volunteers. (www.clinicaltrials.org NCT01496989)

Vaccine combinations: Non-replicating genetic vaccines such as live recombinant viruses deliver the immunogen to the MHC class I presentation pathway either directly or by cross-presentation[184] without producing infectious progeny.[185] Their lower immunogenicity cannot be enhanced by repeated boosts with

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the same vaccine because of a build-up of anti-vector immunity, which dampens insert-specific T cell induction. For this reason, heterologous prime-boost regimens are commonly used. Several combinations have been used so far and include, among others prime-boost combinations of pDNA/MVA[186,187], pDNA/Ad (HVTN505) [188], Ad/MVA[189,190], Canarypox/recombinant protein[156], etc.

The Berlin case: a path forward to gene therapy?

> A case of a successful hematopoietic stem cell transplantation (SCT) in an HIV-1-infected patient suffering from acute myeloid leukemia transferring donor-derived cells from an homozygous for the CCR5-delta32 allele donor, without rebounding of HIV plasma viral load despite cessation of HAART was announced in 2009. Named as the 'Berlin patient' it ressembled the first 'real' cure ever reported. After 4 years of the first SCT, the patient is still not receiving therapy and no viral load or proviral DNA has been detected in peripheral blood cells and different tissue samples, including gut, liver, and brain. As expected, controversy remains open as to whether the patient has achieved complete eradication of HIV or not, but, potential implications and future directions of stem cell-targeted HIV treatments are started to be discussed among scientists, especially representatives from bone marrow donor registries with a view to supporting further attempts to use CCR5-delta32 deleted stem cells for treating probable HIV-1-positive patients with malignancies. [191-193]

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1987 : First preventive HIV vaccine trial testing a gp160 subunit
...more than 40 vaccines have been evaluated in Phase I and Phase II trials
...more than 40,000 HIV negative volunteers have participated in CT
...2 Phase IIb trials were terminated prematurely in 2007
...only 3 Phase III trials have been completed
...only 1 demonstrated a modest efficacy
2012 ??

It is beyond the scope of this introduction to give a detailed description of all the past clinical trials that have tested some of the more than 40 vaccine candidates developed over the last 25 years of HIV vaccine research. Available databases of AIDS vaccine candidates scaled up into clinical trials include IAVI's (International AIDS Vaccine Initiative, www.iavireport.org), HVTN's (HIV Vaccine Trials Network, www.hvtn.org) or the NIH registry of clinical trials (US National Institute of Health, www.clinicaltrials.gov) among others. In addition to preventive vaccine trials, some vaccine candidates have also been tested (alone or in combination with other immunomodulators) such as therapeutic vaccines in already HIV-infected individuals aiming to boost or redirect preexisting immunity against HIV to limit their need of treatment, increase CD4 cell counts and retard disease progression. Candidate vaccines have been tested in the context of HAART or in naive individuals, and in chronic disease stages as well as in early/acute phases, with similar disappointing outcomes. It is worthy of note that most therapeutic vaccine trials conducted in the past have used structured treatment interruptions (STI) to assess the efficacy of tested vaccines, using containment of the viral rebound or CD4 decay upon treatment cessation as the primary trial endpoints. However, after data from clinical trials using STI revealed a striking increase in mortality and non-AIDS associated events[46], treatment interruptions are either planned using very stringent immune and virological criteria or are not allowed at all until better surrogate markers of HIV control from vaccine-induced responses are available. Some investigators, in this context, have moved from so-called therapeutic vaccines to 'eradication' strategies, assessing, in these cases, the reduction of the viral reservoirs as surrogate markers of vaccine efficacy (as in the ongoing ERAMUNE 01 and 02 trials)

In the following section some of the most insightful vaccine trials in the field are listed. The implications of the Manon 02 and STEP failures as well as the modest efficacy of the Thai Trial are discussed in more detail in relation to our own vaccine work.

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Phase	Trial ID	Strategy	Candidate	n
I/II	ACTG 326; PACTG 326	Viral Vector - Pox/Protein	ALVAC vCP1452/AIDSVAX B/B	48
I/II	C060301	DNA	GTU-MultiHIV	28
I/II	EV03/ANRSVAC20	DNA/Viral Vector - Pox	DNA-C/NYVAC-C	147
I/II	F4/AS01	Protein	F4/AS01	
I/II	HVTN 042 / ANRS VAC 19	Viral Vector - Pox/Protein	ALVAC vCP1452/LIPO-5	174
I/II	IAVI 006	DNA/Viral Vector - Pox	DNA.HIVA /MVA.HIVA	120
I/II	RV 132	Viral Vector - Pox/Protein/Protein	ALVAC-HIV vCP1521/gp160 THO23/LAI-DID/rgp120/HIV-1 SF-2	120
I/II	RV 135	Viral Vector - Pox/Protein	ALVAC-HIV vCP1521/gp120 C4-V3	120
I/II	RV 172	DNA/Viral Vector - Adeno	VRC-HIVDNA016-00-VP/VRC-HIVADV014-00-VP	326
I/II	UBI HIV-1MN octameric - Australia study	Protein	UBI HIV-1 Peptide Immunogen, Multivalent	24
I/II	UBI V106	Protein	UBI HIV-1 Peptide Vaccine, Microparticulate Monovalent	24
I/II	VAX 002	Protein/Protein	AIDSVAX B/B/AIDSVAX B/E	120

Phase	Trial ID	Strategy	Candidate	n
II	ANRS VAC 18	Protein	LIPO-5	156
II	AVEG 201	Protein/Protein	rgp120/HIV-1 SF-2/MN rgp120	296
II	AVEG 202/HIVNET 014	Viral Vector - Pox/Protein	ALVAC-HIV MN120TMG strain (vCP205)/rgp120/HIV-1 SF-2	420
II	CM235gp120 and SF2gp120	Protein/Protein	CM235 (ThaiE) gp120 plus SF2(B) gp120/rgp120/HIV-1 SF-2	368
II	HIVNET 026	Viral Vector - Pox/Protein	ALVAC vCP1452/MN rgp120	200
IIa	HVTN 068	Viral Vector - Adeno/Viral Vector - Adeno	VRC-HIVADV014-00-VP/VRC-HIVADV014-00-VP	66
II	HVTN 203	Viral Vector - Pox/DNA	ALVAC vCP1452/AIDSVAX B/B	330
IIa	IAVI 010	DNA/Viral Vector - Pox	DNA.HIVA /MVA.HIVA	115
II	IAVI A002	Viral Vector - Adeno-associated Virus	tgAAC09	91

Phase	Trial ID	Strategy	Candidate	n
III	RV 144	Viral Vector - Pox/Protein	ALVAC-HIV vCP1521/AIDSVAX gp120 B/E	16,403
III	VAX 003	Protein	AIDSVAX B/E	2,500
III	VAX 004	Protein	AIDSVAX B/B	5,400

Phase	SUSPENDED Trial ID	Strategy	Candidate	n targeted
IIb	HVTN 502/Merck 023 (STEP)	Viral Vector - Adeno	MRKAd5 HIV-1 gag/pol/nef	3,000
IIb	HVTN 503 (Phambili)	Viral Vector - Adeno	MRKAd5 HIV-1 gag/pol/nef	3,000

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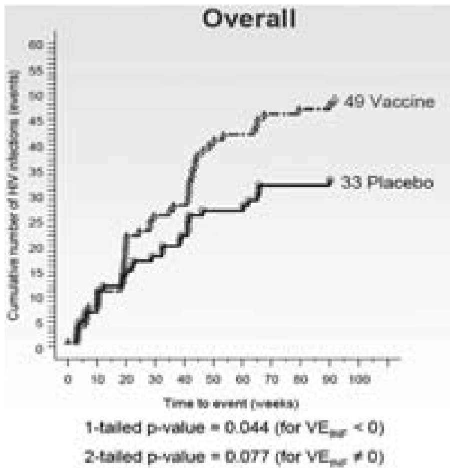
From the pessimism after STEP and Manon02 trials failures...



The STEP trial was a phase IIb test-of-concept trial that aimed to recruit 3,000 volunteers to test the efficacy of Merck's Ad5-Gag/Pol/Nef vaccine in US, Peru, Puerto Rico, Haiti, Dominican Republic, Canada, Australia and in a related trial in South Africa (Phambili trial).[155,194] Immunizations were interrupted in October 2007 after the first interim analysis by the Data Safety Monitoring Board (DSMB) showed pre-determined nonefficacy boundaries: 2,300 individuals had been recruited at that moment, and there was no reduction in the number of HIV acquisitions or decreased early plasma viral load in vaccinees compared with placebo recipients respectively. In fact, post-hoc analyses of men enrolled showed a larger number of HIV infections in the subgroup of vaccinated men who were Ad5-seropositive and uncircumcised compared with a comparable placebo group.[195] Based on that data, both STEP and Phambili trials were unblinded, and participants were informed of their treatment allocation. Long-term follow up of infected patients in both arms did not show any slower disease progression in the vaccinated group either.[196]

Merck's Ad5-Gag/Pol/Nef was however immunogenic, in ELISpot and ICS assays used in the trial[155,197]. In fact, it showed strong immunogenicity, as it had shown in pre-clinical studies in macaques, thus highlighting the need for more reliable immunological correlates of virus control and protection.[135] The increased number of HIV infections in vaccinees associated with higher baseline Ad5 nAb titers was unexpected and had not been predicted by prior preclinical studies. Importantly, the potential enhancement of HIV-1 acquisition appeared to diminish over time. Frahm et al have analyzed samples from the STEP and HVTN 071 trials using the same Ad5 vaccine candidate, showing in both trials that higher frequencies of pre-immunization adenovirus-specific CD4 T cells were associated with the substantially-decreased magnitude of HIV-specific CD4 T cell

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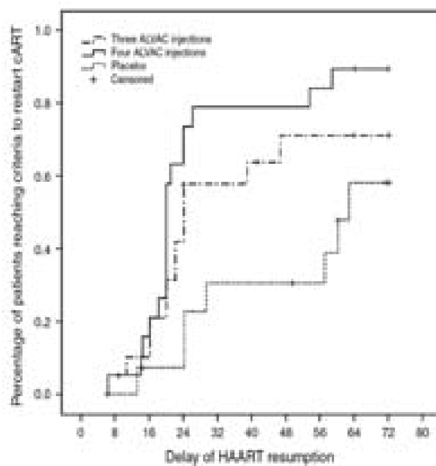
responses and decreased breadth of HIV-specific CD8 T cell responses in vaccine recipients, independent of type-specific preexisting Ad5-specific neutralizing antibody titers. A cause of greater concern was that the epitopes recognized by adenovirus-specific T cells seemed to be very well conserved across many other adenovirus serotypes, including those in the present vaccine pipeline.[171] This may have serious implications in the use of adenoviruses of different serotypes in inducing robust T cell immunity.

As expected, the release of negative STEP results increased global scientific skepticism about the feasibility of ever having an effective HIV vaccine. While other phase IIb trials that were about to start were stopped and/or delayed to be redesigned, adapting them in accordance with the STEP findings, a shift in funding towards more basic aspects of HIV vaccine research was proposed. Some investigators also saw the STEP trial failure as a concept failure of T cell based vaccines and this led to an increase in support for vaccine studies aimed at inducing humoral immunity



This skepticism was not diminished one year later when the results of the Manon02 phase II therapeutical trial were published.[166] An ALVAC-HIV-recombinant canarypox T cell-based vaccine (vCP1452) was tested in a multicentre, randomized, placebo-controlled trial in 66 HIV-infected individuals that were under supressive HAART. Four weeks after the last of 3 or 4 vaccinations of the cararypox-vectored vaccine, patients underwent an STI for 24 weeks. HAART was reestarted earlier in cases where viral loads rebounded to >50,000 copies/mL and/or or CD4 dropped below 250 or 50% from its starting value. Despite favourable immunogenicity achieved in the 4-injection vaccinations arm, higher viral loads were seen in vaccinees compared to placebo recipients after STI and the percentage of patients reinitiating HAART at the end of study was 75% and 48% (in the 4 and 3 injections arm) compared to 23% of patients among the placebo recipients. Post-hoc analyses were conducted with the objective of understanding the shorter time for resuming

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therapy and the higher viral rebounds in vaccinees. Investigators suggested that the vaccinations might have facilitated HIV replication through the amplification of virus niches by creating a 'predator–prey phenomenon'. By generating activated vaccine-specific CD4 cells directed either against the HIV vaccine sequences or against the vector, they might have acted as ideal targets for the virus. In addition, interruptions were made – maybe too early - (4 weeks) after the last immunizations and might have coincided with the peak of the vaccine-induced immune activation, where canarypox vaccine had predominantely stimulated CD4 T cells without/ few HIV-specific CD8 T cell responses.[198]

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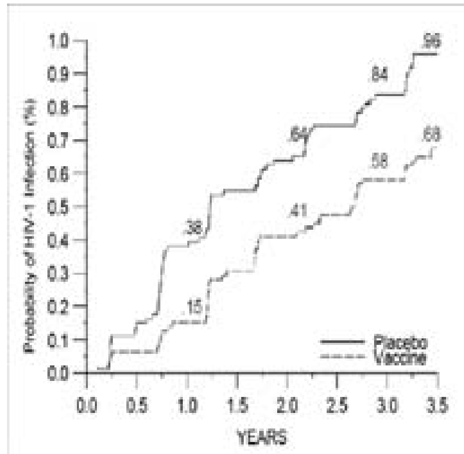
…to 'post-Thai' hope



This past year some encouraging news regarding T cell vaccines were released. First, sieve effects were demonstrated in a post-hoc analysis in volunteers who had been vaccinated in the STEP trial but acquired infection. Breakthrough infections in vaccinees harboured sequences significantly different from those matching the sequences contained in the Ad5-Gag present in the vaccine mixture, meaning that the vaccines 'did work' somehow by preventing infection to few viruses in individuals which the vaccine elicited a cellular response.[199] It is still however unclear whether this "sieve-effect" was mainly driven by blocking the out-growth of HIV variants that were most similar to the vaccine and/or whether the vaccine induced an immune response that shaped a specific viral evolution. Secondly, more recently vaccine strategies to induce effector memory T cell responses against SIV demonstrated the most promising results to date achieved by vaccination, with a rhesus cytomegalovirus (rhCMV) vectored vaccine that was able to control infection in 50% of the vaccinated animals, even in subsequent viral rebounds.[121]

What has most reinforced the field to resume the pursuit of combinational strategies of both cellular and humoral-based vaccine regimens were the results of the RV-144 study in Thailand. It is the first Phase III trial to demonstrate a modest efficacy in preventing HIV infection. The vaccine scheduled included 4 injections of an ALVAC vCP1521 prime (canarypox Env/Gag/Pro) plus 2 injections of an AIDSVAX boost using clade B/E gp120 over a 6-month immunization period. It recruited >16,000 individuals at low risk of HIV infection, and follow-up lasted 3 years until the efficacy of a 31% reduction in new infections (51 infections) compared to the placebo group (74) was shown in the modified intention to treat analyses (mITT).[156] Less positive results included: a) no decrease in viral loads in the individuals who got infected, b) the efficacy waned over time (the reduction of infections was more important during the first year of vaccinations) and c) it was more remarkable in people with lower risk for acquiring HIV. Post-hoc complex case-control analyses to discover the correlates

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of risk and surrogate markers of protection in the vaccinees started soon after the results were launched and involved several well-known international laboratories. One year later, of the six primary immunological variables selected to be studied, only the level of IgG antibodies that bind to scaffolded-V1V2 recombinant protein correlated INVERSELY with infection rates (meaning → Higher V1V2, 43% Lower infection rate) and the Env binding plasma IgA levels correlated DIRECTLY with infection rates (→ Higher IgA to Env, 54% Higher infection rate) (Barton Haynes, AIDS vaccine 2011, Bangkok). It is worth mentioning that neither of these two variables were significant in the multivariate analyses that included:

1) IgA binding to envelope panel	(RR 1.54, p=0.027, Q=0.08)
2) IgG avidity to A244 gp120	(RR 0.92, p=0.37, Q=0.56)
3) ADCC AE.HIV-1infected CD4 cells	(RR 0.92, p=0.68, Q=0.68)
4) Tier 1 neutralizing antibodies	(RR 1.37, p=0.22, Q=0.45)
5) IgG Binding to gp70-V1V2	(RR 0.57, p=0.015, Q=0.08)
6) CD4 T cell Intracellular cytokines	(RR 1.09, p=0.61, Q=0.68)

A potential explanation proposed for these obervations was that monomeric IgA could have blocked IgG from binding to HIV-1 Env on infected cells, preventing a more effective IgG-mediated effector function. Cellular responses were also detected in the Thai trial, but were mainly directed at Env protein, which from our analyses would possibly not mediate effective antiviral immunity. In particular, the low immunogenicity seen towards Gag could be based on having induced cellular immune responses to variable targets (such as Env in RV144 and Nef in STEP) that could have diverted the response away from more protective responses.

Despite many questions on correlates of risk and surrogates of protection in the Thai trial effectiveness remain unanswered, a new private-public consortium has been created aiming to promote research into and scaling up of pox-vectored vaccines. Named P5, (Pox Protein Public Private Partnership), it embraces the participation of NIAID, the Bill&Melinda Gates Foundation, HVTN, MHRP and Sanofi Pasteur to enhance the vaccine efficacy of pox-candidates. The following milestones are included in the roadmap of this initiative:

1) Re-analyzing of previous trials with the same vaccine candidates as those used in RV144: Phase III trials VAX003 (MSM in USA) and VAX004 (IDU in Thailand) in order to search for the presence of the same correlates of risk found in RV144. Although both trials showed no protection against HIV infection,

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the populations differed greatly from the RV144 volunteers—in terms of both the mode of transmission and the level of risk.

2) New non-human primates studies to assess whether the administration of antibodies of the type that were elicited by the RV144 vaccine regimen are capable of protecting monkeys against infection.

3) Thai trial RV306, with an estimated 300 participants who will receive similar vaccinations as those in RV144, PLUS an additional booster immunization at 12 months to assess whether the waning protective effect can be improved over time.

4) New Phase IIb trial (expected in 2014) testing the same vaccination schedule BUT in high risk MSM population in Thailand and South Africa (estimated n=8,000 participants) to determine whether the protective effect observed in the RV144 is translatable to high risk populations.

Apart from this programme focused on pox-vaccines, many other groups are working intensively on the development of novel and refined HIV-1 vaccine candidates, particularly to contend the extensive viral diversity.[200] Different approaches to improving the cross-reactive potential of vaccine-elicited cellular immune responses include the use of ancestral strains[201], creating 'mosaics' as multiple antigen cocktails[202] of antigens or designing immunogens based on conserved regions.[203,204] Our own work is very much related to this and focussed on new strategies for handling the issue of viral diversity and how to translate these findings into a rational HIV vaccine immunogen design. In the following pages, the three articles compiled in the thesis aim to better characterize the HIV specific T cell responses, the mechanisms by which they mediate viral control (including conservation, functional avidity and cross-reactivity) and how we have incorporated this information into a reductionistic HIV vaccine design.

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HYPOTHESIS

1) Natural control of HIV infection is mediated by the interaction of host's genetic, viral factors and the adaptative immunological reponse.

2) Viral targets of HIV-1-specific T-cell responses with potent antiviral activity can be identified along the proteome.

3) HIV-1 infected individuals with better control of viral replication have a broader HIV-specific cytotoxic T cell response against conserved regions.

4) Functional properties of HIV-1 specific cytotoxic T cell responses particularly functional avidity and cross-reactivity play a crucial role in the viral control of HIV.

5) A reductionistic polypeptide T cell immunogen could potentially break immunodominance to non-beneficial targets and focus the immune response on selected protective specifities.

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CHAPTER 1

Virological, Immune and host genetics markers in the control of hiv infection

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Dis Markers. 2009;(3):105-20. Review.

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ABSTRACT

HIV infection, if left untreated, leads in most cases to the development of wide immune deterioration, opportunistic infections and eventually AIDS and death. The identification of individuals who despite persisting infection show no or few signs of HIV disease progression has spurred great hopes that an effective HIV vaccine could be attainable. The design of such a vaccine will greatly depend on the precise definition of disease markers, host genetic and immune characteristics that mediate relative *in vivo* control of this virus. Accordingly, a number of viral factors and host genetic characteristics have been shown to play a crucial role in the control of HIV disease by delaying progression to AIDS or even preventing infection. There is also an improved understanding of humoral and cellular immune responses in terms of specificity, functional repertoire, longevity and tissue distribution and their ability to contain HIV replication. However, the definition of good immune correlates unequivocally and causally associated with protection or disease progression remains elusive. Here we review work on viral factors, host genetic markers and immunological determinants that have been identified in individuals with superior control of HIV infection or in subjects who remain uninfected despite clear exposure to the viral pathogen.

Keywords: HIV-1, long-term non-progressors (LTNP), Elite Controllers (EC), Highly exposed persistently seronegatives (HEPS), CCR5, CTL, Innate immunity, HIV control, immune correlates, HLA allele frequency

INTRODUCTION

The AIDS pandemic is one of the greatest global health crises of our time. Since HIV was identified 25 years ago, 23 million people have already died and 33 million more are living with HIV (1) Despite advances in education, HIV prevention and improvements in access to antiretroviral drugs, the pandemic continues to outpace global efforts at prevention and control. According to the United Nations Development Program (UNDP), HIV has inflicted the "single greatest reversal in human development" in modern history (2).

AIDS vaccine research and development is of highest priority today and, at the same time, one of the biggest scientific challenges the immunology field faces. The enormous viral variability together with human host genetic diversity throughout the world are primary factors hampering the development of efficient strategies to control and prevent HIV infection and the design of potentially effective immune-based therapeutic interventions and prophylactic vaccines. The recent failure in 2007 of a phase III trial of a prophylactic vaccine candidate further highlighted how far the field is still away from reliable markers of HIV control and how to best design an effective vaccine. Thus, an improved understanding of the immunopathogenesis of HIV infection and the role of host genetic markers and viral diversity in this control is urgently needed. However, it will likely not be sufficient to link certain more or less random markers with clinically well-established parameters of disease progression, such as plasma viral RNA levels and nadir CD4+ T-cell count or CD4+ T-cell count decline over time. Rather, specific markers that are directly mediating viral control need to be identified so that vaccine design is not misled by focusing on epiphenomena and functionally unlinked markers. This is certainly easier said than done as determinants of an effective viral control will likely need to be identified in multifactorial models that comprise viral variability, environmental particularities (such as co-infections) and host genetics.

Here we review the role of viral factors, host genetics markers and HIV specific immune responses in the control of HIV infection and their possibly underlying mechanisms, which comprise determinants of viral attenuated strains, specific HLA class I and II alleles, certain polymorphisms in co-receptor genes and ligands, the specificity and functionality of virus-specific CD4+ and CD8+ T-cell responses, as well as new insights into factors of the innate immune response in HIV control.

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Insights from studies in individuals with self-controlled viral replication and HEPS cohorts

Infection with HIV leads to a devastating erosion of the immune system clinically characterized by a progressive rise in HIV viral load and decrease of the number of CD4+ T-cells. This decline in CD4+ T-cells heralds the progression to AIDS with its associated opportunistic infections and cancers and ultimately ends in death.

Among the HIV-infected population, several groups of individuals have been identified that remain clinically stable and free of any AIDS defining conditions for decades after infection in the absence of antiretroviral therapy (3). These individuals (estimated to be 5-8% out of the total HIV-infected population) have been referred to as long term survivors (LTS) or long-term non-progressors (LTNP) and are generally able to control viral replication to low levels – plasma RNA levels <2,000 copies/ml (or <5,000-10,000 copies/ml, depending on cut-offs decided upon by the investigators) and to maintain normal CD4+ T-cell counts over time with a reduced rate of CD4+ T-cell loss (18 cells/µl/year) compared to that in normal progressors (around 60 cells/µl/year) (4). A subset of such LTNP is able to maintain undetectable plasma viral loads for extensive period of times and is known as elite controllers (EC). The clinical and scientific interest in such individuals is great as they may indeed hold the key of spontaneous control of HIV infection. Current efforts have allowed for the establishment of large international cohorts of controllers and elite-controllers and an intensive search for host genetic, virological as well as immunological markers of HIV control is ongoing in these subjects.

Some recent work on elite controllers (estimated to represent less than 1% of the HIV infected population) has shown wide heterogeneity in the immunological and clinical course of HIV infection despite certain similarities in genetic determinants (5), again suggesting that HIV control needs to be seen in a combined system that integrates host genetics, immune function as well as virological diversity. Nevertheless, these and earlier analyses have directly allow to identify a number of properties of the immune response to HIV (specificity, T cell polyfunctionality), as well as host genetic markers (mainly HLA class I alleles) that are strongly associated with disease control and which will prove helpful in the quest of developing a broadly applicable HIV vaccine.

Similarly, highly Exposed Persistently Seronegatives (HEPS) individuals, such as some commercial sex workers or discordant couples who are multiply exposed to HIV yet remain uninfected, may also provide important clues into potential mechanisms of HIV control and even prevention of infection (6, 7).

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However, these studies need to always be well documented for real exposure to HIV, even more so now that studies in discordant couples sometimes include HAART treated subjects which put the seronegative partner at clearly reduced risk of HIV acquisition. Some recent studies have identified genetic polymorphisms in the SDF1 gene, specific CD8+ T cell responses and IgA production as factors associated with a reduced risk of HIV acquisition in these groups (8-12). However, T cell response rates and antibody production have not emerged consistently in all studies as potentially protective (13, 14) and further studies are needed to clarify these findings. In addition, immune analyses conducted to identify potentially protective immune responses need to be based on sensitive but specific assays and stringent cut-offs (15). Given these considerations, it can reasonably be expected that future analyses on HEPS may provide further valuable information.

Viral determinants in control of HIV-infection

While individuals with apparently effective control of HIV infection (LTNP, elite controllers) have been studied exhaustively for virus-specific immune responses, less is known about potential virological determinants that could be driving the observed control. A number of studies have identified single individuals or small cohorts (such as the Sydney Blood Bank Cohort –SBBC- a unique collection of individuals infected with an attenuated HIV-1 virus from a common donor) that harbor partially defective viruses that seem to have a decreased replication competency. Others have also been able to isolate virus with impaired replicative capacity from PBMC cultures generated from LTNP, supporting the hypothesis that primary infection by 'attenuated virus strains' with slow replication kinetics may facilitate control of viremia (16). This is not only of relevance to the replication fitness of potentially partly defective viruses as it could also reduce the kinetics of viral evolution and CD4+ T cell depletion over prolonged period of time.

A certain 'acquired' degree of attenuation in terms of impaired replicative capacity could be partially induced by antiretroviral therapy, as individuals who were efficiently treated for long periods of time showed a prolonged delay in restoration of pretreatment viral diversity after therapy interruption (STI). This suggested that punctuated antiretroviral therapy may cause a considerable evolutionary bottleneck leading to the emergence of viral populations with overall reduced viral fitness (17). However, it could also reflect improved immune competence and at least partial immune restoration after prolonged treatment periods, which could on its own impact viral repopulation dynamics.

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Aside from treatment-induced viral variants, several viral genetic defects and polymorphisms that impair replicative capacity have been implicated in mediating relative viral control. Viral genomes carrying deletions or inactivating variants in the nef gene or in the overlap of nef and the U3 region of the long terminal repeat (LTR) were among the very first viral defects associated with control. These cases were identified in 1995 in a group of 6 individuals from the SBBC cohort who had become infected after obtaining blood transfusions from the same HIV-infected blood donor but remained free of HIV-related disease (18). Although still unclear, one potential underlying mechanism suggested for the beneficial effects of this nef deletion is that Nef is no longer available to down-regulate HLA class I molecules and infected cells thus would be more readily recognized by HIV-specific CD8+ T cells (19). However, longitudinal analysis of viral evolution of the *nef*/LTR sequences over time in the SBBC cohort, were unable to fully explain mechanisms that could have contribute to slow progression to HIV disease in 2 (out of the 6) individuals of the cohort, suggesting that nef gene deletions are not necessarily mediating life-long protection from disease progression. Thus, other viral and/or host factors plus immune pressure was likely contributing to the long-term control in these individuals (20).

Aside from Nef-mediated effects, other mutations in structural proteins have been associated with slower disease progression as well. These include unusual, difficult-to-revert polymorphisms and 1-2 amino acids deletion in gp41 and Gag or a four amino acids insertion in Vpu among others (21). In addition, replication defective strains have been identified when constructing viral clones with mutations at the rev activation domain, which were seen more frequently in controller individuals than in subjects with regular HIV disease progression (22). In addition, to these mutations of unknown origin in terms of specific selective force (apart from reduced replication capacity), viral mutations evolving under strong immune selection pressure have been show to lead to reduced viral replication (see below).

Despite these reported cases of reduced replicative viruses and slower disease progression, the frequency and contribution of such defects on the maintenance of undetectable viremia is not well established. In particular, conflicting data exists partly due to the fact that the identification of such attenuated virus strains *in vivo* are limited to small number of subjects and that sequence analyses have been often based on analyzing cellular proviral DNA, thus possibly also including some level of grave-yard sequences. Today, with more sensitive assays, some elite controllers have been shown to have persistent viral replication detectable in plasma at levels below 50 copies/ml (23). In addition, a recent report by Miura et al for instance did not reveal any gross genetic defects in HIV-1 coding gene

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sequences derived from plasma viral RNA from a large cohort of elite controllers (24). This observation suggests that active viral suppression by the immune system rather than shared viral genetic defects or polymorphisms is driving viral control in HIV controllers. This conclusion is also in line with work on accessory genes from HIV controllers, where replication-competent viruses from CD4+ T-cell co-culture supernatants were analyzed and did not reveal any consistent defects in either vpr or vif genes (25).

Impact of host genetics on *in vivo* HIV control

An extensive number of host genetic markers have been identified over the last 20 years that are associated with either rapid or slow HIV disease progression or with protection from infection. Many older studies however were carried out using single gene approaches with small number of samples and the genetic associations found were not always confirmed in subsequent functional studies. Thanks to the advances of the Humane Genome Project, the use of whole-genome association scans, and the establishment of international consortia such as CHAVI, EuroCHAVI or the HIV International Controllers consortium, it has been possible to uncover certain genetic factors that might play a relevant role in the control of HIV. This availability of large samples number and the possibility to sequence 650,000 single-nucleotide polymorphisms within the human genome should further enhance studies examining the contribution of multiple genetic factors (26). However, unlike studies on genetic markers associated with for instance autoimmune diseases, the search for host genetic polymorphisms in HIV infection also needs to take into consideration the viral diversity in regions of different host genetics/ethnicities. An earlier report on the effect of a single genetic (HLA) marker and viral evolution has recently been confirmed in a massive international effort, addressing the inter-relationship between host genetics and viral evolution (27, 28). These findings highlight that viral diversity is likely be shaped by differences in the frequency of different host genetic markers and, based on viral evolution, can lead to opposite effects of a specific genetic marker on HIV disease control (27). Thus, whole human genome approaches are severely complicated by viral diversity in different host ethnicities making comparisons across different clades of HIV and various geographically distinct human populations difficult. This consideration also points to the possibility that different clades of HIV may possess inherently different replication fitness and may drive disease development at variable levels, as recently considered as a possibly contributing factor in a case of severe acute HIV infection (29).

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Association of HLA polymorphisms with HIV disease outcome

Many host genetic polymorphisms associated with levels of disease control involve genes encoding for receptors for viral entry and molecules expressed on the surface of the cells of the innate or acquired immune system, such as HLA, CCR5 and KIR receptors. Moreover, it seems that in some cases their potential protective influence might have a cumulative effect as seen for the synergic effect of some KIR receptors and HLA-B complexes (30). Likely the most robust analyses have focused on the Human leukocyte antigen (HLA) genes and their polymorphisms. The HLA class genes form highly polymorphic loci in the Major Histocompatibility Complex (MHC) located in the short arm of chromosome 6 and encode for cellular surface molecules that present foreign antigenic epitopes to T lymphocytes. There are two groups of HLA molecules including HLA class I and HLA class II antigens. The HLA class I molecules are divided into HLA-A, HLA-B, HLA-C all of which bind peptides derived from intracellularly processed proteins and present them to CD8+ cytotoxic T-cell lymphocytes (CTL). Among these, the HLA-B alleles, while most diverse (more than 1,000 HLA-B alleles have been identified to date) have also been shown to carry the bulk of the anti-viral T cell immune response in HIV infection (31). Accordingly, the number of well-defined HLA-B restricted epitopes exceeds the number of epitopes identified HLA-A and, particularly, HLA-C alleles. However, especially the HLA-C alleles are currently under more intensive investigation as larger HIV infected cohorts with more complete and high-resolution HLA-C typing have become available.

HLA alleles are grouped into 9 supertypes based on their structure, peptide-binding motif, epitope representation and sequence similarity (32, 33). Particularly alleles included in the HLA-B7 (B*5101, B81, B35Px/Py), HLA-B27 (HLA-B27, B*1503) and HLA-B58 supertypes (HLA-B57, B*5801, B*1516, B*1517) have been associated with improved or impaired levels of HIV control. Of note, almost all the alleles in the HLA-B58 supertype appear to mediate superior control of HIV infection (34); with the exception being the HLA-B*5802 allele, which is highly prevalent in South Africa and which is associated with elevated median viral loads (35). The reasons how subtle changes in the HLA sequence (HLA-B*5802 only differs in three amino acids from the "good" HLA-B*5801 allele) can so profoundly affect HIV disease outcome are still unclear and are not in all cases simply attributable to different CTL epitope repertoires presented on these alleles (35, 36). The fact that this allele, as well as the HLA-B*1503 allele are present at high frequency and are both associated with higher viral loads in HIV infected individuals (HLA-B*1503 is a "good" allele

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in the North American population where it is rare; ref (27)) is in line with earlier reports that found an advantage of expressing rare HLA supertype alleles in controlling HIV (37).

In addition to HLA allele frequency, the homocygous expression of individual HLA alleles has been associated with reduced viral control. This heterozygote advantage has been widely observed in several cohorts, including Caucasian and non-Caucasian populations (38, 39) and has been reproduced in Hepatitis C infection for which HLA-associated markers of viral clearance and virus control have been identified as well (40). Furthermore, the effects of particular HLA supertypes or of individual alleles have also been reported to provide the basis for immunologically mediated resistance to infection (41, 42). It will be interesting to confirm the potential protective effects of such alleles in additional cohorts with variable allele frequencies and to assess other mechanisms and markers present in genetic linkage to these alleles that may possibly be involved to at least some levels in protection from HIV infection (43).

Associations between HIV control and specific polymorphisms in the HLA class II loci have been less well-defined, maybe reflecting a possibly only indirect anti-viral effect of HLA class II restricted CD4+ T cells. Nevertheless, some cohort-based studies have reported that DRB1*13 allele expression is associated with partial protection from HIV disease progression, although this has not consistently been observed (44). The DRB1*13/DRQ1*06 haplotype has also been found at increased frequency in individuals who were treated early in HIV infection and who maintained virus suppression after treatment interruption (45). Furthermore, a protective role of DQB1*06 alleles, irrespective of their DR haplotype co-expression, has been identified (46). While the HLA class II associations have not produced as strong markers as HLA class I analyses, the representative studies given above highlight the importance to further explore the contribution of the specific CD4+ T cell responses and their genetic basis in the control of HIV.

Specific HLA class I B alleles associated with variable levels of HIV control

HLA-B*5701 and, to a lesser extent HLA-B27 are HLA-B alleles overrepresented in North American and European cohorts of LTNP and EC individuals (26, 38, 39, 47, 48) and reviewed largely in (49)). Similarly, the HLA-B*5703 allele (which is the prevalent B57 subtype in Africans) is also significantly enriched among African subjects that control HIV replication (31, 50). These associations are further supported by survival analyses of HLA-B57 and HLA-B27 expressing individuals (51). How these alleles mediate their beneficial effect has

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however not been entirely clear, although in the case of HLA-B27 and HLA-B57 compelling evidence suggests it may be due to their presentation of multiple immunodominant epitopes located in HIV Gag and reverse transcriptase (5, 52, 53). The broad epitope repertoire and the wide cross-recognition of epitope variants presented by HLA-B57 suggest that effective viral escape from HLA-B57 restricted CTL responses may be difficult to achieve. Of note, HLA-B*5701 is in strong linkage disequilibrium with the polymorphism located in the HLA complex P5 gene (HCP5), located 100 kb centromeric from the HLA-B locus and who has been identified as a protective marker of HIV infection, independent HLA-B57 expression (26). However, the mechanistic explanation of this protective effect remains elusive and probably is a result of a combined haplotypic effect with HLA-B*5701. This is also in agreement with recent studies showing significant fitness cost of HIV escape variants that affect HLA-B57 restricted CTL epitopes (54). Similar results have recently also been reported for CTL responses targeting HLA-B13 restricted epitopes, which support the notion that the nature of the presented epitope, more than the restricting HLA molecule allele may determine the beneficial effects of a specific HLA allele (27, 55). However, evolutionarily closely related alleles with subtle sequence differences and comparable binding motifs but associated with opposite rates of HIV disease progression also indicate that the presenting HLA molecule may have a modulating effect on the effectiveness of the restricted T cell response.

Analogous to the HLA-B58 and -B13 alleles above, the protective effect of HLA-B27 allele is also thought to be due to its restriction of an immunodominant CTL response to a conserved HIV epitope located in p24 Gag. This 10mer epitope sequence contains the arginine residue at position 264 of HIV Gag which, once mutated weakens the epitope binding to HLA-B27 (56). However, the classical mutation to lysine has detrimental effects on viral replication capacity and requires a complex series of compensatory mutations in partly distant sites in Gag to restore viral fitness (57-59). Thus, the mutation is less likely to revert and to restore full replication fitness and individuals with the escape mutation may still present with lower viral loads than the rest of the population.

Apart from HLA alleles that mediate relative protection from HIV disease progression, a number of alleles have been identified that are linked to accelerated disease courses. Among these, the HLA-B35/Cw04 haplotype has been consistently found at increased levels in individuals with rapid progression to AIDS (38). Subsequent studies revealed different peptide-binding specificities for the various HLA-B35 subtypes, which prompted their discrimination into Py and Px alleles, respectively (36). Accelerated HIV disease progression has been associated with the HLA-B35 Px (HLA- B*3502/3503/3504) alleles but not Py

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alleles (B*3501). This could explain why HLA-B35 associations were not observed in African-Americans as this ethnic group often expresses HLA-B35-Py alleles. It also confirmed that the HLA-Cw04 association with rapid disease progression was due to its linkage disequilibrium with HLA-B35-Px alleles rather than exerting a deleterious effect by itself (36).

Aside from HLA linkage disequilibrium and other polymorphisms in the MHC region, the potentially synergistic effects of specific HLA type and NK inhibitory receptor (KIR) have recently obtained much attention. KIR receptors are polymorphic receptors that interact with HLA class I molecules and regulate the NK activity, either by mediating activating or inhibitory signals. A number of studies have associated the expression of specific HLA and KIR combinations with different diseases, such as cervical neoplasia and infectious diseases, including HIV (30, 60-62). The insight into potential mechanisms of these favorable combinations is most advanced in the case of HLA-B/KIR allele combinations. HLA-B molecules contain one of two mutually exclusive serological epitopes, Bw4 and Bw6, which differ by five amino acids spanning positions 77-83 of the HLA-B heavy chain, including the crucial Isoleucine residue at position 80 (63). HLA-Bw4- but not HLA-Bw6-molecules have been considered ligands for KIR3DL1 and possibly KIR3DS1 (62, 64, 65). In a proportional hazard model that included all known genetic predictors of HIV progression, co-expression of KIR3DS1 and HLA-Bw4 was found to be an independent predictor of decreased time to AIDS. Interestingly, this beneficial effected was observed despite the fact that KIR3DS1 alone in the absence of its HLA ligand was associated with more rapid disease progression. Thus, the findings highlight the potential for KIR/HLA interactions to be important independent predictors of HIV progression and may help shed light on the relative contribution of these interactions compared to HLA-restricted CTL activity (62, 66).

Non-MHC encoded genetic markers of HIV control

Although the CD4 antigen is the main receptor for HIV entry into susceptible cells, effective viral infection requires the presence of one of two major co-receptors, referred to as CCR5 and CXCR4, respectively. These two co-receptors belong to the superfamily of 7-transmembrane G-protein–coupled chemokine receptors and determine viral tropism, allowing for the differentiation of R5, X4 or R5/X4 viruses that can use either one or both of these receptors (67, 68).

Chemokines are a superfamily of small molecules (8-15 kDa) that exert many roles in inflammatory and in homeostatic immune processes (69, 70). They are divided into four subfamilies based on the structural cysteine motif located in

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the amino-terminus of the mature protein (CXC, CC, CX_3C and C chemokines) and their receptor usage shows a considerable level of redundancy (71). The chemokine Regulated on Activation Normal T-cell Expressed and Secreted (RANTES or CCL5), Macrophage Inflammatory Protein-1α (MIP-1α or CCL3) and 1β (MIP-1β or CCL4), were first identified as natural ligands for the CCR5 receptor and were subsequently shown to be potent inhibitors of R5 viruses *in vitro*. The natural ligand of CXCR4 is Stromal Cell Derived Factor-1 (SDF-1 or CXCL4), which also possesses potent inhibitor function of X4 viruses *in vitro* (72-74). Other chemokine receptors that can act as HIV co-receptors have been described, including CCR3, CCR2b and CCR8 (75).

Shortly after their identification as crucial HIV co-receptors, genetic polymorphisms in the various chemokine receptors were reported. In particular, a 32 base pair deletion in the CCR5 gene (CCR5-Δ32) was identified that generates a non-functional protein that is not expressed on the cell surface. The homocygous expression of the CCR5-Δ32 variant provides *in vivo* resistance to infection by R5 HIV isolates. These observations were made in different cohorts of men who have sex with men and hemophiliacs with documented exposure to HIV. When present in a single copy only, the heterocygous expression of the wildtype CCR5 receptor was found to be sufficient to enable infection, although the lower levels of CCR5 on the cell surface were associated with reduced viral replication and a delayed onset of AIDS (76-79). Spurred by the effects of heterocygous expression of CCR5-Δ32, mutations in the promoter region of CCR5 were identified that are also associated with altered transmission or delayed disease progression, although to a lesser extent than the CCR5-Δ32 mutation (80, 81).

In addition to the CCR5-Δ32 mutation, the V64I substitution in the CCR2A protein sequence (CCR2-V64I) has also been found to delay HIV disease progression; however without preventing HIV transmission. Intriguingly, CCR2 is rarely used as a co-receptor in HIV infection and its impact on global epidemic is unclear. Furthermore, it has been demonstrated that the CCR2-V64I allele is in strong linkage disequilibrium with a point mutation in the CCR5 regulatory region (82). Together with CCR5 mutations, approximately 29% of LTNP phenotypes in large cohorts have been estimated to be due to a mutant genotype for CCR2 or CCR5 (83). Interestingly though, relative protection against AIDS provided by CCR5-Δ32 heterocygosity appears to be continuous during HIV disease over time, whereas the protective effects of the CCR2-64I variant was most pronounced in early infection (84).

The identification of CCR5 and CCR2 as crucial molecules for HIV-infection has also offered new treatment targets to inhibit viral replica-

tion and CCR5-based HIV entry-inhibitors have been developed are now part of effective rescue treatment strategies (85, 86). More recently, a single case of stem-cell transplantation from a homocygous CCR5-Δ32 donor to an HIV-infected individual with acute myeloid leukemia showed no signs of rebounding viremia in plasma, bone marrow or rectal mucosa 20 months post-transplantation and in the absence of antiretroviral treatment. This is even more surprising given that minor X4-variants were identified in the pre-transplantation viral population, given rise to a number of questions on how viral populations with different cell tropism are controlled *in vivo* (87).

Polymorphisms involving other chemokine receptors and/or other chemokine receptor ligands have been identified as well. Especially, plasma levels of RANTES, which can significantly vary among healthy individuals, were found to be modulated by two single nucleotide polymorphisms in the RANTES gene promoter region. These changes have also been associated with delayed progression of HIV disease and experimental over-expression and increased promotor activity of RANTES functionally link the polymorphisms with reduced HIV replication capacity as a consequence of increased RANTES production (88-90). Similarly, a genetic variant consisting of a transition (G-A) in the 3´untranslated region of the SDF-1 gene (SDF-1 3´A) has also been associated with a delay in AIDS onset when present as homocygous variant. Its underlying mechanism and effect on X4 viral populations *in vivo* is however not well understood (91-94). Noteworthy, no mutations in the CXCR4 receptor gene have been proposed as markers of relative HIV control, probably because CXCR4 and SDF-1 are essential at embryonic development stages and, consequently, such mutations might be potentially lethal (95).

A genetic determinant that has recently been associated with rate of HIV disease progression is the copy number of the chemokine gene CCL3L1 (MIP-1α). Individuals with higher CCL3L1 copy numbers than the population race-adjusted average showed lower steady-state viral load; suggesting an increased rate of HIV disease progression in subjects with lower CCL3L1 copy number (96).

Finally, several polymorphisms in the DC-SIGN (dendritic cell specific intracellular adhesion molecule -3-grabbing nonintegrin, i.e. CD209) promoter have been found to be linked to an increase or decrease in susceptibility to HIV infection, particularly also in parenterally acquired HIV infection (97). As DC-SIGN has also been associated with tuberculosis infection and outcome, combined studies, as a recent one in an Indian population, may help employing DC-SIGN based strategies for the combined fight of these two major human pathogens (98).

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Immune markers in the relative control of HIV infection

An extensive amount of data indicates a potentially crucial role of HIV-specific cellular and humoral immune responses in the *in vivo* control of viral replication and HIV disease progression. As discussed above, a number of host genetic markers have been identified, particularly specific HLA class I alleles, suggesting an important contribution of the HLA class I restricted CD8+ T-cell immunity in virus control. Despite an increasingly more detailed understanding of the interplay between host immunity, viral evolution and the impact on viral control, clearly defined immune correlates of controlled HIV infection remain elusive. As a consequence, effective vaccine design is still hampered by the availability of well-defined immune parameters that actively mediate *in vivo* viral control. While much of the current investigations focus on the detailed characterization of individuals with exceptional ability to control their virus, it is important to notice that the immune markers associated with this control can often be biased towards subjects expressing specific host genetics and are thus not necessarily translatable to the general population. Nevertheless, a number of specific characteristics of the host immunity against HIV that have been identified clearly extend beyond these limitations and will provide important guidance to vaccine development and offer new immune-based therapeutic treatment options.

HIV disease markers associated with virus-specific cellular and humoral immunity

It is generally believed that the cytotoxic T cell (CTL) immune response contributes strongly to the *in vivo* control of viral control. Virus-specific CTL responses have been temporally associated with the initial decline in plasma viremia after acute HIV infection and are thought to determine viral set point in chronic stages of infection (99, 100). This is supported by studies in the SIV macaque model, where transient depletion of the total CD8+ T-cell population in controller animals resulted in 100- to 10,000-fold increases in viremia and where the re-establishment of the $CD8^{+}$ T-cell populations restored the ability of these animals to control SIV replication (101, 102). Further support for an important role of virus-specific CTL in HIV control stems from older studies conducted when tetramer technology became first available in the late 1990ties (103). The use of such tetramer complexes allowed for the direct *ex vivo* visualization of epitope-specific CTL populations and analysis of specific responses against defined epitopes, without prior in vitro expansion and modulation of epitope-specific T cells (104). Initial analyses using SL9 (SLYNTVATL, HIV

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Gag p17) specific tetramers, revealed a significant inverse correlation between SL9-specific CTL frequency and plasma RNA viral loads (105). These analyses were however only based on a limited number of SL9-responding subjects and did not take into consideration the possibly impaired functionality of tetramer-specific T cells. Not surprisingly, the SL9 association with HIV control were not confirmed in studies that used in vitro expanded T cells, possibly due to the differential ability of such cells to expand in vitro (106). Since these earlier studies, novel assays, including the IFN-g based ELISpot assay and in vitro inhibition assays first developed by Yang et al (107, 108) provide additional tools to assess direct *ex vivo* T cell activity and functionality (109, 110). These analyses further support the relevance of HIV specific T cells in HIV control, although in many studies, the precise phenotypic and functional markers of these virus-specific T-cell responses attributed to viral control may reflect the effects rather then the cause of otherwise controlled HIV infection. Dissecting these two possibilities and assigning unambiguously causality to specific immune markers and T cell specificities remains one of the currently biggest challenges in defining functionally relevant immune correlates of HIV control.

Over the years, an number of studies have correlated strong and broad HIV-specific T-cell responses with the delayed progression to AIDS and vaccine success is oftentimes subjected to a quantification of the total breadth and magnitude of induced responses. While a detail characterization of vaccine-induced responses will always need to be conducted, it is also clear from a growing number of reports (111) that total virus specific immunity is not necessarily the best measure of *in vivo* immune control of HIV and that more detailed analyses of these response data are needed. Indeed, re-analyses of earlier total-virus specific CTL data suggest that T-cell responses preferentially targeting Gag or other highly conserved epitopes are most relevant specificities for the enhanced antiviral efficacy of T-cells seen in those individuals (109, 112). On the other hand, CTL responses against Env or accessory and regulatory proteins have been shown to have the opposite effect, and are directly correlated with elevated viral load (50). These findings are in line with more recent studies in clade B as well as clade C infection and analyses that either assessed total viral immunity in peptide pools rather than individual peptide preparations or that focused on responses restricted by specific individual HLA alleles only (50, 52, 113-117).

However and despite strong associations between Gag-specific T cell immunity and relative HIV control, the causative relationship between the observed response patterns and viral control is still outstanding. Plausible explanations for how dominant Gag specific cytotoxic T-cells could mediate relative virus control stem from the ability of certain HLA class I molecules such as HLA-B57

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to present a broad number of HIV Gag peptides and to induce high-magnitude CD8+ T-cell responses in early infection (52, 113). In addition, rapid re-presentation of epitopes derived from the Gag proteins contained in the incoming, infecting virus particles (possibly within less than 2hr after infection) may provide Gag-specific T cells with an decisive advantage to eliminate infected cells before massive virus production has been initiated (118). However, not all dominant Gag-protein specific responses may be equally effective and comparative studies in clade C and B infection have identified subdominant Gag responses as well as responses outside of Gag as crucial components in relative virus control (27). The important role of subdominant responses has also been confirmed in studies in the SIV macaque model and is also supported by the detection of subdominant CTL responses in groups of HEPS (11, 101).

Apart from CTL specificity, the *in vivo* antiviral efficacy of HIV-specific CD8+ T-cell immunity has also been tightly linked to the functional competence of these responses. In particular, proliferative capacity with high perforin expression and secretion of multiple cytokines such as IFN-γ, IL-2, TNF-α, MIP-1b and/or CD107a surface expression after antigen contact characterize the responses seen in LTNP (119, 120). In addition to polyfunctionality, the avidity of virus-specific T-cell responses is also considered a potentially important measure of an effective immune response and has been shown in HCV infection, to be associated with viral clearance and higher levels of cross-variant recognition (121, 122). Thus, the quality of the CD8+ T-cell response to HIV serves as a better marker of controlled infection than the quantity (i.e. breadth and magnitude) of these responses. In addition, a HLA-DR+, CD38- activation CD8+ T-cell phenotype was more frequently found among virus-specific T-cells in HIV controllers than in non-controllers and may represent a T cell population with superior ability to expand upon exposure to antigen and capacity to exert effector functions (110). Whether full-differentiation into CCR7-/ CD45- effector cells and broad functional CTL is only a hallmark of controlled HIV infection in the peripheral PBMC compartment or also extends to CTL in gut-associated lymphoid tissue -where massive initial depletion of CD4+ T-cells occurs- remains an open question. Nevertheless, a rapidly growing set of reports dealing with the emergence and accumulation of CTL escape mutations under appropriate CTL pressure, the transmission of "escaped" variants, implications of fitness costs incurred by CTL escape mutations and the global adaptation of HIV to HLA class I polymorphisms further document the crucial role that HIV-specific CTL overall play in the control of HIV infection escape (28, 54, 58, 123-130).

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An additional factor for an effective CD8+ T-cell response includes the presence and function of CD4+ 'helper' T-cells. HIV-specific CD4+ T-cell population have been shown to be required for long-term maintenance of antigen-specific CD8+ memory T-cells, both in the human setting as well as in the monkey model (101, 131-133). The relevance for functional CD4+ T-cell help in the maintenance of effective CTL populations has recently also been reported for viral infections other than HIV, including EBV and CMV infections (134, 135). The potential importance of virus-specific T helper cell activity is further highlighted by studies that have associated the presence of gp41-specific antibodies with CD4+ T-cell responses to Gag-p24 (136, 137). Regardless of the well-documented anti-viral effects of neutralizing Ab responses (138) general antibody-responses have not emerged as strong markers of HIV control. Some of the existing data have been inconsistent as some studies have associated higher titers of heterologous nAb in LTNP whereas more recent studies indicate the presence of lower Nab activity among elite controllers (5, 16, 139). It will be interesting to investigate whether possible residual viral replication in the former group of patients may drive additional Ab production or whether additional, unaccounted factor and assay differences are responsible for the observed differences.

Innate Immunity

During primary HIV infection, there is a massive destruction of the CD4+ T-cell population in the gut-associated lymphoid tissue (GALT) impairing local cellular immunity at mucosal sites and causing translocation of microbial products which in turn contributes to a deleterious persistent inflammation (140-143). The potential damaging effects of chronic inflammation by continuous bacterial translocation are also highlighted by similar studies in HCV infection, where it has been implicated in the progression to advanced stages of cirrhosis (144). Most importantly however, the massive depletion of CD4+ T-cells, general inflammation and immune activation occur at times when the adaptive immune system has not mounted an effective immune response. As mentioned above, some markers associated with the innate immune system, particularly KIR and Toll-like receptors (TLR) have been associated with variable levels of HIV control in these early stages of infection. As such, recent host genetic studies indicate that individuals co-expressing KIR3DS1 and HLA-Bw4-80I (family of HLA alleles that presumably bind to KIR3DS1 and activate NK cells) have lower viral loads and show a reduced risk of progression to AIDS (62, 145). In addition, polymorphisms in toll-like receptor 9, which mediates innate immune response against DNA motifs common in bacteria and viruses, have recently been

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shown to impact clinical outcomes as well (146). However, significant functional data supporting the innate immunity and its linkage to disease pathogenesis is still scarce and needs to be further explored. In addition, extensive cohorts of individuals captured in earliest period of acute HIV infection will need to be comprehensively studied to assess the impact of these markers on initial peak viremia and the level of CD4+ T-cell depletion. However, the recent identification of immune memory mediated by NK cell populations may offer novel approaches for preventative and therapeutic interventions in HIV infection (147).

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CONCLUSIONS AND IMPLICATIONS FOR VACCINE DESIGN

Control of viral replication in HIV infection is a multifactorial process. Polyfunctional CD8+ T-cell immunity against particular viral proteins along with virus-reactive CD4+ T-cell help have been most consistently implicated in modulating HIV infection *in vivo*. Viral factors such as specific mutations often emerging as a consequence to immune selection pressure and entire gene segment deletions have also been associated with reduced viral burden and slower progression of HIV disease. In addition, specific host genetic markers, particularly HLA, has been most compellingly linked to relative control of HIV replication. While such host genetic markers may provide great help in understanding the (immune)-pathology of HIV, they will likely not be directly informative for HIV vaccine development. However, they can guide vaccine immunogen design, although care needs to be taken that such immunogen design is not overly guided by observations made in individuals with favourable host genetics. To avoid the resulting vaccine product to be tailored unreasonably strongly towards individuals with beneficial genetics, subjects who do not express these markers yet control HIV on their own will be most informative. In addition, while immune parameters that could mediate sterilizing immunity, i.e. resistance to infection, still need to be defined, the development of vaccines that are able to induce partial *in vivo* control, albeit not prevent infection, would have significant impact on individual health by slowing HIV disease progression and would help to contain the HIV pandemic by reducing transmission rates.

In this regard, the early assessments of vaccine success in phase I and phase II trials will be based on immune read-outs, rather than prevention of infection (which would be the central end-point in a phase IIb/III trial). Thus, the definition of precise immune correlates of controlled HIV infection is crucial since vaccine induced responses will be compared to these parameters. If their definition is flawed or represents epiphenomena of otherwise controlled HIV infection, valuable vaccine candidates may be discarded prematurely. Together with immune parameters of controlled infection, the identification of host genetic markers may in the future facilitate the design of gene therapy approaches that would try to either block expression of unfavourable genes or introduce beneficial components. Although not based on gene-therapy, the case of the CCR5-Δ32 stem-cell transplanted individual referred to above, points towards the potential feasibility of such approaches.

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ACKNOWLEDGMENTS

Financial support: Supported in part by the Red de Investigación de Sida (RIS) (RD06/04). Instituto de Salud Carlos III, Madrid, Spain and HIVACAT

Potential conflict of interest: none

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CHAPTER 2

Definition of the viral targets of protective HIV-1-specific T cell responses

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J Transl Med. 2011 Dec 7;9(1):208.

ABSTRACT

Background: The efficacy of the CTL component of a future HIV-1 vaccine will depend on the induction of responses with the most potent antiviral activity and broad HLA class I restriction. However, current HIV vaccine designs are largely based on viral sequence alignments only, not incorporating experimental data on T cell function and specificity.
Methods: Here, 950 untreated HIV-1 clade B or -C infected individuals were tested for responses to sets of 410 overlapping peptides (OLP) spanning the entire HIV-1 proteome. For each OLP, a "protective ratio" (PR) was calculated as the ratio of median viral loads (VL) between OLP non-responders and responders.
Results: For both clades, there was a negative relationship between the PR and the entropy of the OLP sequence. There was also a significant additive effect of multiple responses to beneficial OLP. Responses to beneficial OLP were of significantly higher functional avidity than responses to non-beneficial OLP. They also had superior in-vitro antiviral activities and, importantly, were at least as predictive of individuals' viral loads than their HLA class I genotypes.
Conclusions: The data thus identify immunogen sequence candidates for HIV and provide an approach for T cell immunogen design applicable to other viral infections.

Keywords: HIV-1 specific CTL, clade B, clade C, HLA, vaccine immunogen design, functional avidity, epitope, entropy, correlates of immune protection

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BACKGROUND

HIV-1 infection induces strong and broadly directed HLA class I restricted T cell responses for which specific epitopes and restricting HLA class I alleles have been associated with relative in vivo viral control [1]. The bulk of the antiviral CTL response appears to be disproportionately HLA-B restricted, but the relative contribution of targeted viral regions and restricting HLA molecules on the effectiveness of these responses remains unclear [2,3,4,5]. In addition, the impact of HIV-1 sequence diversity on the effectiveness of virus-specific T cell immunity in vivo is unclear, as functional constraints of escape variants, codon-usage at individual protein positions, T cell receptor (TCR) plasticity and functional avidity and cross-reactivity potential may all contribute to the overall antiviral activity of a specific T cell response [6,7,8,9,10,11,12,13]. Of note, T cell responses to Gag have most consistently been associated with reduced viral loads in both clade B and clade C infected cohorts [14,15,16]; however, the specific regions in Gag responsible for this effective control remain poorly defined. In addition, it is unclear whether the relative benefit of Gag is due to any other specific characteristic of this protein, such as rapid antigen-representation upon infection, protein expression levels, amino acid composition and/or inherently greater processability and immunogenicity, particularly in the context of selected HLA class I alleles [17,18]. Thus, concerns remain that a purely Gag-based vaccine might mainly benefit those people with a particular HLA genotype and will not take advantage of potentially beneficial targets outside of Gag [4,16,17,19]. In addition, CTL escape and viral fitness studies have focused largely on Gag-derived epitopes presented in the context of protective HLA class I alleles such as HLA-B27 and –B57 [7,20,21], yielding results that may not be generalizable to the genetically diverse majority of the human population. Furthermore, many studies have focused on immunodominant targets only, despite some studies in HIV-1 and SIV infection demonstrating a crucial contribution of sub-dominant responses to targets outside of Gag to the effective in-vivo viral control [4,22]. Thus, the current view on what may constitute a protective cellular immune response to HIV-1 is likely biased towards a immunodominant responses and those restricted by frequent HLA class I alleles and HLA alleles associated with superior disease outcome.

To overcome these potential limitations, the design of an effective and broadly applicable HIV-1 vaccine should to be based on information gained through comprehensive analyses that extend across large portions of the population's HLA class I heterogeneity. Here we focus on three cohorts totaling more than 950 untreated, chronically HIV-1 infected individuals with clade B and C in-

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fections, from which responses to certain regions of the viral genome and specific T cell response patterns emerge as correlates of viral control. Importantly, the analyses identify functional properties unique to these responses and control for the impact of HLA class I alleles known to be associated with superior control of HIV-1 infection, thus providing vaccine immunogen sequence candidates with potential usefulness in a broadly applicable HIV-1 vaccine.

METHODS

Cohorts: A HIV clade B infected cohort of 223 chronically infected, treatment naïve individuals was recruited and tested at IMPACTA in Lima, Peru. The majority (78%) of enrollees were male and all recruited individuals considered themselves to be of a mixed Amerindian ethnicity [14]. The cohort had a median viral load 37,237 copies/ml (range <50->750,000) and a median CD4 count of 385 cell/ul (range170-1151). A second clade B infected cohort was established at the HIV-1 outpatient clinic "Lluita contra la SIDA" at Hospital Germans Trias i Pujol in Badalona (Barcelona, Spain) consisting of 48 treatment-naïve subjects with viral loads below 10,000 and CD4 cell counts >350 cells/mm^3 ("controllers", n=24) or above 50,000 copies/ml and CD4 cell counts <350 cells/mm^3 ("non-controllers", n=24). The HIV-1 clade C infected cohort has been described in the past and consisted of 631 treatment naïve South African with a median viral load of 37,900 copies/ml (range <50 - >750,000) and a median CD4 count of 393 cells/ul (range 1-1378) [16]. An additional 78 from a recently published cohort in Boston were included in the analyses of functional avidities [39]. HLA typing was performed as previously described using SSP-PCR[58]. For Hepitope and FASS analyses, 4digit typing was used for the Lima cohort and 2-digit typing for the Durban cohort. Protocols were approved in Lima by the IMPACTA Human Research Committee, in Durban by the Ethical Committee of the Nelson R. Mandela School of Medicine at the University of KwaZulu-Natal and in Barcelona by the Human Research Committee at Hospital Germnas Trias i Pujol. All subjects provided written informed consent

Peptide test set and ELISpot assay: Previously described peptide sets matching HLA-clade B and C consensus sequences were used in all experiments for which the OLP-specific entropies have been calculated in the past, based on available sequence datasets [23,24,49] and (http://www.hiv.lanl.gov/content/immunology/hlatem/index.html). The peptides were clade-specific sets of adapted 18mers, overlapping by 11 residues designed using the PeptGen tool available at the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html). The individual OLP in the peptide sets

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for clade B and clade C had all the same starting and ending position relative to the source protein and follow the same numbering across the entire viral proteome for both clades. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation and used directly to test for $CD8^+$ T cell responses in vitro. IFN-γ ELISpot assays were performed as described previously, using Mabtech antibodies (Mabtech, Stockholm, Sweden) and a matrix format that allowed simultaneous testing of all 410 overlapping (OLP) peptides in the respective test set [14]. Thresholds for positive responses were defined as: exceeding 5 spots (50 SFC/10^6) per well and exceeding the mean of negative wells plus 3 standard deviation or three times the mean of negative wells, whichever was higher. Stimulation with PHA was used as a positive control in all ELISpot assays.

Definition of functional avidity: Responses targeting 18mer OLP in HIV-1 Gag p24 were assessed for their functional avidity using OLP-specific sets of 10mer peptides overlapping by 9 residues that span the 18mer peptide sequence. Functional avidity was defined as the peptide concentration needed to elicit half maximal response rates in the Elispot assay and was calculated as a sigmoidal dose response curve fit using GraphPad Prism software [13].

***In vitro* viral replication inhibition assay:** A double mutant virus containing a Nef M20A and Integrase G140S/Q148H raltegravir (integrase inhibitor) resistance mutations was tested for replication in CD4 T cells in the presence or absence of autologous T cell lines targeting protective or non-protective OLP. Use of the raltegravir-resistant virus allows to prevent potential replication of autologous virus in the inhibition assays [38], excludes potential negative impacts on antigen processing or CTL functions attributed to protease inhibitors [59] and avoids overlap between the resistance mutations sites (i.e. G140S/Q148H) and location of beneficial and non-beneficial OLP sequences. In brief, the p83-10 plasmid containing mutations for a methionine to alanine substitution at position 20 of the Nef protein and the p83-2 plasmid engineered to contain the G140S and Q148H mutations in the integrase were combined to produce a virus that is replication competent, highly resistant to raltegravir and does not downregulate HLA class I in infected cells [60,61]. Although not entirely physiological, this approach was chosen to potentially increase the signal in the in vitro inhibition assay, even when responses were restricted by Nef-sensitive HLA class I alleles. Plasmids were co-transfected into MT4 cells and virus was harvested after 7 days [60,62,63]. Autologous CD4 cells were enriched by magnetic beads isolation (Miltenyi) and expanded for 3 days using a bi-specif-

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ic anti-CD3/8 antibody and IL-2 containing medium (50 IU r-IL2) before infecting them at multiplicities of infection (MOI) between 0.01 and 1. Effector cells were obtained by stimulating PBMC with either beneficial or non-beneficial OLP for 12 days before isolating specific OLP-reactive cells by IFN-γ capture assay according to manufacturers' instructions (Miltenyi, Bergisch Gladbach, Germany). The effector T cells were analyzed by flow cytometry for the specificity to their respective targets after capture assay and quantified to adjust effector-to-target ratios. Since the NL4-3 backbone sequence differed in several positions in beneficial and non-beneficial OLP, the epitope specificity was predicted based on the HLA class I genotype of the tested individual and responses confirmed to efficiently recognize variant sequences in the NL4-3 backbone sequence. Culture supernatant was harvested and replaced by raltegravir containing medium 0.05μg/ml after 72 h. Levels of Gagp24 in the culture supernatant were determined by ELISA as described [25].

Statistical Analyses: Statistical analyses were performed using Prism Version 5 and R Statistical Language [64]. Results are presented as median values unless otherwise stated. Tests included ANOVA, non-parametric Mann-Whitney test (two-tailed) and Spearman rank test. The significance of differences in viral load distribution between OLP-responders and OLP-non-responders was assessed by a two-sided Student's T Test with multiple tests addressed using, instead of a Bonferroni correction, a q-value approach to compensate for multiple comparisons [25]. The multivariate analysis was based on a novel multivariate combined regression method known as FASS, a forward selection method combined with all-subsets regression [29,30,31]. Briefly, the FASS approach works by iteratively performing the following procedure: Let 'V' be the set of all variables and 'M' be the set of variables included in a model. In the first step, those variables that are not already in the model are divided into equal-sized blocks of variables (the last block may have less than 'g' variables). Then, for each block of variables, 'm' is a new estimated and evaluated model using the Bayesian Information Criterion (BIC). The best model 'm' according to its BIC is retained and the procedure starts all over again until in one step or more the model is not improved.

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RESULTS

HIV-1-specific T cell responses targeting conserved regions are associated with lower viral loads: In a first analysis, HIV-1-specific T cell responses were assessed in a cohort of 223 HIV-1 clade B infected individuals recruited in Lima, Peru using IFNg ELISpot assays and a previously described set of 410 clade B overlapping peptides (OLP) [14,23]. For each OLP, a protective ratio (PR) was calculated as the ratio of the median viral loads between OLP non-responders and OLP responders, such that OLP with PR>1 were reflective of OLP predominantly targeted by individuals with reduced viral loads. OLP-specific PR were a) compared between OLP spanning the different viral proteins and b) correlated with the viral sequence heterogeneity in the region covered by the OLP. The data showed highest median PR values for OLP spanning the Gag protein sequence, whereas Nef, Env and Tat had the lowest median PR values (Figure 1A, $p < 0.0001$, ANOVA). A protein-subunit-breakdown of PR values showed the p15 subunit of Gag and RT in Pol to score less favorable than the remainder of the respective proteins (Figure 1B, $p=0.0032$ and $p=0.0025$, respectively). While these data confirm the association between HIV-1 Gag-specific responses and lower viral loads, it is important to note that all proteins contained OLP with PR>1, suggesting that some beneficial responses can be located outside of Gag; data that has not emerged from any of the previous studies linking Gag responses to relative viral control. At the same time, all proteins contained OLP with PR<1, indicating that proteins considered overall beneficial may contain non-beneficial regions as well. In addition, when the OLP-specific PR was compared to the sequence entropy of the region spanned by the individual OLP, a significant negative correlation between PR and entropy was observed ($p=0.0028$, $r=-0.15$; Figure 1C). Although rarely targeted OLP may have introduced statistically less robust data points in this comparison and caused a wide scatter of data points, the results show a relative absence of OLP with high entropy and high PR values, suggesting that responses to more variable regions are less effective in mediating in vivo viral control.

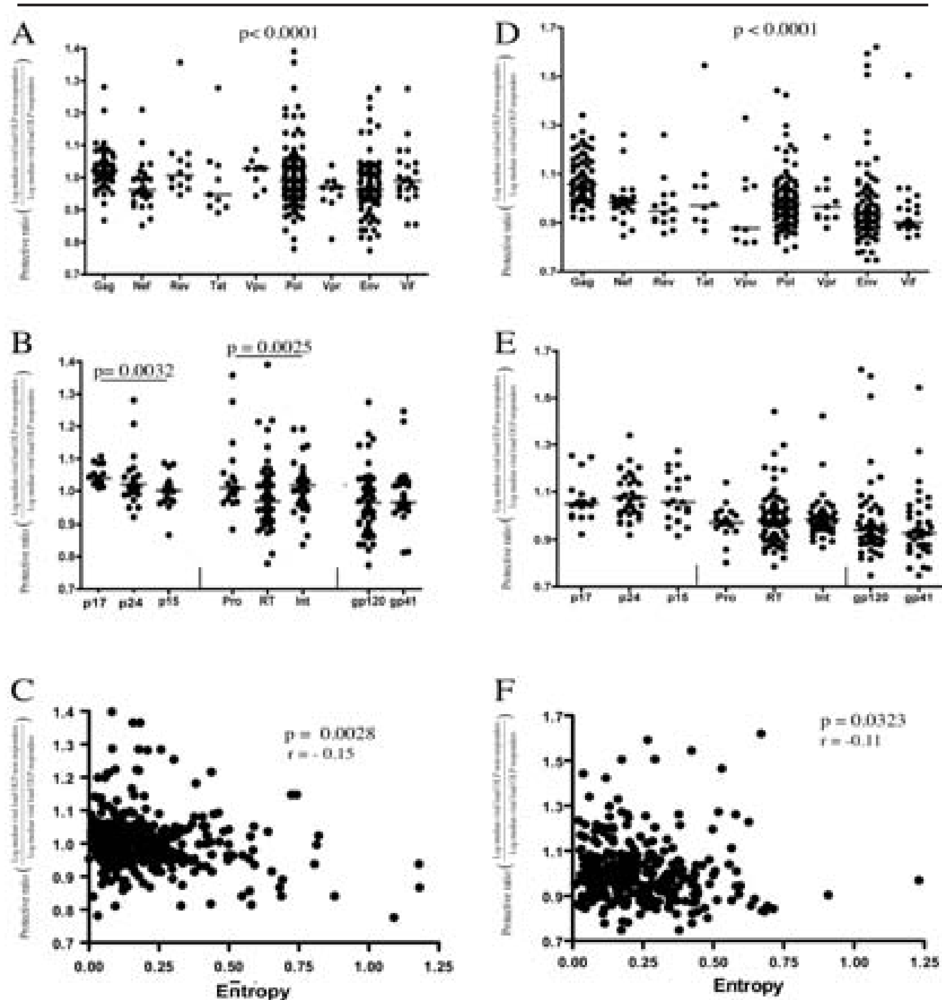

Figure 1. **Localization and conservation of beneficial and non-beneficial OLP in HIV-1 clade B and C cohorts.** Total HIV-1-specific T cell responses were assessed in a cohort of 223 chronically HIV clade B infected, untreated individuals in Lima, Peru (**graphs A-C**) and in 631 chronically HIV clade C infected, untreated individuals in Durban, South Africa (**graphs D-F**) using peptide test sets of 410 18mer overlapping peptides (OLP) spanning the consensus B and C sequences, respectively [2,23]. For each OLP, the protective ratio (PR, defined as "the ratio of the log median viral load in OLP non-responders divided by log median viral load in OLP responders") was determined. Each symbol represents an individual OLP, grouped either by (**A, D**) proteins or (**B, E**) protein-subunits for OLP located in Gag, Pol and Env (p-values in A, D based on ANOVA, in B, E on Mann-Whitney by pariwise comparing the different protein subunits, red lines indicating median PR values). In (**C and F**), the OLP-specific entropy (a measure of the viral diversity in the region the OLP spans) is compared to the OLP-specific PR and shows an inverse association between the sequence conservation and PR (Spearman rank).

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To assess whether the above observations would also hold true outside of clade B infection, the same analyses were conducted in a cohort of 631 clade C HIV-1 infected subjects enrolled in Durban, South Africa and tested for responses against a clade C consensus OLP sequence as described previously [24]. As in clade B infection, the OLP specific PR values were highest for OLP spanning Gag without any significant differences between the Gag and Pol protein subunits (Figure 1D and 1E). As in the clade B cohort, the PR values were negatively correlated with the OLP-specific entropy (p=0.0323, Figure 1F), confirming the findings in the clade B cohort and further pointing towards the importance of targeting conserved segments of the viral proteome for effective in vivo viral control.

Identification of individual beneficial OLP sequences in clade B and C infection: In order to identify individual OLP that were significantly more frequently targeted in individuals with relative viral control and to compare the beneficial OLP in clade B and C infection, the viral load distribution in OLP-responders and non-responders was analyzed individually for each OLP. For the clade B cohort in Peru, the analyses yielded 43 OLP sequences for which the median viral load differed between the two groups with an uncorrected p-value of <0.05. Of these 43 OLP, 26 were OLP with a PR >1 (referred to as "beneficial" OLP), and 17 OLP with a PR <1 ("non-beneficial" OLP, Table 1). The distribution of OLP with PR>1 among viral proteins was biased towards Gag and Pol, while Env produced exclusively OLP with PR<1 (Figure 2A).

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Table 1: Beneficial and non-beneficial OLP identified in Lima clade B cohort ($p < 0.05$)

OLP #	Protein	Protein sub-unit	OLP clade B consensus sequence	Median viral load in OLP responders	Median viral load in OLP non-responders	Protective Ratio (PR)*	p-value
3	Gag	p17	EKIRLRPGGKKKYKLKHI	22947	39014	**1.053**	0.037
6	Gag	p17	ASRELERFAVNPGLL	15380	43189	**1.107**	0.001
7	Gag	p17	ERFAVNPGLLETSEGCR	25939	38974	**1.040**	0.049
10	Gag	p17	QLQPSLQTGSEELRSLY	16285	37237	**1.085**	0.031
12	Gag	p17	SLYNTVATLYCVHQRIEV	23855	37113	**1.044**	0.037
23	Gag	p24	AFSPEVIPMFSALSEGA	22947	37113	**1.048**	0.036
31	Gag	p24	IAPGQMREPRGSDIA	3563	35483	**1.281**	0.028
34	Gag	p24	STLQEQIGWMTNNPPIPV	6127	37360	**1.207**	0.002
48	Gag	p24	ACQGVGGPGHKARVLAEA	12975	35755	**1.107**	0.041
60	Gag	p15	GKIWPSHKGRPGNFLQSR	16266	36434	**1.083**	0.044
75	Nef	-	WLEAQEEEEVGFPVRPQV	13407	37360	**1.108**	0.026
76	Nef	-	EVGFPVRPQVPLRPMTYK	59618	29855	0.937	0.001
84	Nef	-	NYTPGPGIRYPLTFGWCF	55402	30538	0.945	0.006
85	Nef	-	RYPLTFGWCFKLVPV	69890	29903	0.924	0.002
90	Nef	-	SLHGMDDPEKEVLVWKF	89687	32650	0.911	0.042
159	Pol	Protease	KMIGGIGGFIKVRQYDQI	14736	36434	**1.094**	0.020
160	Pol	Protease	FIKVRQYDQILIEICGHK	3682	35755	**1.277**	0.031
161	Pol	Protease	QILIEICGHKAIGTVLV	9117	35483	**1.149**	0.050
163	Pol	Protease	LVGPTPVNIIGRNLLTQI	25965	45637	**1.055**	0.007
171	Pol	RT	LVEICTEMEKEGKISKI	1865	35483	**1.391**	0.014
181	Pol	RT	LDVGDAYFSVPLDKDFRK	65858	32871	0.937	0.041
195	Pol	RT	LRWGFTTPDKKHQKEPPF	5624	37113	**1.219**	0.006
196	Pol	RT	DKKHQKEPPFLWMGYELH	10103	35483	**1.136**	0.044
210	Pol	RT	EIQKQGQGQWTYQIY	18155	35483	**1.068**	0.045
222	Pol	RT	PPLVKLWYQLEKEPIVGA	412599	34640	0.808	0.030
230	Pol	RT	IHLALQDSGLEVNIV	85102	34117	0.919	0.030
237	Pol	RT	VYLAWVPAHKGIGGNEQV	85102	34117	0.919	0.029
240	Pol	RT	SAGIRKVLFLDGIDKA	116902	32761	0.891	0.019
269	Pol	Integrase	TKELQKQITKIQNFRVYY	6629	35755	**1.192**	0.030
270	Pol	Integrase	TKIQNFRVYYRDSRDPLW	18171	37360	**1.073**	0.019
271	Pol	Integrase	YYRDSRDPLWKGPAKLLW	25939	35755	**1.032**	0.043
276	Pol	Integrase	KIIRDYGKQMAGDDCVA	6629	35755	**1.192**	0.021
279	Vpr	-	GPQREPYNEWTLELLEEL	60222	32650	0.944	0.042
307	Env	gp120	DLNNNTNTTSSSGEKMEK	179419	34117	0.863	0.044
311	Env	gp120	IRDKVQKEYALFYKLDVV	179419	32871	0.860	0.008
314	Env	gp120	YRLISCNTSVITQACPKV	58206	31273	0.943	0.008
315	Env	gp120	SVITQACPKVSFEPIPIH	61011	32871	0.944	0.034
320	Env	gp120	TNVSTVQCTHGIRPVV	341587	34640	0.820	0.034
355	Env	gp120	VAPTKAKRRVVQREKRAV	161602	34117	0.870	0.042
399	Env	gp41	VIEVVQRACRAILHIPRR	388089	34640	0.812	0.026
405	Vif	-	VKHHMYISGKAKGWFYRH	16458	37237	**1.084**	0.021
406	Vif	-	GKAKGWFYRHHYESTHPR	16458	37237	**1.084**	0.022
424	Vif	-	TKLTEDRWNKPQKTKGHR	10319	36434	**1.137**	0.014

* PR values in bold indicate PR > 1, i.e. OLP-responses seen more frequently in individuals with reduced viral loads

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The same analyses were repeated for the clade C cohort in Durban, which due to its larger size allowed to apply more stringent statistical criteria to identify beneficial and non-beneficial OLP. To compensate for multiple statistical comparisons, we employed a previously described false-discovery rate approach [25], resulting in the identification of 33 clade C OLP with q-values of <0.2 (i.e. OLP with significantly different viral load distributions between OLP-responders and non-responders with a false positive discovery rate (q-value) of 20%). The 33 OLP identified were comprised of 22 beneficial OLP and 11 non-beneficial OLP, with the beneficial OLP being again located in Gag, Pol and Vif, similar to what was seen in the clade B cohort (Figure 2B).

Table 2: Beneficial and non-beneficial OLP identified in Durban clade C cohort (q<0.2)

OLP #	Protein	Protein sub-unit	OLP clade C consensus sequence	Median viral load in OLP responders	Median viral load in OLP non-responders	Protective Ratio (PR)*	p-value	Q-value
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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In both cohorts, the total breadth and magnitude of responses did not correlate with viral loads as reported for parts of these cohorts in the past [14,16]. The OLP with significant differences in median viral loads (43 OLP in clade B and 33 OLP in clade C, Tables 1 and 2, respectively, i.e. "scoring OLP"), were more often targeted in their respective cohort than OLP that did not score with a significant difference in viral loads (p=0.0015 Lima; p<0.0001 Durban). However, beneficial and non-beneficial OLP were equally frequently targeted in either cohort. Also, there was no difference in the median magnitude of the OLP-specific responses, regardless whether it was a beneficial, non-beneficial or not-scoring OLP (all p>0.7, data not shown). Finally, there was no correlation between the number of total OLP responses (against all 410 OLP) and the magnitude of responses to beneficial OLP in either cohort, indicating that the strength of beneficial OLP responses was not diminished by other responses to the rest of the viral proteome.

In the clade B cohort, the 26 beneficial and 17 non-beneficial OLP showed a significant difference in their median entropy (p=0.0327, Figure 2C), in line with the overall negative association between higher PR and lower sequence entropy seen in the comprehensive screening including the entire 410 OLP set (Figure 1C). While this comparison was not significant in clade C infection, a detailed look at Gag showed that beneficial Gag clade C OLP had a lower entropy values than the rest of the Gag OLP, suggesting that targeting of the most conserved regions even in Gag provided particular benefits for viral control (Figure 2D, p=0.0172). These beneficial OLP were also more frequently targeted (median of 36 responders) compared to the rest of Gag OLP (median 12 responders, p=0.0099), likely reflecting the high epitope density in these regions [24,26].

Finally, the two cohorts showed a partial overlap in the targeted beneficial and non-beneficial OLP, despite the vastly different HLA genetics in these two populations [4,23,27,28]. As Gag was enriched in beneficial OLP scattered throughout the entire protein sequence, we used the available reverse transcriptase (RT) protein structure to assess whether beneficial responses were targeting structurally related regions of the protein, even though the linear position of beneficial OLP did not precisely match between the two clades. Indeed, superimposing the locations of beneficial OLP in the RT protein indicates that in both clades, beneficial OLP fell in structurally related domains of the RT protein (Figure 2E and 2F). This suggests that despite differences in response patterns between ethnicities and clades, viruses from both clades may be vulnerable to responses targeting the same structural regions of at least some of their viral proteins.

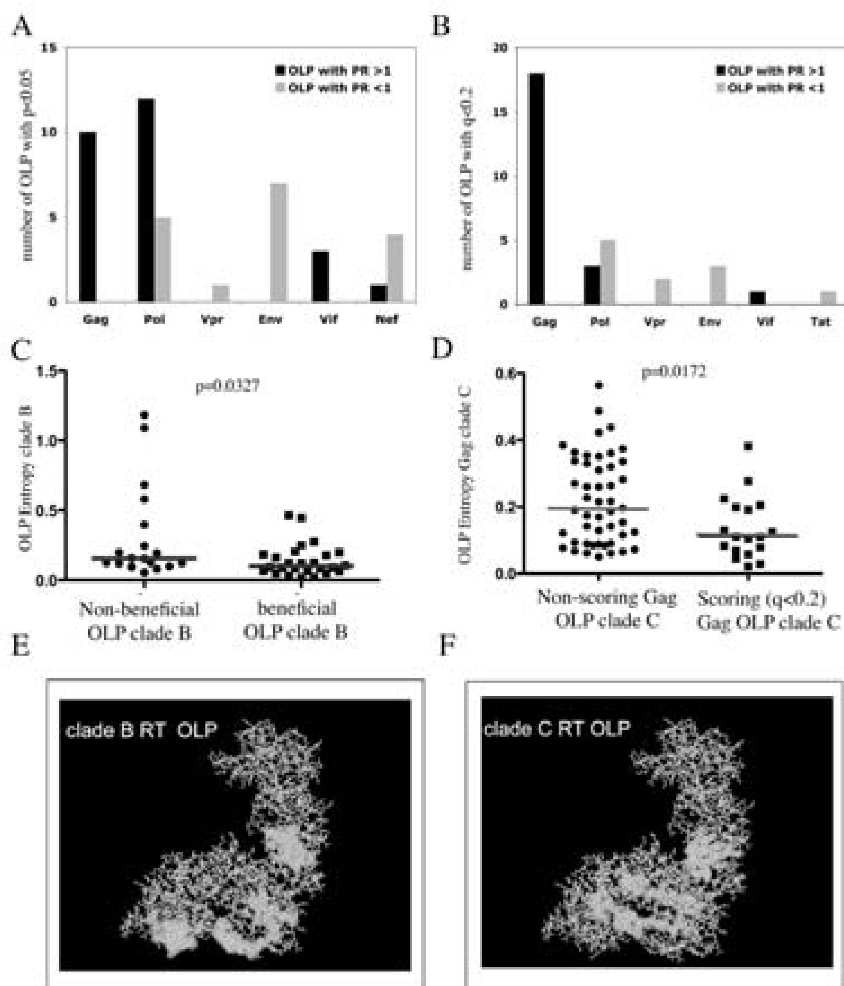

Figure 2. **Genome distribution, entropy and RT localization of OLP with significant impact on viral loads in HIV-1 clade B and C infection:** The distribution of OLP with significantly elevated or reduced PR across the viral proteome is shown in **A)** for clade B infection (cut-off uncorrected p-value of $p<0.05$) and in **B)** for clade C infection (cut-off $q<0.2$). The entropy of beneficial and non-beneficial clade B OLP is compared in **C)** while in **D),** the entropy of beneficial OLP in HIV clade C Gag is compared to the remainder of Gag OLP (p-values based on Mann Whitney, red lines indicating median sequence entropies). In E and F, protein structures for HIV-1 reverse transcriptase (Protein databank structure ID 3IG1) were loaded into the Los Alamos HIV Database "protein feature accent" tool (http://www.hiv.lanl.gov/content/sequence/PROTVIS/html/protvis.html) and locations of beneficial RT OLP identified in clade B (Table 1) and in clade C (Table 2) marked by red highlights.

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Increased breadth of responses against beneficial OLP is associated with decreasing viral loads, independent of Gag-specificity or the presence of protective HLA class I alleles: To assess whether individuals targeting more than one beneficial OLP profit from a greater breadth of responses to these targets, subjects in both cohorts were stratified by the number of responses to beneficial OLP and their viral loads compared. In both cohorts, negative correlations between the number of responses to beneficial OLP and viral loads were observed (p<0.0001, r=-0.33 for Lima; p<0.0001, r=0.-25 for Durban; data not shown), suggesting that there is a cumulative benefit of responses to these particularly effective targets. Similarly, when individuals in the clade C cohort were grouped based on mounting 1-2, 3-4 or five and more beneficial OLP responses, a gradual reduction in median viral loads was seen. This reduction was close to 20-fold when 5 or more of the 22 beneficial OLP were targeted (median viral load 5,210 copies/ml) compared to individuals without a response (98,800 copies/ml, Figure 3A). Importantly, this observation was not driven only by individuals expressing HLA class I alleles associated with relative control of viral replication (including HLA-B27, -B57, -B*5801, -B63 and -B81) as their exclusion still showed a strong association between increased breadth of responses to beneficial OLP and a gradual suppression of viremia (Fig 3B). This was further supported when translating the clade B data from Peru to a second clade B infected cohort in Barcelona, Spain where HIV-1 controllers also mounted a significantly greater proportion of their responses to the beneficial Peruvian OLP compared to the HIV-1 non-controllers (61% vs. 29%, p=0.0011; Figure 3C); this despite the fact that the Barcelona cohort was genetically different and excluded individuals expressing HLA-B27, -B57, -B58 and B63. Thus, despite the frequent targeting of Gag and the inclusion of individuals expressing HLA alleles such as HLA-B*5701 and –B*5801 in the two larger clade B and C cohorts, the present data identify regions of the viral genome that serve as the targets of an effective host T cell response, largely independent of the presence of HLA alleles known to influence HIV-1 viral replication.

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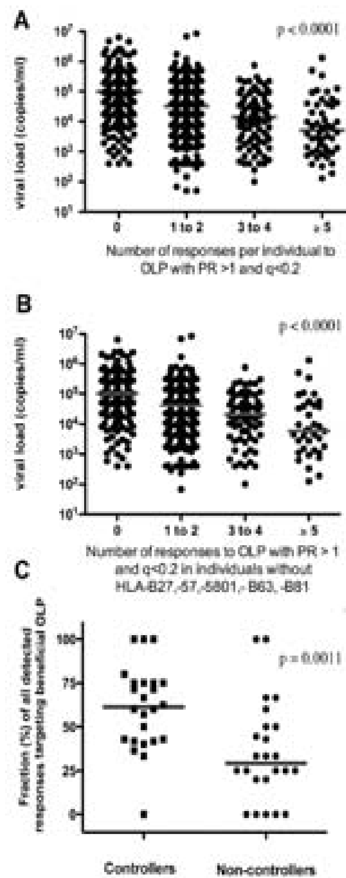

Figure 3 **Increased breadth of responses to beneficial OLP results in gradually reduced viral loads and is independent of cohort and HLA-B27, -57, -B58, -B81 and –B63.** (**A**) The number of responses to beneficial OLP in the clade C cohort in Durban was determined for each individual and compared to viral loads. An increased breadth of responses to the 22 beneficial OLP was associated with a reduced viral loads (ANOVA, p<0.0001). (**B**) This association remained equally stable after removing all individuals expressing known beneficial HLA allele (HLA-B27, -B57, -B5801, -B63, -B81) from the analysis (ANOVA, p<0.0001). (**C**) The set of 26 beneficial and 17 non-beneficial OLP identified in the clade B infected cohort in Lima, Peru was tested in a second clade B infected cohort in Barcelona. HIV controllers showed a significantly higher focus of responses on the 22 beneficial OLP (61% of all responses to the 43 OLP) while non-controllers reacted predominantly with the non-beneficial OLP (only 29% of all responses targeting beneficial OLP). The Barcelona cohort did not included subject expressing any HLA allele previously associated with relative control of HIV-1 (p=0.0011, Mann Whitney).

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PR-values are mediated by individuals with broad HLA heterogeneity: To further assess the contribution of specific HLA class I alleles on the PR of individual OLP, the statistically significant OLP in the clade C cohort were further analyzed. In a first step, median viral loads in the OLP-responder and non-responder groups were compared after excluding individuals with specific HLA class I alleles. If the statistical significance of the comparison was lost, the excluded HLA class I allele was assumed to have significantly contributed to the initially observed elevated or reduced PR value and to restrict a potential CTL epitope in that OLP. In a second step, a "Hepitope" analyses (http://www.hiv.lanl.gov/content/immunology/hepitopes) was conducted to identify HLA class I alleles overrepresented in the OLP responder group; providing an alternative approach to identify specific epitopes that may contribute to relative viral control. Together, the two strategies permit to estimate the HLA diversity in the OLP responders and to identify the most likely alleles that restrict the epitope-specific responses to the OLP. Both are important measures when determining the relative usefulness of a selected beneficial OLP in a potential immunogen sequence as it should provide broad HLA coverage. The data from these analyses are summarized for beneficial and non-beneficial OLP in Table 3A and 3B, respectively. The results demonstrate that with a few exceptions, for each OLP, several HLA alleles appeared to be mediating the observed effects as their removal caused the statistical significance to be lost. However, for the most frequent HLA class I alleles, the loss of significance may be due to a reduction in sample size rather than the actual allele, since the exclusion of many allele carriers could reduce the number of OLP responders (and non-responders) sufficiently to lose statistical power. The "Hepitope" analysis controlled for this effect and confirmed the obtained results, strongly indicating that responses to all beneficial OLP were mediated by responder populations with heterogeneous HLA allele distributions.

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Table 3: Impact of individual HLA alleles on the statistical significance of observed PR values (clade C OLP)

OLP #	Protein	Protective Ratio	p-value PR	Removed HLA allele(s) abolishing statistical significance[1]	Alleles over-represented in the OLP responder group[2]
A) Beneficial OLP (PR > 1)					
3	Gag	1.09	0.0002	A30,B42,C17	A30,B08,A03,A74,C17,A43,B42,B07
6	Gag	1.22	0.0000	B15	B49,B82,C14
7	Gag	1.25	0.0000	-	B42,C17,B45,A30
22	Gag	1.18	0.0000	B57,C07	B57,A74,B45,C07,C16,B13
25	Gag	1.06	0.0021	A30,B15,C04,C07	B42,C17,B81,B39,A01,C12,C18,A30,B67
26	Gag	1.23	0.0061	A02,A23,A68,B07,B14,B58,C07,C08	C03,B15,A68
27	Gag	1.16	0.0015	B15,C07	B15,A68,C03,C08
29	Gag	1.07	0.0045	A68,B15,B58,C02,C03,C06,C12	B35,B39,C12,B40,B07,C04
31	Gag	1.20	0.0146	A02,A11,A23,A29,A32,A34,A68,B07,B13,B15,B42,B44,B58,C04,C06,C07,C17	B13,A29,C06,A11
33	Gag	1.13	0.0025	A02,A23,B44,B57,B58,C07	B58,B57,A02,C07,C03,A68
37	Gag	1.17	0.0004	A30,B42,B58,C17	C18,B42,C17,A01,B81
39	Gag	1.34	0.0182	A02,A03,A23,A29,A30,A68,A74,B08,B15,B18,B42,B45,B53,B57,B58,C02,C03,C06,C07,C08,C16,C17	A02
41	Gag	1.07	0.0020	A23,C06	C03,B14,A68,C08,B15
42	Gag	1.09	0.0078	A23,A30,B08,B15,B42,B53,B58,C03,C04,C07	B53,C03
55	Gag	1.19	0.0092	A02,A24,A29,A30,B07,B15,B39,B42,B44,B58,C02,C06,C07,C17	B42,B08,C17
59	Gag	1.16	0.0046	A02,A30,B08,B42,B44,B58,C04,C07,C17	A02,B13,A29
60	Gag	1.21	0.0066	A02,A30,B42,B58,C06,C07,C17	A02,B41,C07,C17
63	Gag	1.21	0.0093	A02,A23,A29,A30,A68,B08,B15,B44,B58,C02,C03,C06,C07	A23
181	Pol	1.19	0.0186	A01,A23,A29,A30,A34,A68,A74,B14,B15,B18,B35,B44,B45,B57,B58,C02,C03,C04,C06,C07,C08,C16	B57,C18
199	Pol	1.20	0.0198	A02,A03,A23,A24,A26,A30,A31,A34,A36,A66,A68,A80,B08,B13,B15,B18,B35,B40,B41,B42,B44 B45,B49,B50,B51,B53,B57,B58,B81,C01,C02,C03,C04,C05,C06,C07,C08,C15,C16,C17	B53,A23,C04
216	Pol	1.09	0.0026	A02,A30,B58,C07,C17	B53,B58,C07,B57
417	Vif	1.50	0.0178	A03,A23,A30,A34,A36,A68,B08,B14,B15,B44,B53,B58,C03,C04,C06,C08	B14,C08,A36
B) non-beneficial OLP (PR < 1)					
116	Tat	0.91	0.0033	A02,A34,B15,C04	B15,C02
178	Pol	0.84	0.0033	A03,A68,B15,B58,C04,C06,C07	A68,C06,B58,B82,A03
190	Pol	0.92	0.0043	A03,A30,A66,B18,B42,B45,B58,C06,C07	A02,B18,B35,C05,C16,B45,A80,C12,B67,B39
239	Pol	0.82	0.0205	-	C05,A03
253	Pol	0.92	0.0082	A03,A68,B15,B39,B42,B44,B58,C02,C04,C06,C08,C17,C18	A68,C03,B15,B07,C15,B41
265	Pol	0.94	0.0178	A02,A03,A23,A24,A26,A29,A30,A32,A33,A34,A66,A68,B07,B08,B14,B15,B27,B40 B41,B42,B44,B53,B54,B55,B57,B81,C01,C02,C04,C06,C07,C12,C17,C18	B15,C02,A43,A74
283	Vpr	0.93	0.0126	A02,A03,A23,A30,A66,A68,A74,B07,B14,B18,B39,B41,B42,B45,B57,C02,C04,C07,C08,C15,C17	A68,C03,B07,C17,B41
284	Vpr	0.92	0.0099	A03,A23,A30,A66,A68,A74,B07,B14,B18,B39,B42,B45,B57,C07,C08,C15,C17	A68,C03
312	Env	0.84	0.0208	-	B08,C07
365	Env	0.88	0.0001	B58,C06	C06,B58,A43,B45,C16,A66
393	Env	0.78	0.0007	A30,B58,C06	A31,C06,B45

[1] in italics HLA alleles that do not emerge from the Hepitope analysis [2] cut-off in Hepitope analyses for p<0.05, alleles sorted according to strength of association

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Effects of T cell specificity on in vivo viral load are at least as strong as those associated with host HLA genetics: To assess whether specific response patterns and/or HLA combinations could be identified that mediated synergistic or superior control of viral infection in clades B and C, multivariate combined regression analysis was conducted on either OLP only, HLA only or the combination of OLP and HLA variables [29,30,31]. The OLP-only analysis for Lima identified 7 OLP of which 4 were associated with lower median viral loads and 3 with increases in viral loads, respectively (Table 4). Targeting at least one of these beneficial clade B OLP was associated with significantly reduced viral loads (median 11,079 copies/ml) compared to the subjects who did not target any of these four OLP (median 52,178 copies/ml; $p<0.0001$, Figure 4A). As seen in the univariate analysis (Figure 2C), the four beneficial OLP emerging from the Lima FASS analysis were more conserved than the rest of the OLP (median entropy 0.0759 vs. 0.1649, $p=0.0267$) or the three non-beneficial OLP (0.0759 vs. 0.1228, $p=0.0571$, data not shown). In contrast to OLP-only FASS analysis, only one HLA allele (HLA-C04) emerged from the HLA-only multivariate analysis. The analysis for the combined variables (OLP and HLA) controlled for the potential bias in this result due to more OLP variables (n=389) than HLA (n=146) being included in the statistical tests; yet still identified more OLP variables (n=9) than HLA class I alleles (n=3). In addition, the relative co-efficients of these associations were stronger for the OLP than the HLA variables, suggesting that T cell specificity influenced viral loads to at least the same degree as host HLA class I genetics. Of note, the identified OLP and HLA variables did not reflect responses to known optimal CTL epitopes, as none of the OLP contained described epitope(s) restricted by any of the identified HLA alleles [26].

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Table 4: Multivariate analysis of OLP and HLA variables for clade B and C cohorts

OLP variables only (Lima, clade B)

	change viral load (co-efficient) *	p-value
Beneficial		
OLP.6	-0.4591	0.0008
OLP.31	-1.4055	0.0002
OLP.171	-2.5981	0.0000
OLP.276	-1.1270	0.0007
Non-beneficial		
OLP.76	0.2486	0.0067
OLP.306	3.2968	0.0001
OLP.411	1.3329	0.0120

OLP variables only (Durban, clade C)

	change viral load (co-efficient) *	p-value
Beneficial		
OLP.7	-0.6256	0.0000
OLP.21	-0.6663	0.0000
OLP.22	-0.4926	0.0006
OLP.25	-0.2822	0.0002
OLP.27	-0.4719	0.0053
OLP.33	-0.3396	0.0024
OLP.398	-1.8179	0.0027
OLP.417	-1.6535	0.0008
Non-beneficial		
OLP.38	0.9045	0.0022
OLP.84	0.1947	0.0091
OLP.116	0.6156	0.0039
OLP.183	0.6610	0.0013
OLP.224	0.2508	0.0036
OLP.265	0.5782	0.0000
OLP.365	0.4911	0.0009
OLP.393	1.2624	0.0013

HLA variables only (Lima, clade B)

	change viral load (co-efficient) *	p-value
Non-beneficial		
HLA-C0401	0.35652	0.00024

HLA variables only (Durban, clade C)

	change viral load (co-efficient) *	p-value
Beneficial		
HLA-A74	-0.3553	0.0025
HLA-B13	-0.6443	0.0004
HLA-B57	-0.5195	0.0007
HLA-B81	-0.3619	0.0015
HLA-C12	-0.6544	0.0001
Non-beneficial		
HLA.B.15	0.2506	0.0012
HLA.B.18	0.5521	0.0005
HLA.C.6	0.3958	0.0000

HLA and OLP variables together (Lima)

	change viral load (co-efficient) *	p-value
Beneficial		
OLP.6	-0.5792	0.0000
OLP.31	-1.1607	0.0005
OLP.171	-2.7948	0.0000
OLP.276	-0.9609	0.0011
Non-beneficial		
OLP.2	0.3945	0.0180
OLP.237	0.7211	0.0035
OLP.288	1.5537	0.0016
OLP.311	0.7197	0.0091
OLP.411	1.5306	0.0024
Beneficial		
HLA-B1502	-1.4688	0.0164
Non-beneficial		
HLA-B0801	0.6600	0.0049
HLA-C0401	0.2894	0.0006

HLA and OLP variables together (Durban)

	change viral load (co-efficient) *	p-value
Beneficial		
OLP.6	-0.4798	0.0023
OLP.7	-0.4528	0.0015
OLP.27	-0.4676	0.0049
OLP.59	-0.4196	0.0115
OLP.417	-1.3870	0.0041
Non-beneficial		
OLP.148	2.5215	0.0029
OLP.183	0.6108	0.0023
OLP.393	1.1442	0.0023
Beneficial		
HLA-A74	-0.3744	0.0007
HLA-B57	-0.4887	0.0007
HLA-B81	-0.3859	0.0004
HLA-C12	-0.6003	0.0002
Non-beneficial		
HLA-B15	0.2797	0.0001
HLA-B18	0.5316	0.0003
HLA-B49	0.9713	0.0007

* negative co-efficient values indicate reduction in median viral loads

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Results from the clade C cohort in Durban confirmed the clade B findings in Lima as the FASS analyses identified 16 OLP but only 8 HLA variables that had an impact on the individual viral loads. As in Lima, the impact of OLP specificity was at least as strong than HLA genotype (trend for higher coefficients for OLP than HLA; data not shown, $p>0.05$). In addition, targeting at least one of the eight beneficial OLP in Durban was associated with strongly reduced viral loads ($p<0.0001$, Figure 4B). This effect was, as in the univariate analysis, additive for more than one response ($p<0.0001$, Figure 4C) and included OLP that were, aside from Gag, located in Pol and Vif. Also, the combined (OLP and HLA) analysis suggests the effect of OLP specificity on viral loads to be at least as strong as HLA genetics as 8 OLP and 7 HLA variables were identified. This especially since among the 7 HLA alleles, two (HLA-B57 and HLA-A74) are expressed in linkage disequilibrium [32], further reducing the number of HLA variables with a significant impact on viral loads.

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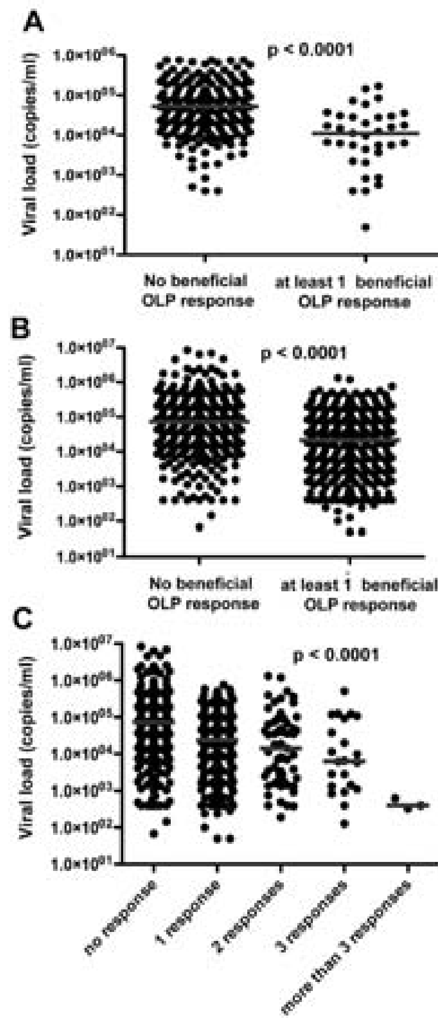

Figure 4. **Responses to OLP identified in multi-variate analysis are associated with reduced viral loads**: Response patterns and HLA class I genetics in the clade B cohort in Lima and clade C cohort in Durban were subjected to FASS multivariat analysis [29,30,31]. Viral loads in individuals mounting zero vs. at least one response to beneficial OLP identified by the FASS multi-variate analysis were compared for (**A**) the Lima clade B cohort and the (**B**) Durban clade C cohort. The larger data set for the clade C cohort allowed for a further stratification of the responder group by increasing numbers of targeted OLP emerging from the FASS analysis (**C**). A gradually declining median viral load in relation to an increasing breadth of these responses was seen (ANOVA, $p < 0.0001$).

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Responses to beneficial OLP are of higher functional avidity and suppress viral replication in vitro more effective than responses to non-beneficial OLP: Functional avidity and the ability to suppress in vitro viral replication have emerged as two potentially crucial parameters of an effective CTL response against HIV-1 [33,34,35,36,37,38,39]. To assess this potential functional characteristic of beneficial CTL populations, we determined the functional avidity of responses to the four beneficial OLP located in Gag p24, a region that has been most consistently associated with eliciting relatively protective CTL responses. As 18mer peptides are suboptimal test peptides to determine functional avidity, 10mer overlapping peptide sets were synthesized to cover the four beneficial OLP and all detected responses were titrated. The SD50% was determined for a comparable numbers of responses detected in controllers (n= 21 responses) and non-controllers (n= 24 responses) and showed a statistically significant difference between the two groups (median 3,448 ng/ml vs. 25,924 ng/ml, p = 0.0051, Figure 5A). This reduced avidity in HIV non-controllers to beneficial OLP could possibly explain why HIV-1 non-controllers did not control their in vivo viral replication despite targeting these regions in some instances and with responses of comparable magnitude as HIV controllers (278 SFC vs 305 SFC/10^6 PBMC, p= 0.55, data not shown).

To more directly assess whether responses to beneficial OLP were of particularly high functional avidity, regardless of HIV controller status, we determined SD50% of responses to 17 optimal epitopes from beneficial, neutral and non-beneficial OLP (Figure 5B). Median epitope-specific SD50% were determined from an average of 7 titrations per epitope and compared to the OLP specific PR. A strongly significant, negative association between the PR and the SD50% was noted (p=0.002, r=-0.69), indicating that beneficial OLP are targeted by high-avidity responses. To control for inter-individual differences due to disease status and viral load, we identified 10 individuals who targeted optimal epitopes in beneficial and non-beneficial OLP and determined their functional avidity. As in the cross-sectional analysis before, this matched comparisons showed in all cases a higher functional avidity for the epitopes located in the beneficial OLP compared to the responses targeting non-beneficial OLP (Figure 5C, p=0.0020). Lastly, to relate the higher functional avidity to potential superior anti-viral effects in vivo, the ability to inhibit in vitro viral replication was assessed in three individuals who mounted robust responses against both beneficial and non-benefi-

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cial OLP. The in vitro inhibition assay first developed by Yang et al [40], was modified so that the NL4-3 based test virus contained a single nucleotide mutation in Nef (M20A) that blocks the Nef-mediated downregulation of HLA class I molecules as well as two mutations in the integrase gene that mediate raltegravir-resistance to permit the suppression of potentially replicating autologous virus in the assay. Indeed, CTL specific for the beneficial OLP(s) were up to 2 logs more effective inhibiting viral replication than CTL targeting non-beneficial OLP (Figure 5D), in line with recent data demonstrating different suppressive ability of HIV-1 specific CTL populations targeting Gag and Env-derived epitopes [34]. Although the in vitro inhibition assays were limited to few individuals with suitable response patterns, these data together with the results from the extensive titration assays in Figure 5B and 5C indicate that responses to beneficial OLP are of particularly high functional avidity and inhibit in vitro viral replication more effectively than responses to non-beneficial OLP. Of note, higher avidity responses to beneficial OLP compared to non-beneficial OLP were seen in all 10 tested individuals, ruling out that inter-individual variability in viral loads, duration of infection and HIV disease status could have biased the analyses.

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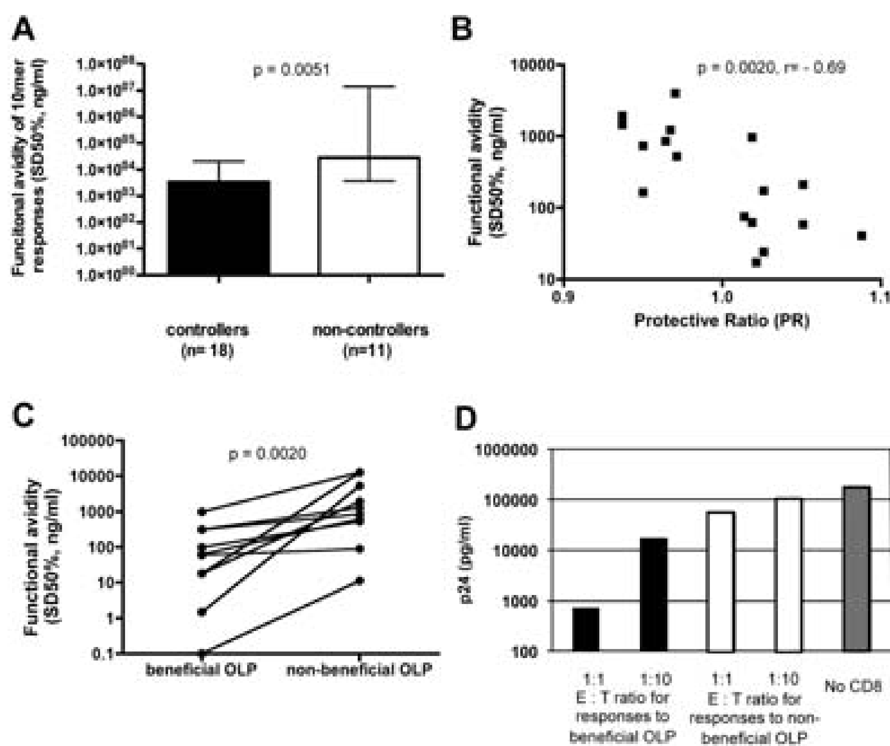

Figure 5 **Responses to beneficial OLP are of higher functional avidity and suppress in vitro viral replication more effectively.** (**A**) Responses to the four beneficial OLP located in HIV-1 clade B Gag p24 were retested using a peptide set of 10mers overlapping by 9 residues. A total of 21 responses in HIV-1 controllers and 24 responses in HIV-1 non-controllers were titrated and the SD50% compared between the two groups, showing a significantly higher functional avidity in the controllers (p=0.0051, Mann Whitney). (**B**) Responses to 17 different optimally defined CTL epitopes located in beneficial, neutral and non-beneficial OLP were titrated in samples from 78 HIV infected individuals with variable viral load and disease status. The median SD50% (ng/ml) was defined for each epitope and compared to the OLP-specific protective ratio (Spearman Rank test, p=0.0020). (**C**) Ten individuals who mounted responses to well-defined optimal CTL epitopes located in beneficial as well as in non-beneficial clade B OLP were identified and their responses titrated. The SD50% for responses detected in the same individual were compared (Wilcoxon matched pairs test, p=0.0039). (**D**) In-vitro viral replication inhibition assays [40] were performed using a Nef modified and raltegravir resistant test virus and purified CTL effector populations from the same individual targeting beneficial and non-beneficial OLP. One representative experiment of three assays conducted in different individuals is show. Levels of Gag p24 were determined after 4 days of co-culture of effector cells and auologous CD4 T cells used as target cells. Target cells were stimulated 3 days prior with dual-specific anti-CD3/8 mAb and infected at a MOI of 0.1. The negative control contained wells with target cells only ("no CD8").

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CONCLUSIONS

Defining functional correlates of HIV-1 immune control is critical to the design of effective immunogens. T cell responses to specific HIV-1 proteins and protein-subunits have been associated before with relatively superior viral control in vivo[14,16,41], but evidence from recent clinical trials suggests that including maximal immunogen content into various vectors does not necessarily induce more effective CTL responses [42,43]. In fact, it has been argued that the existence of potential "decoy" epitopes may divert an effective CTL response towards variable and possibly less effective targets in the viral genome [44]. Thus, the definition of a minimal yet sufficient immunogen sequence that can elicit CTL responses in a broad HLA context is urgently needed. Thereby, focusing vaccine responses on conserved regions could help induce responses towards mutationally constrained targets and provide the basis for protection from heterologous viral challenge.

We present here the results of an extensive analysis that included more than 950 HIV-1 infected individuals with diverse HLA genotypes, from three different continents and including clade B and C infections. In both, the analysis in clade B in Lima and clade C in Durban, individual OLP were identified that are predominantly targeted by individuals with reduced or elevated viral loads, although the different size of the cohorts required different statistical approaches for their identification. In general, most of these OLP were among the more frequent targets in the HIV proteome, possibly due to both, the need for sizable responder groups to achieve statistical significance in the viral loads comparison as well as the high epitope density in these OLP. The identified OLP were frequently located in HIV-1 Gag and Pol, but rarely in the more variable proteins such as Env and Nef. With one exception, Nef and Env featured only non-beneficial OLP, thus arguing against their inclusion, at least as full proteins, in a CTL immunogen sequence [16]. In addition, in both cohorts, the Vif protein yielded few, yet exclusively beneficial OLP, which may warrant a renewed look at the inclusion of regulatory proteins in vaccine design [45,46]. Also common to both clades, (and despite the wide scatter possibly due to the inclusion of less-frequently targeted OLP), an negative correlation between sequence entropy and PR was observed providing strong rationale for vaccine approaches that focus on conserved viral regions where T cell escape may be complicated by structural constrains [47]. This was particularly evident in the clade C cohort, where even within the relatively conserved Gag protein, a lower entropy was seen for the beneficial OLP compared to the remainder of the OLP spanning the protein. On the other hand, while beneficial and non-beneficial OLP showed a sig-

nificant difference in their median entropy in the clade B cohort, this comparison was not significant in the clade C cohort. It is possible that the immunogen sequence, designed in 2001, did not optimally cover the circulating viral population in Durban throughout the enrollment period (until 2006), leading to missed responses particularly in the more variable segments of the virus [48,49]. The study may have thus failed to identify beneficial as well as non-beneficial OLP in the more variable genes of HIV. This should have preferentially affected highly variable OLP due to a more frequent mismatch between autologous viral sequence and in vitro test set in these regions. However, even if scoring as beneficial OLP, such high-entropy OLP may from an immunogen-design point of view be of less interest as they would possible contribute only little to protection from heterologous viral challenge. It needs however also to be considered that the OLP-specific entropy values are based on variable numbers of sequences in the Los Alamos HIV database covering the different OLP, introducing potential further bias into these analyses, particularly for less covered proteins such as vpu and other viral genes. Such differences between autologous viral sequences and in vitro test sets may also have impacted the assessment of functional avidities. These determinations included responses in the same individual towards epitopes located in beneficial and non-beneficial OLP; with the former overall being more conserved. Thus, the higher functional avidity towards epitopes located in beneficial OLP could be biased by the higher chance that these epitopes matched the autologous viral sequence compared to epitopes located in non-beneficial OLP and which may thus have induced a more robust, avid response. Apart from covering autologous sequences, future studies will ideally also include comparable analyses in individuals identified and tested in acute infection that go on to control the infection at undetectable levels of viral replication (i.e. elite-controllers) so that the selective early emergence of responses to beneficial OLP could be linked to relative control of viral replication in chronic infection. As is, the identified beneficial responses may be particularly important to maintain low viral replication in chronic stages of infection, which in theory could be different (for instance due to more accelerated intra-individual viral evolution in variable genes) from responses determining viral set point during acute infection. However, the existing HLA bias in such cohorts and the small number of responses identified during earliest stages of infection may make such analyses a formidable undertaking that will require large numbers of individuals to be tested longitudinally.

A broadly applicable T cell immunogen sequence should include T cell targets restricted by a wide array of HLA class I alleles. Although broad representation of HLA-B alleles may be particularly important in this regard, emerging

data on the effects HLA-C alleles in these cohorts may warrant a broad HLA-C representation as well [2,32,50]. In the present study, the 26 beneficial OLP from Lima and the 22 beneficial OLP from Durban covered 26 described, optimally defined CTL epitopes restricted by 20 different HLA alleles for the clade B cohort and 33 epitopes presented by 34 alleles for the clade C cohort, respectively [26]. As this is likely to be an underestimate of the true diversity in HLA restriction (Table 2 and ref [51]), it is reasonable to predict that the inclusion of identified beneficial OLP, or even a subset thereof, could evoke potential responses in a widely diverse HLA context. This could also provide the basis for the induction of polyspecific T cell responses with increased breath, which the present data clearly associates with progressively lower viral loads and which emerge as a potentially important parameter from several recent vaccine studies showing superior protection from SIV challenge in animals with a broad vaccine induced responses to Gag p17 [52,53].

Recent studies have suggested a global adaptation of HIV-1 to its various host ethnicities [4,28]. The consequence of such adaptation has led in some cases to the elimination of protective CTL targets, causing a profound absence of responses to these epitopes and detrimentally changing the association between HLA allele and HIV-1 disease outcome [4]. It is thus not surprising that the two main cohorts tested here yielded only partially overlapping sets of beneficial OLP as the impact of host genetics and viral evolution in the studied populations cannot readily be overcome. In fact, given studies by Frahm et al [4], the past and current adaptation of HIV-1 to common HLA class I alleles will likely still call for somewhat population tailored vaccine approaches, especially if the immunogen sequences should be kept short to avoid regions of potentially reduced immunological value [44]. Such approaches will also profit from more extensive structural analyses that may identify specific domains of viral proteins that are or are not enriched in valuable T cell targets; of which the latter could possibly be ignored for the design of T cell immunogen sequences. Additional analyses in other genetically unrelated cohorts of HIV-1 infected individuals and studies in SIV infection may further help to guide such selective immunogen design and to understand the factors defining the effectiveness of different epitopes in mediating relative HIV-1 control. Of note, the beneficial OLP identified here, 24 in clade B and 22 in clade C infection matched other immunogen design based on conserved elements in some parts as well, i.e. of the 14 conserved elements proposed by Hanke et al, eight (57%) overlapped at least partly with beneficial OLP identified here [54]. Similarly, among the highly conserved elements proposed by Rolland et al [44], 35% (5/14) were covered by our beneficial OLP in clade B infection. These differences possibly emerge because

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the present analysis is based on functional T cell data rather than viral sequence alignments, which may not take into consideration epitope density and processing preferences of certain regions. Nevertheless, the partial overlap with these other immunogen design support the focus on conserved regions and offers the opportunity for alternative or combined vaccine approach that elicit responses to regions where the virus is and possibly remains vulnerable [4,28,47,55].

Finally, we used the extensive data set available to approach the question of relative effects of host genetics (i.e. HLA) and CTL specificity on HIV-1 control. While the two factors cannot be entirely disentangled, our data suggest that CTL specificity has an at least equal if not stronger effect on viral control than HLA class I allele expression. These findings are also in line with data by Mothe et al [56] showing that targeting key regions in p24 surrounding the dominant epitopes restricted by known protective alleles (KK10 for HLA-B27 and TW10 for HLA-B57/58) in HLA-B27, -57 or B58 negative individuals is associated with significantly reduced viral loads. In addition, the presence of individuals not expressing known beneficial alleles in HIV-1 elite controller cohorts [57], further indicates that HIV-1 control is not necessarily bound to a few specific HLA class I alleles. A detailed study of the total HIV-1-specific CTL response of subjects not expressing these alleles yet effectively controlling HIV-1 can be expected to provide further and crucially needed insight into the importance of targeting specific (conserved) regions of the viral genome for HIV-1 control. Similarly, the characterization of functional attributes of these responses, including functional avidity and the ability to suppress *in vitro* viral replication will need to be further assessed in such individuals. Building on experimentally derived and potentially promising immunogen sequences as defined here may thus provide a suitable basis for further immunogen design and iterative clinical trials in the human setting.

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COMPETING INTEREST

The authors declare no financial or commercial conflict of interest

AUTHORS CONTRIBUTION

BM conducted conducted cellular immune analyses, in vitro inhibition analyses and drafted the first version of the manuscript. AL, JZ, VB generated the recombinant test virus, performed viral inhibition analyses and did the OLP screening of the Barcelona patients. JI and MD conducted OLP data analyses and HLA-epitope predictions. CM recruited patients and provided samples, RZ conducted and coordinated the screening of the Lima cohort subjects, SPA conducted statistical analyses and developed the multivariat approach, CTB performed functional avidity analyses, MCP, JMP, OOY provided semi-genome plasmids and helped in the construction of the mutant test virus for inhibition analyses. MR, CJB, ZLB analysed beneficial OLP sequences for HLA footprints associated with reduced viral fitness, MF conducted the screening of the Lima cohort subjects, JJS developed HEPITOPE tool and conducted HLA linkage analyses, WH performed HLA typing, VSM provided samples, DH performed initial multivariat analyses, TMA coordinated HLA typing and sequence analyses for the Lima cohort, JIM analysed beneficial OLP sequences for HLA footprints and levels of sequence conservation, GG helped with the statistical analyses and the development of the multivariat analysis, PJG and BDW coordinated all OLP screenings, HLA typing and data collection in the Durban cohort, JMG and BC coordinated sample access and HLA typing in Barcelona, BTK helped with data analysis and writing of the manuscript, JS coordinated patient enrollment, ethical approval for the Lima cohort. CB conceived the study, conducted initial data analyses, and helped writing the manuscript. All authors were involved in the writing of the final manuscript and have given final approval of the version to be published.

ACKNOWLEDGMENTS

This work was supported by NIH contracts N01-AI-30024 and N01-AI-15422 (CB, TA, BDW, BTK, JS), NIH-NIDCR R01 DE018925-04 (CB), grant MTM2008--06747--C02-00 (GG) from the Ministerio de Ciencia y Tecnología and a grant from the Fundacion para la Investigacion y Prevencion del SIDA en España (FIPSE) # 360737/09 CB), Spain as well as a grant from the European Community FP7 ("CUTHIVAC"). BM holds a research fellowship grant from the FIS (Rio Hortega, CM08/00020), Madrid, Spain. ZB is supported by a New Investigator Award from the Canadian Institutes of Health Research (CIHR). CB and JMP are ICREA (Institució Catalana de Recerca i Estudis Avançats) Senior Research Professors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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CHAPTER 3

CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control

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PLos One. 2012;7(1):e2977. Epub 2012 Jan 4.

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ABSTRACT

Cytotoxic T lymphocyte (CTL) responses targeting specific HIV proteins, in particular Gag, have been associated with relative control of viral replication in vivo. However, Gag-specific CTL can also be detected in individuals who do not control their virus and it remains thus unclear how Gag-specific CTL may mediate their beneficial effects in some individuals but not in others. Here, we used a 10mer peptide set spanning HIV Gag-p24 to determine immunogen-specific T-cell responses and to assess functional properties including functional avidity and cross-reactivity in 25 HIV-1 controllers and 25 non-controllers without protective HLA class I alleles. Our data challenge the common believe that Gag-specific T cell responses dominate the virus-specific immunity exclusively in HIV-1 controllers as both groups mounted responses of comparable breadths and magnitudes against the p24 sequence. However, responses in controllers reacted to lower antigen concentrations and recognized more epitope variants than responses in non-controllers. These cross-sectional data, largely independent of particular HLA genetics and generated using direct ex-vivo samples thus identify T cell responses of high functional avidity and with broad variant reactivity as potential functional immune correlates of relative HIV control.

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INTRODUCTION

Several studies in cohorts of clade B and clade C-infected individuals have shown that cytotoxic T-cell (CTL) responses against HIV-1 Gag correlate with relative control of HIV-1 [1,2,3,4]. The rapid re-presentation of epitopes derived from the Gag proteins contained in the infecting viral particles and structural constraints of the Gag protein that complicate CTL escape have been suggested as possible mechanisms that lend Gag-specific CTL responses this superior effectiveness in controlling HIV-1 [5,6]. However, in all studies reporting beneficial effects of Gag-specific responses, some HIV-1-infected non-controllers mount detectable responses against Gag as well, raising the question as to why these individuals are unable to control their viral replication. A possible answer to this question is that functional characteristics [7,8,9], including functional avidity and variant cross-reactivity are distorted in the CTL population in HIV non-controllers. However, some of these characteristics may not be captured reliably when using some standard in vitro antigen test sets and assay systems [10,11].

In the present study, we analyzed HIV Gag-p24 specific T cell responses in HIV-1 controllers and non-controllers using 18mer and 10mer peptide sets to compare relative response rates using either longer or shorter test peptides and to determine the functional avidity of these responses as well as their ability to react with naturally occurring sequence variants. Furthermore, the data also allowed to assess whether the most conserved regions within p24 are differentially targeted by HIV-1 controllers and non-controllers in order to provide in vitro relevance for vaccine approaches focusing on such conserved elements (CE) in the viral genome [12,13].

Although responses to Gag p24 were of comparable breadth and magnitude in HIV-1 controllers and non-controllers when using the 10mer peptide set, significantly higher avidity responses were seen in controllers, who also showed broader epitope variant cross-reactivity than non-controllers. The data suggest that the maintenance of high avidity responses with broad variant recognition potential is a potential hallmark of controlled HIV-1 infection; a finding that may have important implications in the development of preventative as well as therapeutic vaccine strategies.

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RESULTS

Gag p24 specific T cell responses in controllers and non-controllers are significantly increased when using 10mer peptides sets. Chronically HIV-1 infected individuals with controlled HIV infection (n=25; median viral load 810 RNA copies/ml and median CD4 cell count 642 cells/mm^3) and non-controlled viral replication (n=25; viral load median viral load 200,000 RNA copies/ml and median CD4 cell counts 98 cells/mm^3) were recruited from the HIV Unit in Hospital Germans Trias i Pujol, Badalona, Spain. The study was approved by the Institutional Review Board of the Hospital Germans Trias i Pujol and all individuals provided written informed consent. Median age of individuals was slightly higher for the non-controllers group compared to controllers (44 years-old (24-55) vs 38 years-old (26-56), p=0.04) but individuals did not significantly differ in time since HIV diagnosis (p=0.07) (Table 1). The participants were mostly of Caucasian ethnicity (79% Caucasian, 17% Hispanic, 2% African and 2% Asian) and the ethnic origin did not differ between the two groups. HLA diversity was heterogeneous in both groups and individuals expressing HLA-B27, HLA-B57, or HLA-B58 were intentionally excluded from the cohort to avoid bias due to the presence of dominant Gag p24 CTL epitopes restricted by these alleles and to overcome the limitations of past studies in which these alleles were highly over-represented (**Table S1**).

In a first step, the distribution of total HIV-1-specific T-cell responses using a 18mer overlapping peptide (OLP) set covering the full HIV-1 proteome was assessed in the 50 individuals included. The majority of responses were directed against OLP located in the HIV-1 Gag, Pol and Nef proteins with a relative dominance of Gag/p24 in HIV-1 controllers (p=0.0336 for Gag, p=0.0486 for Gag p24), These data confirmed the expected distribution of responses from earlier reports in HIV-1 controllers even though the present cohort was smaller and did not include individuals expressing known protective HLA class I alleles [4,14]. Of note, the peptide concentrations used were relatively high (14ug/ml), and in our hands saturating [15], to avoid missing responses due to suboptimal peptide concentrations.

To increase the sensitivity of the assay and to discern potential functional differences of Gag responses in non-controllers unable to mediate relative viral control in these subjects, all individuals were tested against a set of 223 10mer peptides (overlapping by 9 residues) spanning the group M Center-of-Tree (COT-M) Gag p24 sequence (**Figure S1**). Significantly more responses were identified by using the 10mers in both groups **(Figure 1A,B**; p=0.0002 for controllers, p=0.0006 non-controllers). 20 of the 25 individuals in each group showed an in-

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crease in the detected responses with the 10mer test set; while only 3 had equivalent breadth and 2 individuals in each group had one response less compared to the 18mer peptides. Controllers and non-controllers showed a 2-3-fold increase of their responses which abolished the broader response rates seen in controllers when using the 18mer peptides (p=0.4260). Responses detected with the 10mer peptide set were of comparable magnitude in the two groups, both in terms of total magnitude (median 4,250 vs. 2,600 SFC/10^6 PBMC in controllers and non-controllers, respectively; p=0.6004, **Figure 1C**) and the average magnitude of individual responses (median 614 vs. 657 SFC/10^6PBMC, p=0.9178, **Figure 1D**).

	C (n=25)	NC (n=25)	P value
Age, years	38 (26.2-55.7)	44.5 (24.3-54.8)	0.04
Time since HIV-1 diagnosis (years)	9.3 (3.5-26.3)	15.9 (1.5-23.3)	0.07
Gender (Female/Male)	F 40%/M 60%	F 40%/M 60%	
HIV risk group			
Heterosexual [b]	6 (24%)	10 (40%)	0.36
Men who have sex with men [b]	8 (32%)	4 (16%)	0.32
Injecting drug users [b]	7 (28%)	9 (36%)	0.76
Other [b]	4 (16%)	2 (8%)	0.66
Last CD4+ T cell counts (cells/mm³)	642 (434-1114)	98 (11-361)	<0.001
% CD4 cells	32 (16-50)	9 (1-27)	<0.001
Last HIV-1 RNA levels (copies/ml)	810 (UD[c]-10,000)	200,000 (52,000-1,200,000)	<0.001
HLA alleles representation			
HLA-A (n=24 alleles)	20 alleles	15 alleles	
HLA-B (n=34 alleles)	27 alleles	17 alleles	
HLA-C (n=20 alleles)	17 alleles	15 alleles	

[a] Data are expressed as median (min-max range), [b] n, (%), [c] UD: undetectable viremia (<49 copies/ml)

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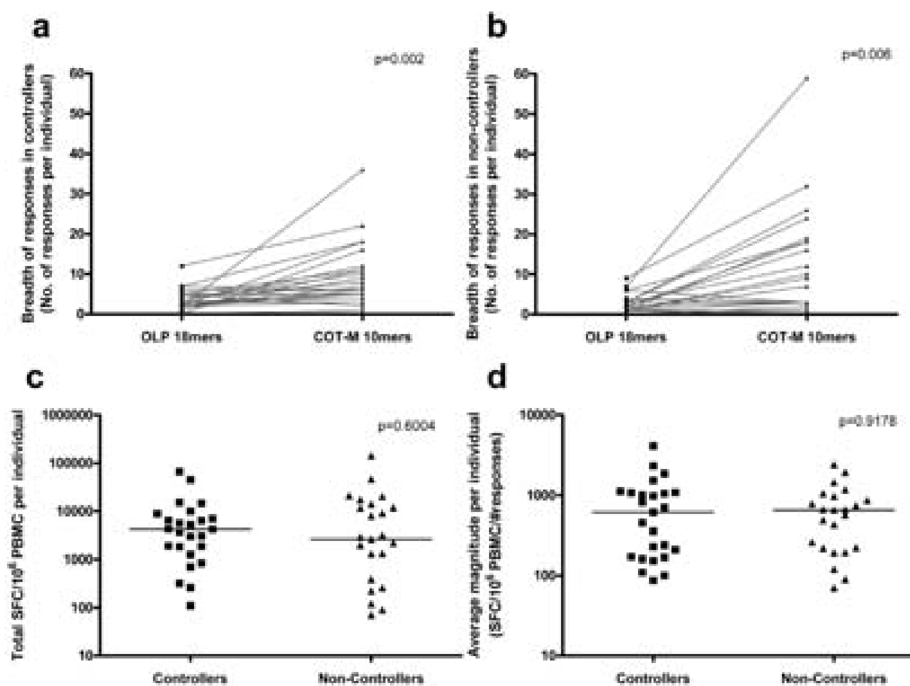

***Figure 1*. Increased detection of Gag p24 specific responses using a 10mer peptide set.** IFN- ELISpot responses against Gag p24 elicited either by consensus B overlapping 18mer or COT-M 10mer peptide sets in 25 HIV-1 controllers **(A)** and 25 HIV-1 non-controllers **(B)** P-values reflect the increase in median breadth of responses when using 10mer peptide sets instead the 18mer peptides (two-tailed Wilcoxon matched paired test). Total magnitude of responses **(C)** and average magnitude of responses **(D)** to COT-M Gag p24 10mer peptides are shown for 25 controllers and 25 non-controllers, respectively. Lines represent median values and indicated p values are based on Mann-Whitney t-tests.

These results demonstrate that Gag p24 specific responses are readily detectable in HIV-1 non-controllers when using a sensitive 10mer peptide set and that they are unlikely to represent spurious, nonspecific reactivities. The data also show that using 18mer peptides may potentially miss up to 2/3 of responses, al least in some of the HIV non-controllers and the antigen (i.e. p24) tested here.

Responses in HIV-1 controllers are of higher functional avidity than in non-controllers and mediate better variant recognition. Data from animal studies and our own analyses in HCV infection suggest that T cell responses of high functional avidity are superior in mediating viral control [3,16,17,18]. We thus tested whether HIV-1 controllers and non-controllers differed in the over-

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all functional avidity of their responses. Based on cell availability, the functional avidity was determined for a total of 474 individual positive responses (219 in controllers and 255 in non-controllers). Controllers indeed showed responses of higher functional avidity (median 6,110 ng/ml, range 0.05-7.6x10^7) compared to non-controllers (median of 13,548 ng/ml, range 0.64-4x10^9; p=0.0101, **Figure 2A**). This difference was more pronounced when the analyses was limited to the 52 10mer-specific responses that were titrated in both groups (6,998 ng/ml vs. 46,637 ng/ml, respectively; p=0.0173, **Figure 2B**) While it is possible that even within one 10mer peptide more than one epitope could be located (i.e. 9mer optimal epitopes) and that 10mer responses could be due to presentation of different epitopes on different HLA alleles, the HLA representation between controllers and non-controllers was similar (particularly because HLA-B57, 58, B27 expressing individuals were excluded). Therefore, it is likely that the same epitope in the same HLA context was being targeted in most of the cases included in the matched analysis and that differential allele-effects would not have impacted the comparison between the two groups.

As high avidity responses may be more prone to react with sequence variants in their cognate epitopes, they may provide a crucial advantage in the control of highly variable pathogens such as HIV and HCV [7,17]. To address whether high avidity responses in HIV controllers would indeed react with more epitope variants, naturally occurring sequence variants were tested for cross-recognition in all 50 subjects using a set of 88 additional 10mer peptide variants. The median number of responses to these 88 variants was 2-fold greater in controllers (median of 4 responses; range 2-9) than in HIV-1 non-controllers (median 2 responses, range 0-16, p=0.0236, **Figure 2C**). In particular, controllers showed responses to both the wild-type and variant peptides in half of the cases where a COT-M and a variant peptide was tested (50%) while only 31% in HIV-1 non-controllers reacted to variants (**Figure 2D**). While this did not reach statistical significance, controllers reacted with significantly more variant peptides for which the COT-M sequence did not elicit a response (median of 2 additional responses by inclusion of variants, range 0-7) than the non-controllers (median 1, range 0-5; p=0.0351; **Figure 2E**). The average magnitude of the variant-specific responses was comparable between controllers (median of 742 SFC/10^6PBMC, range 90-3,073) and non-controllers (median 473 SFC/10^6PBMC, range 60-2,707; p=0.5605, data not shown) indicating that cross-reactive responses in HIV non-controllers were robust, when pres

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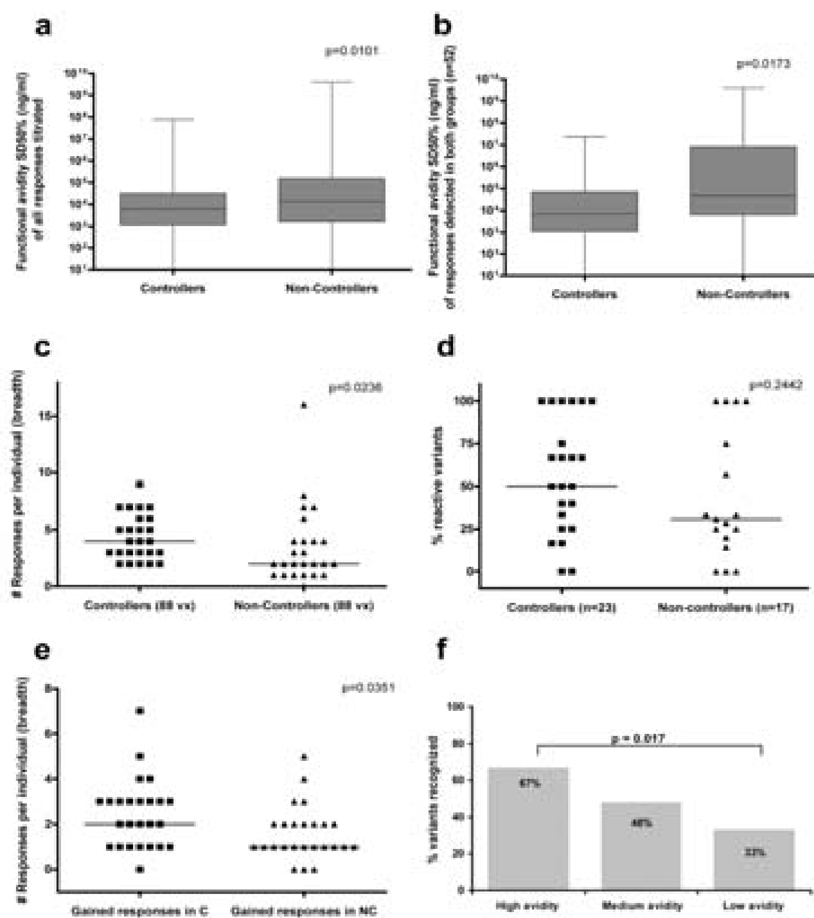

Figure 2. High avidity responses are enriched in HIV-1 controllers and mediate superior variant recognition. (A) Comparison of functional avidity of all COT-M Gag p24 responses titrated in controllers (n= 219 responses) vs. non-controllers (n=255 responses) **(B)** Comparison of functional avidities limited to responses targeting the same 10mer OLP in the two groups (n=52 responses, Wilcoxon). In **(C)** the total breadth (number) of the response to the tested COT-M Gag p24 variant peptides (n=88) is indicated for controllers and non-controllers. **(D)** Shows the percentage of variant peptides that were reactive when the COT-M sequence elicited a response ("cross-reactive responses") and **(E)** indicates responses to variant peptides for which the COT-M sequence did not elicit a response ("gained responses"). The association between functional avidity and cross-reactivity is shown in **(F)** where responses with functional avidities in either the first quartile of all titrated responses (SD50% < 1,401ng/ml) or the second or third quartile (SD50% 1,401-71,594ng/ml) or the fourth quartile (SD50% > 71,594ng/ml) were defined as "high", "intermediate" and low" avidity responses. The percentage of variants that elicited a response was compared between the three groups (Fishers Exact Test).

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In order to directly test whether functional avidity was related to the ability to recognize peptide variants, titrated responses were grouped into high, intermediate and low avidity responses and their variant recognition potentials were compared. Indeed, responses with functional avidities in the first quartile of all titrated responses (SD50% <1,401ng/ml) showed cross-reactivity with their variants in 67% of all cases, whereas fewer (48% and 33%) responses of intermediate or low functional avidity were cross-reactive with their variants, respectively (**Figure 2F**). Collectively, the data demonstrate that high avidity responses were more prevalent in HIV-1 controllers and that these responses mediate superior variant recognition than responses of low functional avidity.

Conserved regions in Gag p24, containing HLA-B14, -B27 and B57 restricted, protective CTL epitopes are frequently targeted by HIV-1 controllers that do not express protective HLA alleles.

The high degree of sequence conservation in HIV Gag p24 makes this protein an interesting vaccine component and many vaccine immunogen designs indeed include Gag p24 [12,19,20]. A recently developed immunogen sequence is based on a strong focus on the most conserved elements (CE) within Gag p24, excluding variable segments that could contain potential decoy epitopes that may divert the host T cell response towards less valuable targets [13]. These CE were defined as sequence stretches of at least 12 amino acids in length that contain only amino acids residues with at least 98% sequence conservation across all available independent group M sequences [13]. Gag p24 contains 7 of such CE segments, ranging from 12 to 24 amino acids in length and corresponding to a total of 124 residues (**Figure S1**). To validate this immunnogen concept, we stratified the T cell data from the 50 controllers and non-controllers based on the location of the targeted 10mer, i.e. whether they were located within or outside of these conserved elements. Both groups showed comparable breadth and magnitudes of total CE-specific responses (**Figure 3A).** Also, controllers reacted with significantly more epitope variants located in CE regions than non-controllers (median of 2 responses in controllers vs. 1 response in non-controllers, p=0.0145, data not shown). Of note, these differences were not due to a suboptimal match between test peptide sequences and autologous viral HIV-1 sequences in the non-controllers as their dominant autologous p24 sequence was in all cases clade B and mostly (99%) represented by the test peptides (**Figure S2**).

Of interest, there were three CE that were recognized by at least 50% more controllers compared to non-controllers (referred to as CE #4, #5, #6), suggesting that these may be preferential targets in HIV-1 controllers **(Figure 3B**). In fact, HIV-1 controllers mounted significantly more responses to at least one of these three CE than did non-controllers (p=0.0006; **Figure 3C**) and showed a

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trend towards these being responses of higher functional avidity, even though only a fraction of the overall data points were included in this comparison (median 7,189 ng/ml, range 0.99-2.5x10^7 vs. median of 17,058 ng/ml, range 16.28-2.62x10^7; p=0.0666, data not shown). In addition, the total magnitude of the responses to CE #4+5+6 showed a statistically significant correlation with HIV-1 viral load (r=-0.5, p=0.0002 by Spearman's rank, **Figure 3D**) across all 50 subjects, suggesting that stronger responses to these three regions may mediate better control of viral replication.

Interestingly, these 3 regions included the well-characterized HLA-B57 restricted TW10 epitope (in CE #4), the HLA-B27 restricted KK10 epitope (in CE #5), and the HLA-B14 restricted DA9 epitope (in CE #6), all of which have been previously associated with containment of in vivo HIV-1 replication [21,22,23,24]. However, as the cohort did not contain any HLA-B57+ and -B27+ individuals and only 2 subjects expressed HLA-B14, the data indicate that mounting responses to these regions is effective even if these protective responses are being restricted by HLA class I molecules different from the originally described restricting HLA molecules [25,26]. Indeed, the HLA class I allele representation of CE #4, #5 or #6 responders was heterogeneous and not limited to individuals with a few shared HLA alleles (**Table S1**) indicating that the CE regions represent a very rich set of epitopes that are not being blocked from presentation in natural chronic infection and that are able to be recognized in a wide HLA class I context.

DISCUSSION

Together, our data strongly suggest that the presence of responses of high functional avidity and with broad variant recognition ability is a potential hallmark of controlled HIV-1 infection. To the best of our knowledge, this is the first demonstration of a direct link between high avidity T cell responses, broad variant recognition and in vivo HIV-1 control using ex-vivo blood samples from a cohort with largely unbiased HLA genotypes. Our data also support that standard approaches using 15-20mer overlapping peptides underestimate the breadth of responses to HIV Gag p24 significantly. While similar findings have been reported in earlier studies, none has addressed this in a systematic way and including a comparably extensive avidity determination as in the present study. More importantly though, since HIV non-controllers profited the most from using a more sensitive peptide set, the data have also important implication for our understanding of HIV immunopathogenesis and vaccine immunogen design: It is not that HIV-1 non-controllers would not mount Gag-specific T-cell respons-

es; rather, they may have either induced low-avidity responses during acute infection or induced originally high functional avidity responses that were lost over the course of HIV-1 infection. This latter interpretation would be in line with results from longitudinal analyses in individuals followed from acute infection time points [27,28]. Although these analyses were based on fewer individuals and included many donors expressing HLA-B27 and -B57 alleles, clonal exhaustion of high avidity cells in the course of chronic HIV replication is certainly a possible explanation why the HIV non-controllers in our study showed responses of reduced functional avidity. On the other hand, it is interesting to note that in a recent report by Berger et al, high avidity responses were not only not restored upon HAART initiation but were actually further diminished [7], suggesting that possibly other factors than duration and extend of antigenimia impact the measurable avidity of an epitope-specific T cell populatio

The wide spread and overlap in the avidity measurements between responses among the two groups is probably the biggest challenge for this kind of study to conclusively demonstrate its potential biological significance. Quite likely, inter-epitope and inter-individual differences hamper a clearer observation. In addition, given that different effector functions are subject to variable activation thresholds [29], the inclusion of additional in vitro read-outs could possibly provide a larger discrimination in the minimal antigen amounts required for responses in HIV controllers and non-controllers. Indeed, as limited effector functions have been previously described for HIV-1 non-controllers, one would expect additional reactivities to occur preferentially in the controllers group. Despite the limitation of assessing a single effector function (IFN   release) though, our findings are supported by a number of previous studies in animal models and in HIV and HCV infection that have assessed the relationship between virus control/clearance, functional avidity and variant recognition [17,30,31,32,33,34,35,36,37]. Moreover, none of earlier studies in humans has been based on the extensive number responses analyzed here (close to 500 titrated responses) and most used either *in vitro* expanded short-term T cell lines or T cell clones [17,32]. While analyzing the functional avidity in clonal T cell populations allows eliminating some of the inherent heterogeneity faced in cross-sectional, directly ex-vivo studies, the *in vitro* selection and expansion of epitope-specific clones may be biasing results too, as shown for HCV specific responses where short-term culture consistently increased the functional avidity compared to directly ex-vivo isolated cells [17]. On the other hand, our data may be limited by the use of 10mers instead of optimal epitopes for the determination of functional avidity. However, many optimally defined epitopes in Gag are 10mers and, it has frequently been shown that 9 and 10mers can have similar SD50%.

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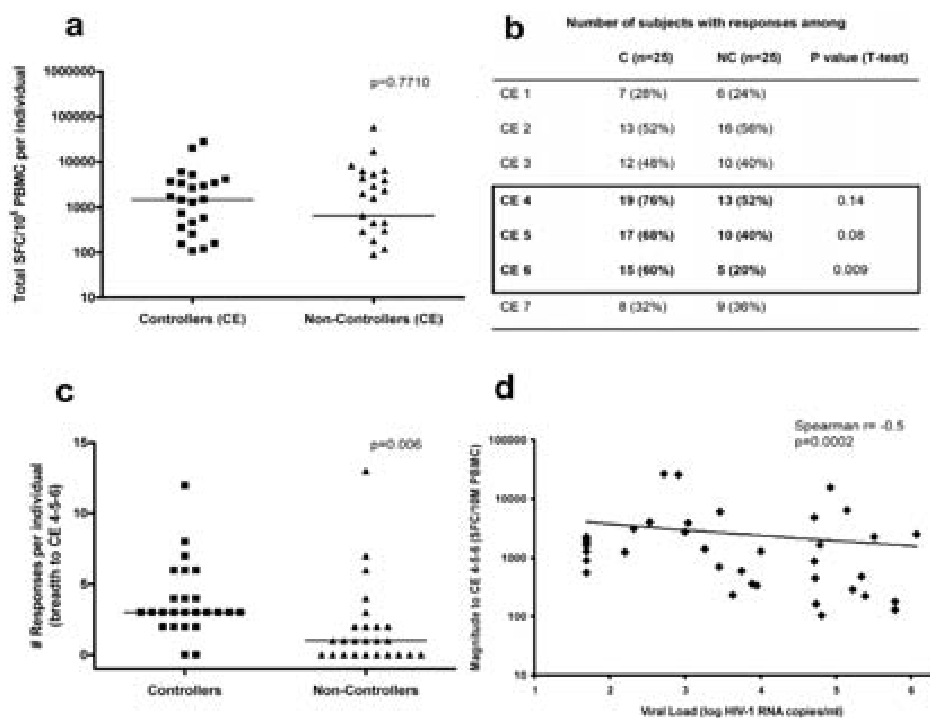

Figure 3. **CE containing HLA-B14, -B27 and B57 restricted, protective CTL epitopes are predominantly targeted by HIV-1 controllers. (A)** The total magnitude of responses to CE regions is compared between HIV controllers and non-controllers. (**B**) The frequency of recognition of the 7 different CE is shown for 25 HIV-1 controllers (C) and 25 non-controllers (NC), respectively. CE regions targeted by at least 50% more controllers than non-controllers are boxed and p-values indicated (T test). **(C)** Breadth of responses to the combination of CE 4+5+6 regions in controllers vs. non-controllers is shown. Horizontal lines represent median values and Mann-Whitney t-test p value is shown. **(D)** Correlation between the cumulative magnitude of responses to CE 4+5+6 and HIV viral loads in all 50 tested individuals is shown (Spearman's rank test)

In fact, in many cases, the definition of optimal epitopes is more driven by the shorter length of 9mers rather than a substantially lower SD50% [26], suggesting that the 10mer approach used here is an acceptable approximation to avoid biases or missing responses by alternative optimal epitope or 9-mer approaches.

In order to control for some of the heterogeneity in our data set, we also compared SD50% between matched responses targeting the same 10mer peptide in the controller and non-controller group. This indeed enhanced the otherwise modest differences in SD50% between the two groups considerably and was statistically significant despite the much smaller number of respons-

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es analyzed. The limited difference in SD50% for the overall analysis may also be due to variable activation thresholds for specific CTL effector functions [29]. As such, the observed difference in the SD50% necessary for IFNγ release may not be directly relevant for the improved viral control in vivo but may still reflect a more avid and thus more effective interaction between the CTL and the antigen-presenting cell, regardless of the ensuing cascade of effector function(s).

As mentioned above, high functional avidity may also render CTL more prone to immune senescence or clonal exhaustion, particularly in individuals with suboptimal control of viral replication. The data from cleared and chronic HCV infection support this idea as only HCV clearers seem to maintain responses of high functional avidity in the absence of possible sources of residual antigen [17]. Thus, while high avidity responses may win out during the induction phase of the virus-specific T cell response, these cells may be preferentially lost over time if viral antigenemia cannot be controlled sufficiently well [28,38]. Whether such losses of high avidity responses correspond to changes in the clonal composition of the CD8 T cell response or to a gradual decrease in their functional avidity due to altered cell reactivity/signaling needs to be further addressed in different clinical settings, as the existing data discussing the 'cause/effect' quandary are conflicting and generally limited to responses restricted by few selected HLA alleles [28,32,39,40,41,42].

Further studies will ideally also include other highly conserved regions in the viral genome outside Gag p24, which may serve as additional components of vaccine immunogens. Such extended analyses would also increase the number of responses per individual, which in the present study is relatively small given that only a short segment of the entire viral proteome was analyzed. Despite this focus on p24, our comparisons reached statistical significance and compared well to the breadth of responses reported in earlier studies looking at responses to the entire HIV proteome or optimally defined HIV CTL epitopes [4,14,15,25]. Together with the results here, these earlier analyses also provide support that the detected responses are generally CD8 T-cell mediated, particularly when testing short 10mer peptides and that they are HIV-specific since testing with even optimally defined short HIV-derived CTL epitopes did not readily elicit responses in HIV negative individuals [15,25].

Given these considerations, our study suggests that HIV-1 controllers mount ex-vivo responses of significantly higher functional avidity than HIV-1 non-controllers. Since the high avidity responses were also more apt to react with epitope variants, their induction by a future HIV-1 vaccine may be cru-

cial to prevent rapid viral escape from the vaccine induced immune response. Finally, as the data presented here confirm findings in HCV infection, they strongly suggest that the ability to maintain T cell responses of high functional avidity is a more general hallmark of effective immune control of infections with highly variable pathogens.

MATERIALS AND METHODS

Synthetic peptides set: An overlapping peptide set of 223 peptides of 10 amino acids in length (overlapping by 9 residues) spanning the entire group M Center-of-Tree (COT-M) Gag p24 sequence was synthesized using 9-Fluorenylmethyloxycarbonyl (Fmoc)- chemistry. Additional 88 10-mer peptides were generated to cover the most frequently occurring variants in the 7 most conserved (CE) regions. We also included a previously described overlapping peptide (410 18mers OLP) set spanning the entire viral proteome [4,14] based on the 2001 consensus-B sequence (http://hiv-web.lanl.gov/content/hiv-db/CONSENSUS/M_GROUP/Consensus.html). Peptides were 18mers varying from 15-20 amino acids in length and overlapping by 10 amino acids, designed using the PeptGen algorithm at the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html).

IFN-γ ELISpot assay: PBMC were separated from whole blood within 4h of venopuncture and used directly for the IFN-γ ELISpot. Each COT-M Gag p24 peptide was tested individually and added at a final concentration of 14μg/ml. For all assays, between 75,000 – 100,000 PBMC per well were added in 140ul of R10 96-well polyvinylidene plates (Millipore, Bedford, MA). The IFN-γ Mabtech kit was used following manufacturer instructions. In parallel, CTL responses to the clade B full proteome were assessed using the 18mer peptide set in a previously described optimized peptide matrix, followed by deconvolution of reactive pools and reconfirmation of each response at a single peptide level on the following day and tested at the same concentration of 14 μg/ml [14]. The number of spots was counted using a "CTL ELISpot Reader Unit" and the magnitude of responses was expressed as spot forming cells (SFC) per million input cells. The threshold for positive responses was defined as at least 5 spots per well and responses exceeding the "mean number of spots in negative control wells plus 3 standard deviations of the negative control wells" and "three times the mean of negative control wells", whichever was higher. As a conservative approach

and not to overestimate the breadth of responses, positive responses to 3 consecutive 10mers in the COT-M Gag p24 peptide set were counted as 1 unique response. Similarly, reactivity to 2 consecutive 18mer OLP was counted as 1 response. The highest magnitude of the sequential responses was taken as the magnitude for each identified response.

Determination of functional avidity: The functional avidity of responses was determined by performing serial 10-fold limiting peptide dilutions ranging from 100μg/ml to 10pg/ml using the 10 mer peptide set; in duplicate whenever enough PBMC were available. Half-maximal stimulatory antigen doses (SD50%) were calculated as the peptide concentration needed to achieve a half-maximal number of spots in the ELISpot assay calculated by a sigmoidal dose response curve fit using GraphPad Prism4.

Gag p24 sequencing: Viral RNA was extracted from 1 millilitre of plasma spun at 25000 rpm for 1 hour (QIAamp Viral RNA Kit™, QIAGEN, Valencia, CA). The whole Gag region was reverse-transcribed and amplified in a One-Step reaction (SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen, Carlsbad, CA) under the following conditions: 30 min at 52ºC for the reverse transcription step; 2 min at 94ºC; followed by 35 cycles at 94ºC during 30 sec, 58ºC during 30 sec and 68ºC during 2 min; followed by a final extension step at 68ºC during 5 min. Primers used for the RT-PCR were: Gag U761 (HXB2: 761-778) 5'-TTT GAC TAG CGG AGG CTA G-3' and Gag D2397 (HXB2: 2397-2376) 5'-CCC CTA TCA TTT TTG GTT TCC A-3'. One microliter of the RT-PCR product was subsequently used as a template for a nested PCR (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen, Carlsbad, CA), using primers p24 U1070 (HXB2: 1070-1088) 5'-TAA AAG ACA CCA AGG AAG CT and p24 D2063 (HXB2: 2063-2044) 5'-TCT TTC ATT TGG TGT CCT TC-3'. PCR cycling conditions were: 2 min at 94ºC; followed by 35 cycles at 94ºC during 30 sec, 54ºC during 30 sec and 68ºC during 2 min; followed by a final extension step at 68ºC during 5 min. The final PCR products were column-purified (QIAquick PCR Purification Kit, QIAGEN, Valencia, CA) and sequenced bidirectionally. Sequences were assembled using Sequencher® 4.10.1 (Genecodes Corp. MI). Assembled sequences were codon-aligned using the Hidden Markov Model implemented in the HIValign tool (www.lanl.hiv.gov). Autologous Gag p24 bulk sequences were obtained for 22 of the 25 HIV-1 non-controllers included

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in our study. Sequences were submitted to Genbank; accession numbers BCN-NC-1.sqn BCN-NC-1 JQ246370-246391.

Statistical analyses

All values are presented as median values unless otherwise stated. GraphPad Prism version 4.0 for Windows (San Diego, CA) was used to compare response rates in both groups and subgroup analyses. Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. Spearman rank correlation was used to assess association.

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Table S1. HLA genotypes of the 25 controllers and 25 non-controllers tested

Gates ID	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
C-1	3001	3601	4201	4201	17MN	17MN
C-2	0201	0205	0702	1402	0702	0802
C-3	0101	3001	1302	3503	0602	1203
C-4	0205	3201	5001	5201	0602	1202
C-5	1101	2402	4403	5201	1202	1601
C-6	2402	3201	3503	4002	202	1203
C-7	3002	6802	1501	5301	0202	0401
C-8	0201	2402	1401	1402	0802	0802
C-9	0201	3301	1402	3801	0802	1203
C-10	0101	2402	1517	5101	0102	0701
C-11	0201	0201	1402	3924	0701	0802
C-12	1101	2402	3501	5201	0401	1202
C-13	0101	6802	1402	3502	0401	0802
C-14	0101	6901	0801	5501	0102	0701
C-15	0202	3002	1516	4403	0401	1402
C-16	3201	3303	1510	4002	0202	0304
C-17	2402	6601	1801	5107	0701	1402
C-18	0201	6801	1501	5101	0304	1502
C-19	1101	3303	3501	5301	0401	0401
C-20	0201	3201	1302	1501	0303	0602
C-21	0211	2601	4402	5202	1502	1502
C-22	0301	1101	0702	5201	0702	1202
C-23	1101	2402	0702	5101	0702	1502
C-24	0301	1101	1301	5501	0102	0403
C-25	0201	2501	3901	4402	0501	1203
NC-1	0201	0301	0702	1801	0702	1205
NC-2	0201	0301	1801	3501	0401	0701
NC-3	0201	0201	3502	4101	0401	17MN
NC-4	2402	2402	4501	5101	1402	1601
NC-5	2301	2402	4402	4405	0202	0501
NC-6	2902	6801	1801	4501	0501	0602
NC-7	1101	2601	0801	4403	0202	0701
NC-8	0101	2601	3503	3701	0602	1203
NC-9	2601	2902	3801	4403	1203	1601
NC-10	1101	3002	1801	3503	0401	0501
NC-11	0201	0205	1801	4402	0202	0501
NC-12	2601	3201	1401	3503	0401	0802
NC-13	3201	74AB	1401	5001	0602	0802
NC-14	0201	2902	3503	4403	1203	1601
NC-15	0201	0201	1503	4402	0202	0501
NC-16	0301	2501	1801	3801	1203	1203
NC-17	0101	0201	1801	4403	0701	1601
NC-18	0205	3002	1801	3502	0401	0501
NC-19	0201	0301	0702	4402	0501	0702
NC-20	0201	0301	0702	3801	1203	1203
NC-21	0201	0301	0702	1801	0501	0702
NC-22	0201	2402	1801	3701	0501	0602

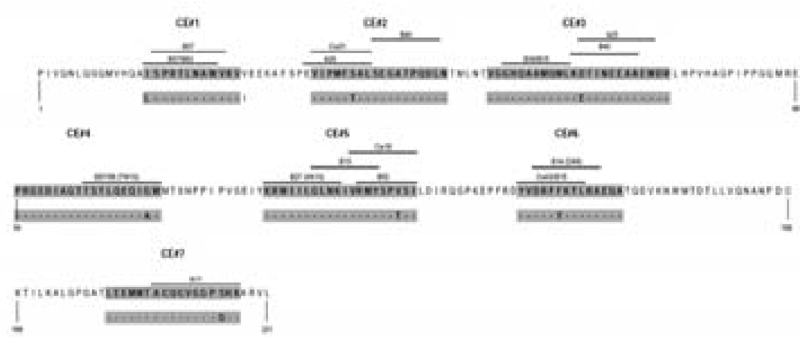

Figure S1. COT-M Gag-p24 sequence and location of CE segments. The Center-of-tree (COT) M sequence is indicated for entire Gag p24. The location of known optimally-defined CTL epitopes listed at the Los Alamos HIV database, are indicated above the protein sequence while the shaded boxes beneath indicate the 7 CE segments and variant (down) residues included in this study.

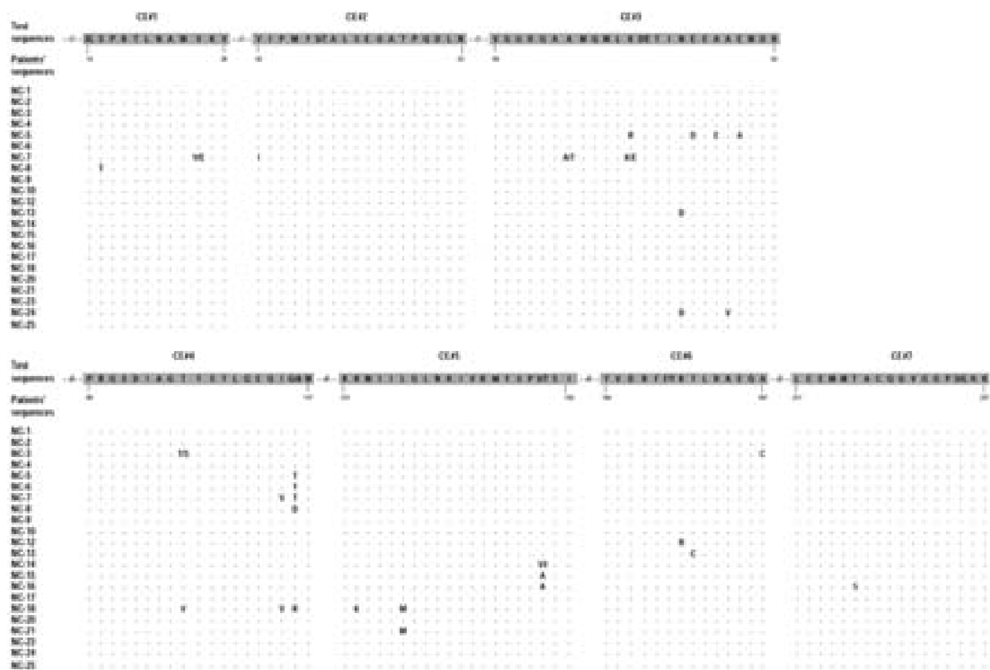

Figure S2. Autologous Gag-p24 CE sequences in 21 HIV-1 non-controllers. Shaded boxes indicate the 7 CE sequences located within in p24 with variant residues included (separated by "/". The amino acid sequences of autologous Gag p24 bulk sequences obtained from 22 HIV non-controllers are shown.

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CHAPTER 4

A novel minimal T-cell immunogen designed to cover beneficial viral targets associated with in vivo control of HIV is broadly immunogenic in mice and is able to break CTL immunodominance.

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RESULTS OF THE ONGOING PROJECT

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ABSTRACT

Clinical trials testing T cell vaccine candidates expressing entire HIV proteins into various vectors have failed so far to induce protective CTL responses. The novel HIVACAT T-cell immunogen is a new reductionist T-cell vaccine candidate whose design was rationally based in large human immunogenicity data and aims to break immunodominance of responses directed to potential "decoy" epitopes that may divert an effective CTL response towards variable and possibly less effective targets in the viral genome. A previous comprehensive screen for beneficial T cell responses yielded 26 protective overlapping peptides (OLPs) in HIV-1 Gag, Pol, Vif and Nef proteins with a broad HLA restriction. These 26 OLP were then a) assembled in 16 regions, b) linked to each other using triple alanines, c) translated into an expression-optimized nucleotide sequence and d) cloned into a CMV basic plasmid. Immunogenicity was first evaluated in C57BL/*6* mice two weeks after two DNA vaccinations through intramuscular electroporation. Cellular immune responses were characterized and compared to a control group of mice immunized with a combination of pDNA expressing complete viral proteins. Our immunogen elicited a more balanced, broad T cell response to all protein subunit components within Gag, Pol, Vif and Nef compared to a narrower and Gag dominated response seen in mice immunized with plasmids encoding for full-length proteins. Less than 25% of the total vaccine induced CTL targeted the identified beneficial regions included in our immunogen in the control group. At present time, preliminary results are being confirmed in the humanized BLT mice model to a) map the vaccine induced responses in the context of different HLA genotypes, b) assess their viral replicative inhibition capacity and cross-reactivity potential as well as c) rule out immunogenicity to potential junction epitopes.

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BACKGROUND

HIV infection induces strong and broadly directed, HLA class I and class II restricted T cell responses for which specific epitopes and restricting HLA alleles have been implicated in the relative *in vivo* control.[1-3] While the bulk of the anti-viral CTL response appears to be HLA-B restricted, the relative contribution of targeted viral regions and restricting HLA molecules on the effectiveness of these responses remains obscure.[4,5] Of note, T cell responses to HIV Gag have most consistently been associated with reduced viral loads in both, HIV clade B and clade C infected cohorts.[3,6] In addition, in terms of CD4 T cells, HIV-1 Gag-dominance has recently also been associated with HIV control.[7]

It is unclear whether the relative benefit of Gag is due to high expression levels, rapid presentation of Gag epitopes[8], conservation[9], viral fitness cost of immune Gag escape[10-12], more resistance in susceptibility to MHC down-regulation by Nef (CROI Abstract #404; Chen manuscript in preparation) or amino acid composition and inherently greater immunogenicity. However, many clinical trials testing T cell vaccine candidates expressing entire HIV proteins (especially entire Gag) into various vectors have failed so far to induce viral control despite being immunogenic.[13,14] In this regard, few previous analyses have assessed the role of responses to shorter regions of the targeted protein(s) that may induce particularly effective responses. It is thus feasible that protein subunits outside of Gag and within these, specific short epitope-rich regions could be identified that induce effective responses predominantly in HIV controllers but not limited to individuals expressing alleles previously associated with effective viral control. While many earlier studies have indeed identified over-representation of Gag-derived epitopes presented in beneficial HLA class I alleles, concerns remain that a purely Gag-based HIV vaccine might mainly benefit those people with an advantageous HLA genotype and will not take advantage of potentially beneficial targets outside of Gag[6,15] or from subdominant responses.[16,17]

To avoid the potential misleading focus on immunodominant regions we have recently identified potential viral targets associated with HIV-1 control through comprehensively screenings in big cohorts of clade B and clade C HIV infected individuals largely independent on HLA genotypes.[18,19] These screens yielded 26 overlapping peptides in HIV-1 Gag, Pol, Vif and Nef proteins that were i) preferentially targeted by individuals with low viral loads, ii) more conserved than the rest of the genome and iii) elicited responses of higher functional avidity and broader cross-reactivity than responses to other regions. These identified results were the basis for the design of a polypeptide sequence for a novel HIV vaccine T cell immunogen aimed to a) provide epitope-rich regions

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in the context of a broad HLA class I coverage, b) break immunodominance of responses with potential non-beneficial effects in viral control and c) focus the vaccine-induced response at the most protective viral targets, including class I and II optimal coverage. Here we will present the details of the construction, in vitro expression experiments as well as the first in vivo immunogenicity tests in C57BL/6 mice. Ongoing experiments are now confirming the results in the humanized BLT mice model to deepen in the characterization of the vaccine-induced responses in the context of HLA genotypes.

METHODS

1. HIVACAT T cell immunogen design

The following approach was followed for the design of the T cell immunogen developed in the local HIVACAT vaccine program: Experimental (IFNγ ELISpot) screening of 232 HIV infected untreated individuals using a consensus clade B peptide set revealed regions of the viral proteome that were predominantly targeted by subjects with superior HIV control.[18-20] The overall test peptide set consisted of 410 18mer overlapping peptides spanning the entire viral proteome. Of these, 26 OLPs were identified as beneficial when the group of individuals who react to these OLP had a significantly ($p<0.05$ uncorrected for multiple comparison) reduced viral load compared to the group of OLP non-responders. These beneficial OLPs had a protective ratio (PR of >1) and were located in HIV Gag protein (n= 10), in Pol (n=12), in Vif (n=3) and in Nef (n=1) proteins of the virus. Of the 26 beneficial OLPs, 15 segments were partially overlapping.

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OLP No.	Protein	sub-unit	OLP clade B consensus sequence
3	Gag	p17	EKIRLRPGGKKKYKLKHI
6	Gag	p17	ASRELERFAVNPGLL
7	Gag	p17	ERFAVNPGLLETSEGCR
10	Gag	p17	QLQPSLQTGSEELRSLY
12	Gag	p17	SLYNTVATLYCVHQRIEV
23	Gag	p24	AFSPEVIPMFSALSEGA
31	Gag	p24	IAPGQMREPRGSDIA
34	Gag	p24	STLQEQIGWMTNNPPIPV
48	Gag	p24	ACQGVGGPGHKARVLAEA
60	Gag	p15	GKIWPSHKGRPGNFLQSR
75	Nef	-	WLEAQEEEEVGFPVRPQV
159	Pol	Prot	KMIGGIGGFIKVRQYDQI
160	Pol	Prot	FIKVRQYDQILIEICGHK
161	Pol	Prot	QILIEICGHKAIGTVLV
163	Pol	Prot	LVGPTPVNIIGRNLLTQI
171	Pol	RT	LVEICTEMEKEGKISKI
195	Pol	RT	LRWGFTTPDKKHQKEPPF
196	Pol	RT	DKKHQKEPPFLWMGYELH
210	Pol	RT	EIQKQGQGQWTYQIY
269	Pol	Int	TKELQKQITKIQNFRVYY
270	Pol	Int	TKIQNFRVYYRDSRDPLW
271	Pol	Int	YYRDSRDPLWKGPAKLLW
276	Pol	Int	KIIRDYGKQMAGDDCVA
405	Vif	-	VKHHMYISGKAKGWFYRH
406	Vif	-	GKAKGWFYRHHYESTHPR
424	Vif	-	TKLTEDRWNKPQKTKGHR

Table 1. List of identified beneficial OLP (PR>1) incorporated in the final T-cell immunogen design. (i.e. OLP-responses seen more frequently in individuals with reduced viral loads) Source: Mothe, B., et al. Definition of the viral targets of protective HIV-1-specific T cell responses. J Transl Med 9, 208 (2011).

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In order to build a continuous immunogen sequence, the 26 beneficial OLPs were aligned and assembled to a total of 16 segments, ranging from 11-78 amino acids in length. The precise starting and end positions of these segments (i.e. shortening or extending them), as well as minor single amino acid substitutions, were modified on analyzing residues in up and downstream of the identified 26 OLPs and was based on a number of considerations:

1) Immunogenicity data from different clade B and C cohorts [3,16,19]
2) Extension or shortening segments for inclusion/exclusion of good or bad known CD8 T cell epitopes[21] as well as flanking crucial regions for escape mutations.[22]
3) HIV Helper CD4 T cell epitopes coverage.[7,21,23]
4) HLA genotypes coverage.[24]
5) Sequence variability (2010 consensus and HBX2 defined epitopes)[25]
6) Creation of neo epitopes or self epitopes by the junctional linkers.[21]
7) Maintenance of natural sequence though not included beneficial OLP, if beneficial regions were close to diminish unnecessary junctions.
8) Introduction of single changes to avoid potential deleterious epitope recognition and cover some of the major antiretroviral resistance mutations sites.[26]
9) Avoid forbidden residues (GPEDQNTSC), PeptGen [21]

This approach resulted in the design of 16 segments (between 11 to 78 aa) which were assembled with single, dual or triple alanine amino acids linkers to ensure optimal processing and to avoid premature epitope digestion. Final polypeptide sequence included the following regions:

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T cell immunogen segments	Length	HIV-1 protein	Position (HXB2)
Seg-1	78	p17	17-94
Seg-2	14	p24	30-43
Seg-3	11	p24	61-71
Seg-4	60	p24	91-150
Seg-5	14	p24	164-177
Seg-6	15	p24	217-231
Seg-7	27	p2p7p1p6	63-89
Seg-8	55	protease	45-99
Seg-9	17	RT	34-50
Seg-10	55	RT	210-264
Seg-11	34	RT	309-342
Seg-12	34	Integrase	210-243
Seg-13	17	Integrase	266-282
Seg-14	23	Vif	25-50
Seg-15	19	Vif	166-184
Seg-16	13	Nef	56-68

Table 2. Final segments of the T cell immunogen sequence. Total length: 529 (including A, AA or AAA linkers)

2 . Inclusion of a leader sequence

Signal peptides are generally highly hydrophobic amino acid sequences (15 to 60 amino acids long) of proteins that must cross through membranes to arrive at their functioning cellular location. By binding to signal recognition particles, these sequences direct nascent protein-ribosome complexes to a membrane where the protein is inserted during translation. Signal peptides direct translational uptake of the protein by various membranes (e.g. endoplasmic reticulum, mitochondria, chloroplast, peroxisome). Leader signal sequences on non-membrane proteins are ultimately removed by specific peptidases. Some signal peptides used include MCP-3 chemokine, for promoting secretion and attraction of antigen presenting cells; a catenin (CATE)-derived peptide for increased proteasomal degradation; and the lysosomal associated protein, LAMP1 for targeting the MHC II compartment.[27] In the present design, the signal peptide from GMCSF (granulocyte macrophage colony-stimulating factor) was introduced at the amino-terminus of the immunogen to enhance secretion of the immunogen from expressing cells, followed by a valine to increase stability.

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3. Inclusion of a tag for in-vitro expression experiments

For the purpose of assessing expression in transfected cells, the immunogen sequence first included a FLAG peptide on the C-terminal region, before the stop codon. The FLAG system utilizes a short, hydrophilic 8-amino acid peptide, which is fused to the recombinant protein of interest. The FLAG peptide includes the binding site for several highly specific ANTI-FLAG monoclonal antibodies (M1, M2, M5; Sigma-Aldrich Corp., Saint Louis, MO, US), which can be used to assess expression of the protein of interest on material from transfected cells. Because of the small size of the FLAG peptide tag, it does not shield other epitopes, domains, or alter the function, secretion, or transport of the fusion protein generally. This sequence was removed afterwards for the final endofree stock of the plasmid DNA for the mice immunogenicity assays.

4. Nucleotide sequence codon optimization

The T cell immunogen sequence was translated into a RNA/codon-optimized nucleotide sequence to enhance expression and secretion (Mr. Gene GmbH, Regensburg, DE). Codon optimization was based on introducing multiple nucleotide changes to destroy the previously identified RNA processing, inhibitory and instability sequences in the mRNA without affecting the encoded protein.[28] This process can also include the elimination of predicted splice sites (score>0.4) from coding sequences by appropriate codon changes, to minimize the possibility of splicing. As a result of the nucleotide changes indicated above, the final GC-content of the T cell immunogen was 63%.

5. Cloning strategy

The codon-optimized T cell immunogen was cloned into the mammalian expression plasmid BV5, which consists of a modified CMV basic plasmid backbone optimized for growth in bacteria that harbors the human cytomegalovirus (CMV) promoter, the bovine growth hormone (BGH) polyadenylation site and the kanamycin resistance gene-–lacking the Xho site.

6. In-vitro expression studies.

Several transient transfections were performed to assess expression, localization and stability of the HIVACAT T cell immunogen. Briefly, $1{\times}10^6$ human 293 cells in complete DMEM plus 10% fetal bovine serum (FBS) were plated on to 60mm tissue culture dishes and allowed to adhere overnight. HEK 293

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cells were transfected by CaPhosphate DNA co-precipitation with a total of 7μg of DNA (100ng or 250ng of the plasmid DNA, 50 ng of GFP expressing plasmid pFRED143 topped up to 7 μg with Bluescript DNA). Six hours after transfection the medium was replaced with 3 ml of DMEM supplemented with 2% of FCS. After 24 and 48 hrs the cells and the supernatants were collected in 0.5X RIPA. Protein expression was analyzed by Western immunoblots. 1/250 of the total of the cell extracts and supernatants were loaded. The proteins were resolved by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels (Nu-Page Bis-Tris, NuPAGE, Invitrogen, Life Technologies Corp., Carlsbad, CA, US) and transferred onto nitrocellulose membranes. Expression of the plasmid was detected upon probing the membranes with horseradish peroxidase-conjugated anti-FLAG monoclonal antibody (Sigma-Aldrich Corp., Saint Louis, MO, US) at a 1:3,000 dilution. Bands were visualized using ECL. Membranes were imaged on a ChemiDoc XRS +. Positive controls were used and included plasmid DNA encoding for complete clade B p55 Gag, which also harbored the FLAG tag.

On a second step, cell extracts from transient transfections using the plasmid encoding for the HIVACAT T cell immunogen without the FLAG-tag were probed with human serum from an HIV-1 infected subject at a 1:3,000 dilution followed by a horseradish peroxidase-conjugated human anti-IgG, dilution 1:10,000.

7. In-vivo immunogenicity in DNA vaccinated mice.

Immunogenicity of the HIVACAT T cell immunogen (called 298H GM-CSF HIVACAT) was evaluated in 6-8 weeks old female C57BL/6 mice (Charles River Labs, Inc., Frederick, MD, US). 20 μg and 5μg of DNA was delivered intramuscularly by electroporation using the Inovio system (Inovio Pharmaceuticals, Inc., Blue Bell, PA, US) in the left and right quadriceps (20 μg/50 μl per dose, 25 μl per site) at week 0 and 4. Mice were sacrificed 2 weeks after the last immunization. Mice splenocytes and serum were harvested for immunogenicity studies. Control DNAs used were 1) 114H p55 gag clade B (expressing complete clade B Gag protein) alone or 2) in combination of 132H NTV (expressing a chimaeric protein of nef, tat and vif) and 133H Pol (expresses full-lenght Pol protein); and 3) BV4 CMV-kan-Basic SHAM control (similar DNA plasmid backbone without any expressed transgene). To allow for comparisons in immunogenicity among groups, sequences of the immunogenic segments contained in the T-cell vaccine were confirmed to be >95% matched with the sequences from the full-length proteins expressed by control pDNAs.

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A total of 35 mice were used in the first immunogenicity experiment, pooling 5 mice per group. Distribution of the immunization per group was as follows:

Groups	Inocula number	Delivery	Dose	N of animals
1	114 p55 Gag	I.M. Inovio	20µg	5
2	114 p55 Gag + 132H NTV + 133 Pol	I.M. Inovio	20µg (each plasmid)	5
3	298H GMCSF-HIVACAT	I.M. Inovio	20µg	5
4	114 p55 gag clade B	I.M. Inovio	5µg	5
5	114 p55 Gag + 132H NTV + 133 Pol	I.M. Inovio	5µg (each plasmid	5
6	298H GMCSF-HIVACAT	I.M. Inovio	5µg	5
7	BV4 CMVKan-Basic (SHAM)	I.M. Inovio	20µg	5

Table 3. Layout of the first in-vivo immunogenicity mice experiment

Cellular immune responses were characterized on a first step using intracellular cytokine staining (ICS) in pooled splenocytes (cells from the 5 mice belonging to a group) Briefly, pooled isolated mouse splenocytes from each group of mice were incubated at a density if $2x10^6$ cells/ml, in 1 ml co-culture overnight, in the presence of peptide pools (15-mers, overlapping by 11aa covering clade B gag, consensus B pol and NL43 nef, tat and vif sequences, 1 µg/ml each peptide, total of about 12 hours, 1 hour without Golgi stop to prevent cytokine secretion). Surface immunostaining was performed with CD3- allophycocyanin-Cy7, CD4-PerCP, CD8-Pacific Blue (BD Biosciences, Inc., Franklin Lakes, NJ, US). Intracellular cytokine staining was performed using interferon gamma-FITC antibody (BD Biosciences, Inc., Franklin Lakes, NJ, US) after permeabilization.

On a second step, at an individual mice level, cellular responses were deconvoluted using frozen splenocytes stimulated with 8 pools of peptides to cover the protein subunits included in the immunogen in an IFNγ mice ELISpot assay. Distribution of peptides in the different protein subunits was as follows: each pool (gag-p17, gag-p24, gag-p2p7p1p6, pol-RT, pol-protease, pol-integrase, vif and nef) contained between 2 and 12 OLP peptides of 18 amino acids based on the 2001 consensus-B sequence and spanning the 16 segments includ-

ed in the HIVACAT T cell immunogen.(http://hiv-web.lanl.gov/content/hiv-db/CONSENSUS/M_GROUP) On the other hand, to assess CTL responses to entire Gag, Pol, and NTV and the relative dominance to our selected regions, a second set of peptide pools was used, and consisted of 18-mers peptides with an overlap of 11 residues spanning the complete 2001 consensus-B sequence of Gag (6 pools, 11 peptides/each), Pol (8 pools, 16 or 17 peptides/each), Nef (2 pools, 13 and 14 peptides/each), Tat (1 pool, 12 peptides) and Vif (2 pools, 12 peptides/each) proteins.

ELISpot assay was performed by using mouse IFNγ ELISpot kit (ALP) (Mabtech AB, Stockholm, SE) following the manufacturer's instructions with minor modifications. For all assays, mice splenocytes were added at an input cell number of $4x10^5$ cells/well in 140μl of Rosewell Park Memorial Institute medium 1640 with 10% fetal bovine serum in 96-well polyvinylidene plates (Millipore Corp., Bedford, MA, US) alone or with HIV-1- specific peptide pools (14μg/ml final concentration for each peptide) for 16hours at 37ºC in 5% CO_2. Concavalin A (Sigma-Aldrich Corp., Saint Louis, MO, US), at 5 mg/ml, was used as a positive control. The plates were developed with one-step 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue Tetrazolium (BCIP/NBT, Bio-Rad Laboratories, Inc., Irvine, CA, US). The spots on the plates were counted using an automated ELISPOT reader system (CTL Analyzers LLC, Cleveland, OH, US) using ImmunoSpot software and the magnitude of responses was expressed as spot forming cells (SFC) per million input splenocytes. The threshold for positive responses was defined as at least 5 spots per well and responses exceeding the "mean number of spots in negative control wells plus 3 standard deviations of the negative control wells" and "three times the mean of negative control wells", whichever was higher.

Humoral responses were first analyzed in pooled mice sera. Binding antibodies to p24, p37 and p55 were detected by western immunoblot by using cell extracts from HEK 293cells transfected with the 1mg of gag and gag-pol expression vectors separated on 12% SDS-Page and probing the membranes with pooled sera from mice (at a 1:100 dilution). Antibody titers to gag p24 were measured by ELISA. Serial 4-fold dilutions of the pooled serum samples were assessed and the optical absorbance at 450nm was determined (Advanced BioScience Lab, Inc., Kensington, MD, US). The binding titers were reported as the highest dilution scoring positive having a value higher than the average plus 3 standard deviations obtained with control sera from the mice immunized with SHAM DNA.

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PRELIMINARY RESULTS

Figure 1. Schematic representation of the gene included in the expression plasmid. Black dots identify start and stop codons.

HIVACAT T cell immunogen is a polypeptide sequence enriched in potential CD8 and CD4 T cell epitopes with a broad HLA coverage. After the final 16 segments were assembled and linked, potential CD8 T cell epitope and HLA coverage were assessed[21]. Despite a clear Gag-p17 and Gag-p24 predominance, a high density of both CD4 and CD8 T cell epitopes in all protein subunits included in the design (n=52 well-characterized optimal defined CTL epitopes). With regard to HLA diversity, 40 different HLA genotypes were covered from an optimal epitope restriction point of view. Of note, if grouped by HLA supertypes, an overrepresentation of B27 or B57 epitopes was not observed.

Protein subunit	N. optimal epitopes
Gag-p17	15
Gag-p24	18
Gag-p2p7p1p6	3
Pol-Protease	5
Pol-RT	8
Integrase	1
Vif	2
Total	**52**

Table 4. CTL optimal epitope coverage of the HIVACAT T cell immunogen, based on the 'A' list of optimal defined HIV-1 CTL epitopes. Yusim, K., et al. HIV Molecular Immunology 2011. Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, New Mexico. LA-UR-12-1007 (2011).

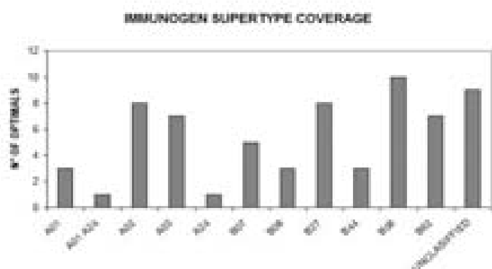

Figure 2. HLA supertype coverage of all the optimal defined epitopes included in the HIVACAT T cell immunogen. Source: Sidney, J., Peters, B., Frahm, N., Brander, C. & Sette, A. HLA class I supertypes: a revised and updated classification. BMC Immunol 9, 1 (2008).

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The HIVACAT T cell immunogen is stably expressed in the cell extract compartment. Plasmids encoding for the HIVACAT T cell immunogen stably expressed the polypeptide construct, which was visualized at the cell extract compartment at 24h and 48h after Ca-P transfection. There was no evidence of secretion of the protein. When expression levels were compared side by side with a control pDNA encoding a full-length clade B Gag at 48h after transfecting 100ng and 250ng of pDNA, the estimated quantization of HIVACAT T cell immunogen produced relative to Gag was shown to be significantly lower, either estimated by the flag signal captured on Western immunoblot analysis and/ or GFP production.

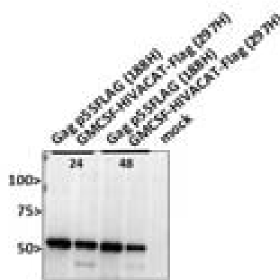

Figure 3a

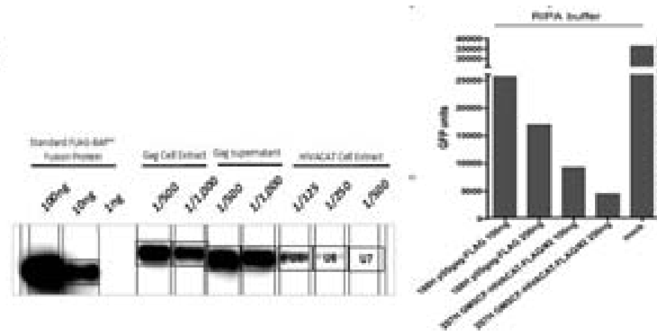

Figure 3b

Figure 3. a) Human HEK 293cells were transfected with 250ng of the plasmids expressing full-length Gag (188H, Lane 1 and 3) and the HIVACAT T cell immunogen (297H, lane 2 and 4) At 24 and 48h later, the cells we harvested and 1/250 of the cell extracts and supernatants (not shown), were visualized by Western immunoblot analysis using 1/3,000 antiFLAG (Sigma) antibody. b) Transfection efficiency and quantization of HIVACAT T cell immunogen relative to gag was estimated by co-transfection of GFP encoding plasmid pFRED143. The relative GFP values (×100) are shown for 100ng and 250ng for the pDNA encoding for complete Gag and the HIVACAT T cell immunogen.

HIVACAT T-cell immunogen was able to elicit a balanced, broad T cell response to all protein components. From the first immunogenicity analyses, both 20 μg and 5 μg of pDNA did generate detectable IFNγ + responses to complete Gag, Pol and Nef-Tat-Vif peptide pools in pooled C57BL/6 mice splenocytes measured by ICS. Detectable CD4+ and CD8+ responses were seen in all groups.

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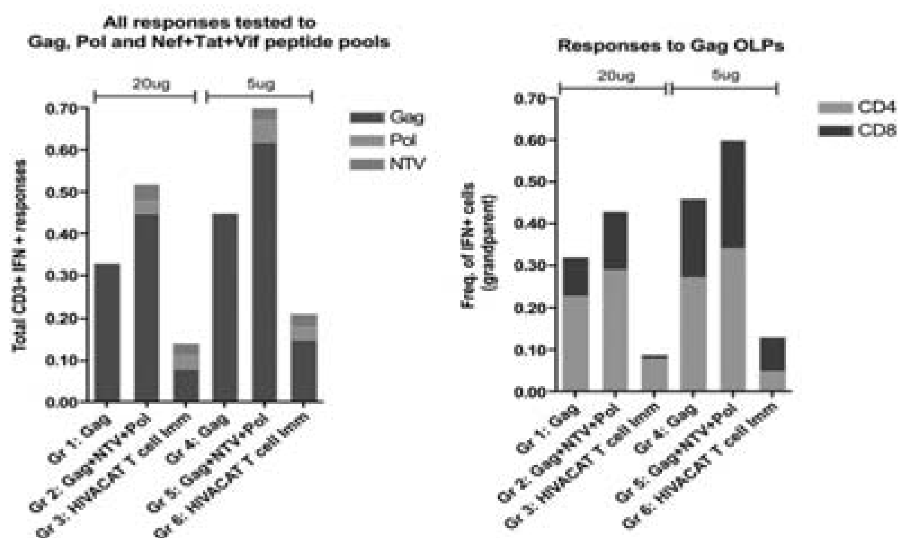

Figure 4. Cellular immune responses analyzed in pooled splenocytes by flow cytometric analysis. Frequency of total Gag, Pol, and Nef-Tat-Vif specific IFNγ responses among groups in (a) and distribution of CD4 and CD8 responses in (b) are shown.

<u>At an individual mice level</u>, responses were deconvoluted using frozen splenocytes stimulated with pools of peptides to cover the eight protein subunits included in the T-cell immunogen as well as with pools of peptides spanning full-lenghtGag, Pol, Nef, Tat, and Vif in an IFNγ ELISPOT assay.

The majority of IFNγ responses detected in mice immunized with plasmids encoding for the entire Gag, Pol and Nef-Tat-Vif polyprotein targeted regions outside the HIVACAT T cell immunogen covered segments (measured by HTI-p1 to p8 peptide pools). In this regard, the median ratio of responses targeting regions included in the T cell immunogen out of the total responses measured to complete Gag+Pol+NTV was 0.26 (range 0.17-0.42) and did not differ among groups immunized with high dose (20μg) or low dose (5 μg) of pDNA.

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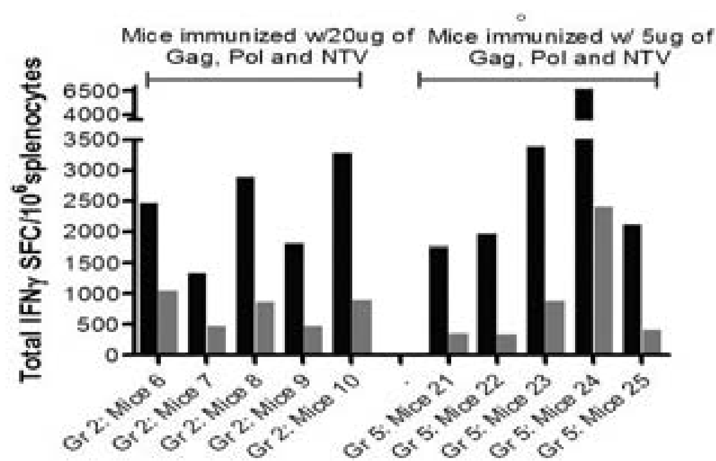

Figure 5. Responses to Gag, Pol, NTV (in black) and to the HIVACAT T cell immunogen sequence (in grey) were measured by IFNγ ELISpot assay in murine splenocytes of mice immunized with the plasmids encoding for the complete proteins (Groups 2 and 5) Contribution of the responses targeting the regions included in the HIVACAT T cell immunogen to the total IFNγ Gag-Pol-NTV IFNγ specific response is shown.

When we focused on the assessment of the IFNγ responses targeting the different protein subunits included in the polypeptide construct, a more balanced, broad T cell response to all protein components was seen in the mice immunized with the HIVACAT T cell immunogen. Median breadth of responses to protein subunits included in the immunogen sequence was 4 (range 2-5) in mice immunized with 20µg of HIVACAT pDNA vs 2 responses (range 1-3) in mice immunized with 20µg of plasmids encoding for entire proteins (ns). Importantly, magnitude of responses to these regions was not significantly different in both groups, despite significant less in-vitro expression of the HIVACAT vaccine than pDNA encoding for Gag. Six out of the eight protein subunits were at least targeted once in the mice immunized with the HIVACAT T cell immunogen, and some mice elicited responses to protein subunits not induced by plasmids encoding for Gag, Pol and Nef-Tat-Vif (i.e. as for Gagp17 or Nef)

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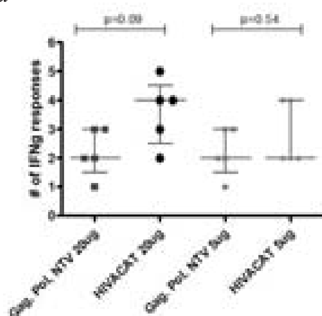
Figure 6a *Figure 6b*



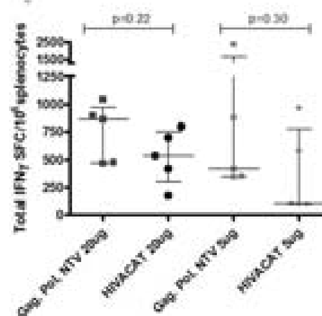


Figure 6. Comparison of the breadth (a) of the IFNγ responses targeting the 8 protein subunits (Gag p17, Gag p24, Gag p2p7p1p6, Pol-Protease, Pol-RT, Pol-Integrase, Vif and Nef) included in HIVACAT T cell immunogen and magnitude in (b) of the total of IFNγ responses targeting the same protein subunits in individual immunized mice. Mice were vaccinated with either the plasmids encoding the full proteins or the minimal T cell sequence.

HIVACAT immunogen segments	HIV-1 protein	Pool peptide Number (#peptides/pool)	Mice making a response (groups Gag-Pol-NTV)	Mice making a response (groups HIVACAT)
Seg-1	gag-p17	HTI-pool1 (10)	0/10	3/10
Seg-2	gag-p24	HTI-pool2 (12)	10/10	10/10
Seg-3	gag-p24			
Seg-4	gag-p24			
Seg-5	gag-p24			
Seg-6	gag-p24			
Seg-7	gag-p2p7p1p6	HTI-pool3 (3)	0/10	0/10
Seg-8	pol-protease	HTI-pool4 (6)	4/10	7/10
Seg-9	pol-RT	HTI-pool5 (11)	5/10	9/10
Seg-10	pol-RT			
Seg-11	pol-RT			
Seg-12	pol-integrase	HTI-pool6 (4)	0/10	0/10
Seg-13	pol-integrase			
Seg-14	vif	HTI-pool7 (4)	3/10	2/10
Seg-15	vif			
Seg-16	nef	HTI-pool8 (2)	0/10	1/10

Table 5. First deconvolution of the breadth of the IFNγ responses detected in mice by protein subunits.

Relative dominance of IFNγ responses in mice immunized with plasmids en-oding the full-length proteins (Gag, Pol, Nef-Tat-Vif) was 89% driven towards

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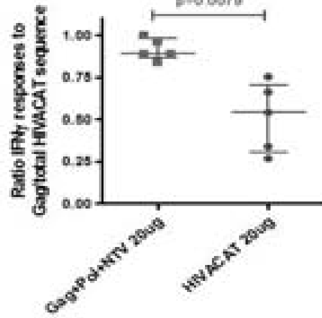
Gag. In the group of mice immunized with the HIVACAT T cell immunogen (at high dose) the pattern of IFNγ responses was shifted to a more balanced hierarchy, being detectable responses to all protein components (Gag, Pol, Vif and Nef) contained in the immunogen.

Figure 7a



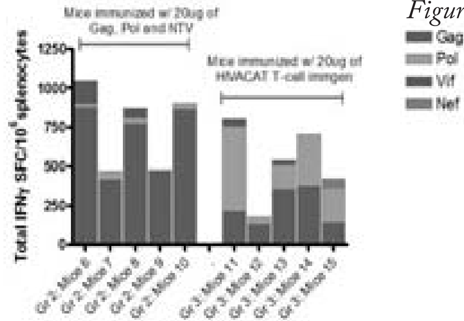
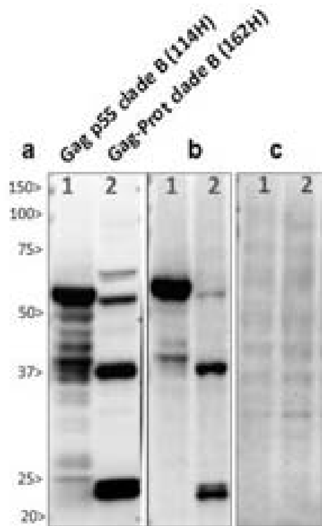


Figure 7b

Figure 7. Balance of IFNγ responses against the regions in Gag, Pol, Vif or Nef included in the polypeptide T cell construct detected in mice immunized with 20 μg of a mixture of plasmids encoding full-length Gag, Pol plus Nef-Tat-Vif or 20 μg HIVACAT T cell immunogen. Dominance of Gag-specific responses in shown in panel (a) for mice immunized with complete proteins whereas a more balanced repertoire in seen in panel (b) for mice immunized with the HIVACAT T cell immunogen.

HIVACAT T cell immunogen induced detectable binging antibodies by Western Immunoblot.



Binding antibody responses to Gag p55, p37 and p24 were detectable by Western blot in pooled sera from the group of mice immunized with 20 μg or pDNA, but not in the mice immunized with a lower doses.

Figure 8. Binding antibodies to p24, p37 and p55 detected by Western immunoblot by using cell extracts from HEK 293 cells transfected with the 1mg of gag and gag-pol expression vectors (showing p55 gag, and processed p24, p37 and p55 gag subunits in lanes 1 and 2 respectively in each panel) separated on 12% SDS-Page and probing the membranes with a) human sera of and HIV-infected patient, b) pooled sera from mice immunized with high doses of the immunogen and c) pooled sera from mice immunized with low doses of the immunogen (all at a 1:100 dilution).

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Binding antibodies to p24 were quantified by ELISA. The endpoint titers of gag-p24 specific binding antibody from the mice that received the plasmids described were determined by ELISA from individual serial 4-fold diluted pooled serum samples. In the high dose group of mice immunized with HIVACAT T cell immunogen at a titre of 1:4,000 which were lower to the titers detected in mice immunized with the full gag construct. As seen by western immunoblot, no binding antibodies to p24 were measurable in the low dose group, which is in accordance with the lack of secretion seen in the in-vitro expression experiments At an individual mice level, in house developed gag p55 ELISA using the HIV-1IIIB pr55 gag recombinant protein (Cat. No. 3276, NIH Reagent Program, Bethesda, MD, US) was performed with mice sera at 1:100 dilution. Low levels of antibody were just detected in 2 out of 3 mice immunized with the high dose of the immunogen, and also, at very low borderline titers

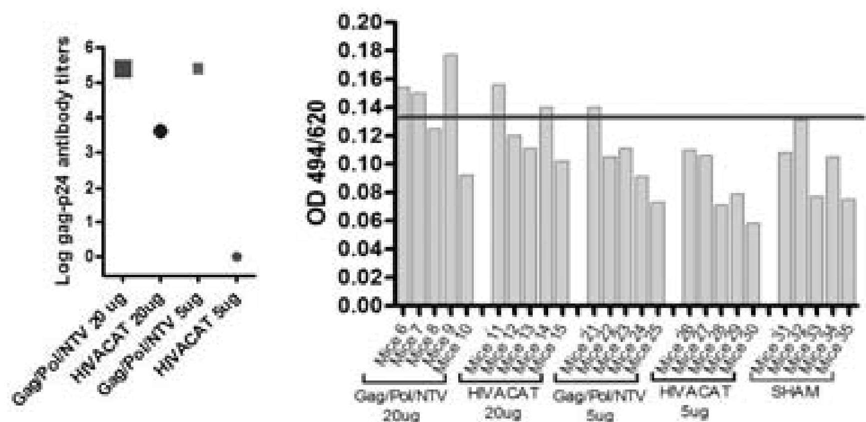

Figure 9. a) Endpoint titers of Gag-p24 specific binding antibody from treated mice. The determination was performed by ELISA from individual serial 4-fold diluted pooled serum samples. b) In house developed gag p55 ELISA using the HIV-1IIIB pr55 Gag recombinant protein (Cat. No. 3276, NIH Reagent Program, Bethesda, MD, US). The determination was performed in individual mice sera at 1:100 dilution.

DISCUSSION

A novel, reductionist HIV T cell immunogen has been designed incorporating an important amount of human immune data that aims to break immunodominance of regions with potential non-beneficial effects in viral control. A polypeptide sequence of 529aa (approximately same size as full-length Gag) largely enriched in potential beneficial CD8 CTL and helper CD4 T cell epitopes was outlined providing a broad HLA class I coverage without a particular dominance of favorable HLA genotypes (i.e. B27 or B57)

A plasmid DNA expressing the HIV T cell immunogen was first engineered. DNA technology allowed us to perform the first proof-of-concept expression and immunogenicity studies, due to its versatility and feasibility. DNA vaccines have great immunogenicity potential, especially if incorporated in heterologous prime/boost regimens, which is our final goal. Its potential relies on their safety, scalability and possibility of repeated administrations without generating immunity against the vector itself. The incorporation of expression-optimized antigens has led to great increases in expression and immunogenicity of the encoded proteins[28] and improvements in delivery methods by in vivo electroporation have shown promising enhancement of DNA uptake.[29,30]

First plasmid DNA constructed expressing the designed HIV T cell immunogen showed to have a stable expression in in-vitro experiments, although its expression levels were significantly lower than other previously engineered DNA expression vectors encoding the full-length clade B Gag protein. No free construct was quantifiable in the supernatants of transfected cells, suggesting a poor secretion of the protein compared to plasmids expressing complete Gag. However, we hypothesized that secretion would not be necessary for CTL immunogenicity purposes, as long as the polypeptide protein was properly being processed and presented. We therefore moved forward to a first in-vivo immunogenicity study in C57BL/6 mice.

Vaccination with pDNA expressing the polypeptide construct generated both CD4 and CD8 IFN-γ+ responses. Interestingly, the T-cell immunogen elicited a more balanced, broad T cell response to all protein components (Gag, Pol, Vif and Nef) contained in the immunogen. Furthermore, a simple deconvolution of responses showed detection of IFNγ responses to six out of the eight protein subunits included in the immunogen. In contrast, control mice immunized with plasmids encoding for complete Gag, Pol, Nef, Tat and Vif proteins[31] developed a stronger and narrower Gag dominant response.

Despite the limitations of the C57BL/6 mice model, our preliminary results suggest that although a lower in vitro expression and no (or very low) secretion

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of the construct, the HIVACAT DNA vaccine was strongly immunogenic and was able to break the immunodominance of CTL responses to targets that do not emerge as particularly beneficial in previous large cohort screenings.[18] In comparison to vaccinations with plasmids encoding for complete proteins, we conclude that our approach allowed for a more balanced and broader immunogenicity to beneficial targets.

We are in the process of confirming the immunogenicity of the same plasmid in the context of different HLA genotypes in the humanized BLT mice model.[32] Humanized BLT mice have a great potential as a platform to assess candidate HIV vaccines such as the one here presented before scaling them up. Whereas immunogenicity -or even challenge- studies in the macaque model could provide complementary supportive/non-supportive information, the here presented T cell immunogen was largely and rationally designed based on human HIV-specific CTL immune data. Therefore, its immunogenicity potential, in terms of proper antigen presentation, it will be better understood if assessed in the context of the human MHC. BLT mice are generated by the cotransplantation of human fetal thymus and liver tissues and $CD34^{+}$ fetal liver cells into non-obese diabetic/severe combined immunodeficiency mice. BLT mice show long-term reconstitution of a functional human immune system, (i.e. with human T cells, B cells, dendritic cells, and monocytes/macrophages repopulating mouse tissues) and sustain high-level disseminated HIV infection resulting in progressive CD4 T-cell depletion and generalized immune activation. Importanly, following infection, HIV-specific humoral as well as CD4 and CD8 T-cell responses are detected after 9 weeks of infection.

Immunogenicity studies in humanized BLT mice can provide a better assessment of the broadness of the effective presentation of several T cell epitopes included in the construct by different and common HLA genotypes. We would like to assess the balance between CD8 and helper CD4 T cell responses induced and rule out the immunogenicity from potential new junctional epitopes. For that purpose, a set of 15mers peptides overlapping by 11 residues spanning the entire T cell immunogen sequence and including the alanine linkers has already been designed and synthesized. If a broad and balanced immunogenicity is further confirmed in the BLT mice, a deeper characterization of vaccine induced responses, in terms of viral inhibition capacity and cross-reactivity potential to epitope variants will be performed.

In conclusion, the preliminary immunogenicity results of the HIVACAT T cell immunogen showed great potential as a candidate T cell vaccine to avoid potential decoy responses and provide a better targeted immunogenicity than previous vaccines encoding for entire proteins.

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DISCUSSION

This doctoral thesis aims to better describe the effectiveness of HIV specific T cell responses, define the viral targets that intervene most consistently in mediating viral control in vivo and discuss how this information can be incorporated into a rational immunogen design for a future HIV vaccine.

1

In a first part (Chapter 1) the complex interplay between viral factors, host genetic and immune characteristics in HIV control were reviewed. Most host genetic polymorphisms associated with better disease outcomes involve the HLA genes, pointing towards a crucial role of HLA class I restricted CD8+ T cells in viral control. However, studies on host genetic polymorphisms and HIV disease control also need to take into consideration the viral diversity in regions with gentically different host populations. If we aim to have an effective global HIV vaccine, we need to better understand how viral evolution is shaped by frequencies of diverse host genetic markers within different regions. For instance, how a global vaccine will overcome the host genetic effect when it can lead to opposite outcomes in HIV disease control in different populations, as for some well described HLAB*1503 restricted epitopes?[1] We will provide some novel insights regarding immune correlates –specially in CTLs- elucidated over the last years to complete the work compiled in Chapter 1. The importance of better discerning between the cause and effect of immune parameters associated with relatively controlled HIV infection is stressed, as factors that are a mere reflection of otherwise contained replication can mislead the vaccine design.

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2

In the second chapter, the most potent viral targets of the virus-specific T cell responses in controling viral replication were identified in large cohorts of HIV infected individuals. The relative effects of host genetics (i.e. HLA) and CTL specificity on HIV-1 control were assessed. Importantly, the impact of T cell specificity on viral loads was shown to be at least as strong as the effects of host HLA genetics. These preferred targets turned out to be inside the most conserved regions of the viral genome, which is in accordance with studies by other investigators also developing novel vaccine concepts focused on conserved elements for universal vaccines.[2,3] However, an important contribution of our work in relation to other approaches is the incorporation of a significant amount of immunogenicity data, as not all conserved regions of the viral proteome are equally immunogenic. Also of importance is the fact that many of the identified regions overlapped in clade B and clade C cohorts and could provide the basis of a broadly applicable HIV T cell vaccine.

3

In the third chapter, functional avidity and the ability to react with viral variants were shown to be associated with controlled HIV infection. As mentioned above, a considerable scientific effort is put into defining better immune correlates of HIV control, mainly derived from basic studies dissecting responses present in LTNP or EC individuals and also directly from vaccinees in clinical trials. In the present work, HIV infected individuals were included in groups of subjects with 'controller' and 'progressor' phenotypes, intentionally excluding all participants with known beneficial HLA genetics to avoid bias due to the presence of dominant CTL epitopes restricted by these beneficial alleles. The main reason behind this approach was to ensure that potential findings from our work would be as independent as possible of the presence of favourable HLA alleles and could be more readily translated to the general population. Our work complements recent data aimed at obtaining a larger picture of CTL functionality. Despite the limitation of assessing a single effector function (IFNγ release), we showed that functional characteristics such as CTL functional avidity is linked to cross-reactivity with epitope variants, possibly preventing the rapid outgrowth of CTL escape variants in vivo. It is important to mention that, the identified beneficial targets in Chapter 2 induced T cell responses of high functional avidity, thus enhancing their inclusion in future HIV vaccines.

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The findings from work described in Chapters 2 and 3 were incorporated into a novel, reductionist HIV immunogen design that provides broad HLA class I coverage and aims to break immunodominance of regions with potential non-beneficial effects in viral control and seeks to focus the vaccine-induced response at the most protective viral targets. A DNA plasmid expressing the HIV T cell immunogen was engineered, shown to have a stable expression in in-vitro experiments and found to induce a particularly broad T cell responses in the first immunogenicity studies in C57BL/6 mice. The main findings as well as potential limitations of our studies will be elaborated in the following section in a broader context of the HIV vaccine field. The discussion of this thesis is then closed with an outlook of the work ahead and future lines of research that have been opened by our studies.

Immune markers and the causality dilemma.

As stated in the Introduction and the first chapter, the interplay between HIV replication immediately after initial infection and the host immune response, both innate and adaptive, may crucially determine the course of HIV disease (Updated reviews in [4-6]). With respect to the different arms of the host immune responses to HIV infection, our studies have focused on the HIV-specific cytotoxic T lymphocyte response (CTL). As mentioned above, the temporal association between the reduction in viral loads after acute infection and the emergence of the virus specific cellular immune response and prior to the appearance of neutralizing antibodies have been considered as point in case that CTL control HIV replication.[7,8] Furthermore, association studies of HIV CTL specificities and viral control[7] and former studies in CD8 depleted SIV infected monkeys[8] supported the notion that CTL are crucial for the control of HIV in vivo and have been probably responsible for fostering the T cell based vaccines in test-of-concept trials, such as the STEP phase IIb trial. However, despite inducing HIV specific T cell responses in 75% of the subjects receiving the vaccine, the trial was terminated prematurely after interim analyses revealed that the vaccine neither prevented HIV-1 infection nor lowered the viral load set points in beak-through infections.[9] Vaccine had been immunogenic, but the immunoassays used had failed to predict vaccine efficacy.[10] An important roadblock in identifying vaccine candidates was still the lack of reliable immune correlates of HIV-1 control.

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Today, there is a better and larger understanding of CD8+ T cell function. New tools have also been developed for evaluating their antiviral capacity. As discussed in Chapter 1 and on incorporating new data, an updated panel of immunological tests to better indentify CD8+ T-cell correlates of HIV protection, as well as appraise candidate HIV vaccines in early phase I/II studies before they are scaled up to phase IIb/III efficacy trials, might include:

a) CTL polyfunctionality[11,12], especially when including 'cytolytic markers such as perforin secretion[13,14]. It has been suggested that rapid perforin upregulation with concomitant degranulation dominates the earliest response in acute stages of infection in LTNPs (with the most prevalent functional subset being perforin+ CD107a+) whereas the later appearance of IL-2+ polyfunctional CD8+ T cells is likely the result of effective control of viremia.[5]

b) Activated cells with a readily effector phenotype, shown by the expression of HLA-DR but not CD38. This phenotype predominates in HIV-specific CD8+ T cells from controllers and has been shown to possess a high potential for expansion upon exposure to the antigen and a capacity to exert multiple effector functions.[15]

c) Although still novel, system biology approaches[16] have identified a superior ability to preserve the T_{CM} compartment in elite controllers and have identified molecular mechanisms involved in T_{CM} survival (FOXO3a, STAT5, and Wnt pathways)[17]. The persistence of a functional T cell memory T is thought to constitute a basis for long-lasting protection from HIV disease progression.

d) Direct antiviral inhibitory capacity of CD8+ T cells, measured by viral inhibition assays. Potent anti-HIV activity has been observed without prior stimulation of CD8+ T cells in controller individuals by different groups[15,18], and more recently, also in longitudinal studies including recently-infected individuals where the antiviral capacity was able to predict CD4 decline over time.[19] Although efforts on assay standardization are still needed, they have a clear potential as a valuable read-out of effective immunity. Such assays were used in Chapter 2 to picture functional differences in inhibitory capacity of CTLs targeting some of the identified beneficial versus non-beneficial viral regions.

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T –cell Aspect	Markers/Assay
Specificity (breadth, dominance) Magnitude Depth (cross-reactivity) Functional avidity	IFNγ ELISpot (importance of peptide sets: size, #peptides/pools, matrix to deconvolute responses, assessment of reactivity to viral variants)
Memory Phenotype	CCR7, CD27, CD28, CD45RA/RO, CD57, CD62L
Activation Status	HLA-DR, CD38, Ki67, CD69, CD95
Cytokine Production	IFNγ, TNFα, IL2,
Cytotoxicity	Perforin, Granzyme A/B/K, Granulysin. Viral Inhibition Assays (using p24+ cells by in flow, or p24 secretion by ELISA)
Miscellaneaous	Degranulation (CD107a exposure), MIP-1α/β, Proliferation (CFSE)
Regulatory Function	CTLA-4, Foxp3, GITR, CD25, CD39, CD73, TGF-β
Exhaustion	PD-1, CD160, Lag-3, TIM3
Tissue Trafficking	α4β7, CCR5, CCR9, CCR10, CD161

Table 1. Potential comprehensive panel of immune parameters (alone or in combination) to assess CD8+ T cell anti-HIV immunity. Source: Adapted from Makedonas, G. & Betts, M.R. Living in a house of cards: re-evaluating CD8+ T-cell immune correlates against HIV. Immunol Rev 239, 109-124 (2011).

The past failures to predict vaccine efficacy may have been crucially dependent on the assays employed. Apart from the already mentioned failure to predict efficacy from T cell vaccines in the STEP trial, the same holds true for B cell components. In the post-hoc RV144 studies, the relative protection in individuals was correlated with IgG binding antibodies to V1/V2 while they were not explained by current knowledge on how these parameters could provide relative protection.

In order to support the validity of a T cell vaccine concept (possibly in combination with bnAb vaccine components), physiologically relevant, specific and sensitive assay systems need to be established. A multi-faceted evaluation of HIV vaccine-specific CD8 T cells potentially offers the greatest odds of predicting and monitoring vaccine efficacy. But even if we incorporate all these new immune parameters, many questions still arise. What threshold of immunogenicity must be crossed for vaccine efficacy? Thus, new studies are needed for integrating continued basic research in HIV-infected, HIV-exposed uninfected (HEPS), but also in vaccinated individuals in early-phase clinical trials to better define such relevant thresholds and correlates of relative protection. Analyses of controller individuals are always of greatest interest, but a deeper focus on acute infection events, longitudinal follow-ups rather than strict cross-sectional approaches as well as studies aimed at elucidating the mechanisms determining the loss of control in later stages of the disease are needed.

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In terms of host genetics and as discussed in the first article, several genome-wide association studies have suggested that genetic polymorphisms can explain at least 13% of the observed variability in HIV-1 viremia.[20] As regards the well established HLA*A and *B polymorphisms[21], recent data have incorporated novel insights on HLA C polymorphisms (a variant 35 kb upstream of the HLA-C gene -35C/T that modulates HLA-C expression levels)[22,23] and a potential involvement of miRNA in the process of HLA-C expression[24] pointing towards a role of interactions with NK cell immunoglobulin-like receptors (KIR). Other genetic associations that link NK cells to enhanced HIV control include KIR3DL1/Bw04 and KIR3DS1/Bw04 associations which seem to determine an increased cytotoxic NK phenotype.[25] (and reviewed in [26]) In terms of MHC class II, although very preliminary, recent data suggest some HLA-DRB1 alleles to be associated with high degree of HLA class II binding promiscuity in HIV controllers.[27]

However, the precise mechanisms of how some alleles mediate superior viral control still remains unclear. In addition to the developed arguments in Chapter 1 for some HLA*B alleles (presentation of multiple immunodominant Gag and RT epitopes of wide cross-recognition for instance), Kosmrlj *et al.* recently modeled the beneficial effects of some alleles to stem from events during in vivo thymic selection.[28] In this scenario, beneficial HLA alleles, for which the human genome encodes relatively few potential self-epitopes, would promote the selection of thymocytes with a particularly broad epitope variant cross-reactivity. This could enable CTL reactivity with more potential CTL escape variants, limiting the escape pathways of the virus and potentially leading to reductions in viral replicative fitness. To extend this concept further, we have recently tested HIV-uninfected individuals with respect to their ability to recognize alanin-substitutions of EBV and CMV-derived CTL epitopes in the context of HLA-A2, -A11, -B7, -B8, -B35, -B53 and B57. We have observed a direct correlation between the number of epitope variants recognized and the reported effect on HIV disease progression for these alleles (manuscript in preparation, AIDS Vaccine Meeting, Atlanta, 2010. #P18.10.) providing a potential functional explanation as to how protective HLA class I alleles may mediate their in-vivo effects in natural HIV infection.

Despite their complexity, some models have previously tried to weight up the influence of different host genetic factors in the disease outcome.[29] Even if a mathematical model is not strictly applied, it is important to have an updated picture of the role of host genetics and account for the most relevant ones when interpreting the results of immune correlates in basic studies or vaccine trials.[30]

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Importance of the targets:
Back to Gag; but not all-Gag or only-Gag.

As previously mentioned an interest in effective T cell components in combination (bnAb + T cells) vaccine regimens is now boosted again[31] Availability of better markers of antiviral activity as well as novel studies on a) the understanding of anti-vector immunity in preventing better immunogenicity from past candidates[32], b) the sieve effects demonstrated in T cell vaccine recipients of the STEP trial[33] c) as well as the promising results from a novel rhCMV-vectored T cell vaccine in controlling viral replication in macaques[34] have altogether helped to re-stimulate the T cell field.

Better T cell immunogens and delivered by better vectors than previous candidates are, however, clearly needed.

Through the compiled work in Chapter 2 and 3, we have tried to strengthen and analyse the idea that HIV is a highly immunogenic virus in terms of T cell immunity, compared to many other viruses, such as EBV, HCV or CMV for instance. HIV infection induces a strong and broad CTL response; all the viral proteins are targets for the anti-viral CTL response although in varying degrees, and even epitopes in alternative reading frames are now known to be immunogenic.[35] In addition, HIV-specific CTL responses leave evolutionary marks on HIV and are thought to be responsible for driving the global viral diversity.[36] However, individuals who do not control their infection can also mount a strong CTL response, even in chronic stages of infection, if sensitive peptide sets are used to measure them as shown in Chapter 3. Therefore many questions arise, which functional characteristics of CTL responses are likely important in discriminating effective from non-effective CTL responses rather than their sheer beadth anf magnitude? If CTLs can effectively inhibit in-vitro viral replication but at very variable levels, could it be that their viral replication suppression is likely, depending on their specificity?

Clinical trials testing T cell vaccine candidates expressing entire HIV proteins off various vectors have failed so far to induce protective CTL responses.[9,37,38] There is growing evidence that the existence of potential "decoy" epitopes may divert an effective CTL response towards variable and possibly less effective targets in the viral genome[3]. In Chapter 2 we show interest in identifying the best viral targets among the 9 encoded HIV proteins. First, as others have

previously shown, in both cohorts of clade B and clade C HIV infected individuals, the total breadth and magnitude of responses did not correlate with the viral loads. However 10 of the identified 26 beneficial OLP in the screened clade B cohort were located in the Gag protein, confirming earlier reports suggesting an important role of Gag-specific CTL (and particularly towards p24) in viral control.[7,39] In addition the beneficial effect of the responses against these 10 Gag-derived OLP was the most prominent in terms of protective ratio (PR) scores and cumulative effects. Comparable results were found in both clade B and clade C cohort.

Numerous studies have tried to elucidate the mechanisms by which Gag-specific T cells could provide such a superior antiviral activity. One possible explanation is that viral fitness costs are reducing in vivo viral replication dramatically when the virus attempts to escape from some of the dominant Gag epitopes restricted by favourable HLA alleles. Other studies in B27+ individuals suggest the rapid re-presentation of Gag and Pol derived epitopes (within 6h following infection) from infecting viral particles as being decisive for the strong antiviral effect of these responses.[40,41] Importantly, in the work of Chapter 2, CTL responses towards the identified beneficial OLP were not simply driven by individuals expressing favorable HLA class I alleles (including HLA-B27, -B57, -B*5801, -B63 and -B81) as the exclusion of such subjects from the analyses still showed the same significant associations. In addition, all the individuals included from the Barcelona cohort harboring such beneficial HLA alleles were intentionally excluded from the study, and not only showed the same Gag dominance CTL pattern but also preferentially targeted the identified beneficial OLP rather than non-beneficial ones compared to non-controller subjects.

Of course, it could be argued whether the specific targeting of HIV Gag demonstrates of a superior ability of the T cells of this specificity to mediate viral control or whether the induction and/or the maintenance of such responses is simply a reflection of otherwise controlled HIV infection. Two recent studies complement our findings and strongly suport the former hypothesis as the direct HIV-suppressive capacity of CD8+ T cells was shown to correlate with the frequency of Gag-specific CTLs, both in clade B and C infection.[42,43]

It is worth noting that despite overall Gag dominance in our analyses, all the HIV proteins harbor beneficial as well as non-beneficial OLP with PR >1 and <1, respectively These data indicate that proteins considered to be generally beneficial may contain non-beneficial regions too, which could be diverting the vaccine induced CTL response in using entire proteins as immunogens. These considerations led to the proposing of a polypeptide-like immunogen design to prevent such non-beneficial viral targets in order to drive immunodominance to-

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wards identified protective regions. The construction details have been further developed in Chapter 4.

An important additional consideration for a rational T cell (and humoral) vaccine design that aims to induce a long-lived immunity is to account on CD4 T cell-mediated helper signals. In this regard, breadth of HIV specific CD4 T cell responses has recently been showed to have a significant inverse correlation with viral loads, but in particular -as we saw for CD8 T cells-, Gag dominance pattern (and particularly p24) distinguished HIV controllers from progressors. {Ranasinghe, 2012 #305} As developed in Chapter 4, these immunogenic regions were included in our polypeptide-like immunogen as they may act as a critical cornerstone for enhancing protective T cell responses.

Towards conservation

An important finding in our work is that beneficial OLP were more conserved that the rest of the genome, particularly when compared to the non-beneficial OLP. These data are in line with results from other studies that propose T cell immunogen designs focused on conserved regions[2,3,44]. Overall, a significant negative correlation between PR and entropy was observed for scored-OLP in both clade B and clade C cohorts and even within the conserved p24, the beneficial OLP identified in the clade C cohort were significantly still more conserved. Thus, even though entire structural proteins such as Gag are among the most conserved regions of the viral proteome, targeting their most conserved fragments provided an advantage to control HIV replication in vivo. These OLP are likely reflecting regions with high epitope density, as seen in the detailed characterization of the responses to conserved regions of Gag-p24 in the work compiled in Chapter 3 and in the description of the final immunogen details in Chapter 4.

Targeting conserved regions of HIV has been proposed as an approach to deal with viral diversity and rapid emergence of escape mutants. ([44] and reviewed in [45]). Conserved regions are by definition, common among the different virus strains and clades to which the vaccinees will be exposed. In addition, potential CTL escape variants in these segments will in many cases carry significant costs for the viral replicative fitness, making them valuable targets for a vaccie induced anti-viral Tcell response.[46-48]. An additional support for more reductionistic immunogens arise from post-hoc analysis in the Merck's Ad5 gag/pol/nef vaccine immunogenicity studies of the STEP trial. The most highly conserved epitopes in Gag, Pol, and Nef were detected at very low frequencies in vaccinees, suggesting potential epitope masking of these responses through the entire proteins

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used.[49] Thus, some of the strategies proposed in the past have been based on the fusion of the most conserved parts of the viral proteome to form artificial protein immunogens[2,3]. A limiting factor in such designs may be that some of the most conserved regions of the virus, particularly in the Pol protein, are immunologically "silent" with diminished recognition in natural infection, as also shown by our work. Thus, not all conserved regions are equally immunogenic or contain beneficial viral targets, an observation that needs to be taken into account when designing subunit vaccine sequences. Also, it has been suggested to link longer conserved regions by specifically designer spacers rather than joining consecutive fusion polyepitope sequences[50]. While this may indeed direct the antigen processing towards the preferred cut-sites, the inserted linkers may generate novel epitopes with no relevance and this need to be handled with caution. As discussed in Chapter 4, our T cell immunogen design reflects all these considerations and potential caveats and, at least in mice, appears to indeed produce broadly directed responses with even magnitudes between different specificities.

An additional strategy to contend with viral diversity, aside the 'conserved elements-like' candidates, is to induce responses of greatest possible ability to react with epitope variants by including the 'variants' themselves in the immunogen. Mosaic antigens are polyvalent cocktails of synthetic but intact proteins that aim to fight antigenic diversity and limit the rapid emergence of viral escape mutants through the induction of an increased depth of CTL responses (more responses to variants of a given epitope).[51,52] Mosaic vaccines are designed using computational strategies that optimize coverage of more potential T cell epitopes. Pre-clinical data in macaques vaccinated have shown promising results, increasing the breadth and cross-reactivity of the vaccine induced responses compared to consensus antigens.[53] As it will be outlined in the Future Research Questions section, we have followed a similar strategy and have now designed a novel Gag sequence that covers the most common CTL escape pathways. We propose to use an entire designer Gag vaccine candidate to be administered as a boost component, especially if co-administered with an envelope immunogen. Having induced responses against the most common forms of the selected epitopes (conserved) and their preferred escape variants should further limit viral escape in vaccinees in the absence of sterilizing immunity. Importantly, as observed in macaques by collaborator investigators, such a prime/boost strategy (priming with a reductionist immunogen plus boosting with a complete protein) helped to boost the first induced responses while maintaining the altered immunodominant hierarchy. (CROI 2012 #427, Kulkarni, manuscript in preparation)

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Cross-reactivity and functional avidity

Three major factors contribute to the enormous viral diversity in infected individuals as well as on a global scale[54,55] and include a) the viral replication speed rate, generating an estimated 10^{10} virions per day in infected individuals[56], b) the error-prone reverse transcriptase which introduces on average one substitution per genome per replication round and c) recombination phenomena which are estimated to occur at a frequency of 7 to 30 recombination events per replication round.[57] In this challenging 'variable' context, high cross-reactivity/depth of vaccine-induced responses is therefore crucial to cope with viral diversity, limit viral escape or at least cripple the viruses by deleterious immune escape pathways. The study presented in Chapter 3 identifies the functional avidity of CD8+ CTL responses to be associated with variant recognition and superior in vivo control in HIV infected individuals from a cohort with largely unbiased HLA genotypes. Complementary, when limiting the analyses from Chapter 2 to the four beneficial OLP identified in Gag-p24, higher functional avidity was also seen in controllers compared to non-controller individuals. In addition, when the functional avidity was determined for 17 optimal epitopes from beneficial, neutral and non-beneficial OLP, the data showed again a strong link between avidity, conservation and protective ratio. Complementary to our results, the collaborative study with Berger *et al* also showed higher functional avidities of Gag-specific and HLA-B-restricted responses in HIV controllers compared to those in non-controllers. Interestentingly, high functional avidity was not restored in individuals with effective viral suppression upon HAART, suggesting that high functional avidity may be a direct immune correlate of viral control rather than a consequence of otherwise controlled HIV infection.[58]

In addition to the previously mentional functional characteristics of CTLs, growing data has also highlighted the beneficial contribution of CTL with elevated functional avidity (or antigen sensitivity) to the control of HIV infection. Potential mechanisims of how CTL responses of high functional avidity could mediate their superior in vivo effects have been suggested:

1) High avidity CD8+ T cells may possess an enhanced ability to inhibit in vitro viral replication at lower antigen levels[59,60], as shown in studies using CTL clones of different specificity, and they may initiate lysis of target cells more rapidly at any given antigen density[61]. In other studies and in addition to CD8+ T cells, the high avidity of some Gag-specific CD4+ T cell responses was explained by a high avidity interaction between the TCR and the peptide-MHC complex, as demonstrated by MHC class II tetramer bindings in controller individuals, which would explain a stable antiviral response in the face of very low viremia.[62]

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2) Our studies are in agreement with data that have established CTL antigen sensitivity as a marker of polyfunctionality[11] and effective sustained suppressive activity.[59,63] Importantly, our data also indicate that high avidity CTL responses may provide greater variant recognition compared to low avidity responses.[58,64] As for the differences in the amount of peptide required to elicit effector function, high avidity responses may be more prone to react with sequence variants in their cognate epitopes, and they may provide a crucial advantage in the control of highly variable pathogens, as it has been also shown for HCV in the past [65] and for HIV in the manuscripts included in this thesis.

3) In a recent report, effects of HLA allele-dependent intrathymic positive selection have been proposed to give raise to high-avidity T cells of superior antiviral activity and also could at least partly explain the advantage of MHC heterozygosity in HIV control.[66] This would suggest that beneficial alleles restrict CTL responses of inherently greater functional avidity, an hypothesis that our data in Berger et al[58] further support as well.

4) A detailed study on the protective HLA-B27 restricted KK10 epitope in HIV Gag p24 also highlights the importance of T-cell receptor (TCR) affinity as a determinant of antigen sensitivity.[67] In their work, Iglesias *et al.* show that the selection of 'public' clonotypes in the epitope-specific T cell repertoire can result in functional advantages that contribute to effective suppression of HIV replication. In this case, HIV controllers mounted responses with relatively narrow TCR repertoires, which were dominated by public TCR sequences in HIV controllers. Similar findings emerged from our studies, where public TCR clonotypes were found at higher frequencies in HIV controllers compared to non-controllers mounting the same epitope specific responses.[58] These findings are of relevance for vaccine design as controlling this selection process would possibly endow the vaccine induced T cell response with a more effective TCR repertoire. How to induce such public-TCR based responses in vivo is still however an open question at this time and will possibly depend on antigen dose and duration of antigen persistence in the vaccinees.

Lastly, with respect to the previously discussed immune correlates, these data also raise the question as to whether the maintenance of high avidity responses by HIV-specific CTL is the consequence rather than the cause of low viral loads in chronically infected subjects. Data by Streeck et al on individuals followed from early time points in acute infection suggests that continuous antigen stimulation may reduce CTL functionality over time[68]. As discussed in Chapter 3, whether such losses of high avidity responses correspond to changes in the clonal com-

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position of the CD8 T cell response or to a gradual decrease in their functional avidity due to altered cell reactivity/signaling is still unclear. Although not conclusive, our avidity data in individuals initiating HAART show that antigen removal does not increase functional avidity in chronic stages of infection, therefore fostering the evidence on high functional avidity as a cause rather than an effect of in vivo HIV control.[58]

Technical aspects of the assays, unclear thresholds and peptides sensitivity.

As pointed out by Bennet *et al.* it is crucial to define avidity thresholds for different T cell effector functions in order to better interpret the significance of functional avidity measurements and to predict CTL efficacy against virus-infected cells.[60] In this regard, in Chapter 3, despite individuals with suboptimal control of viral replication showed CTL responses of significantly lower avidity compared to those detected in controller individuals, a largely overlapping range of functional avidity measurements between responses in both groups was observed and may pose a challenge to conclusively demonstrate its potential biological relevance. However, the sheer extend of tested responses (>450) allowed to observe statistically significant differences between the two groups, although the difference in the median avidity values was quite limited. Evidently, responses to many different epitopes restricted by diverse HLA alleles were being compared and thus introducing a wide range of potential avidities for these responses. Of note, differences in avidity were more pronounced when the comparison was limited to matched responses detected in both groups, by probably reducing the range of potential avidities of the responses tested. In any case, having a defined threshold under which a CTL response in vivo is unlikely to mediate effective immune surveillance woud greatly inform vaccine development. This is also reflected in a study by Keane *et al,* where T-cell responses to early escape mutant epitopes (named neo-epitopes) were shown to have significantly greater functional avidity and higher IFNγ production than T cells for non-adapted epitopes, but were no more cytotoxic.[69]

One major issue that emerged from our analyses was that peptide length in the different in vitro test sets can massively impact the emerging results. Most of currently used peptide sets include 15-18mer peptides overlapping generally by 11 residues.[70] Such well established approaches do not require prior HLA typing, do not depend on the previous knowledge of defined optimal epitopes and allow high throughput screenings. In our work in Chapters 2 and 3, overlapping peptide sets of 18-22 amino acids in length with an overlap of 9-12 residues

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were used spanning the entire HIV-1 proteome. This was complemented by a peptide set spannig Gag p24 that consisted of shorter 10mer peptides overlapping by 9 amino acids. Our data showed that significantly more responses were identified using the 10mers than with the 18mers, with an overproportional loss of responses when using the 18mer peptides in the HIV non-controllers. Importantly, the responses identified by the 10mer peptide sets did not represent spurious or nonspecific reactivities as they did not differ in terms of magnitude to the responses detected using the 18mers. The fact that up to three times the number of responses were detected using the shorter peptides raises serious concerns as to the validity of earlier datasets, which may have missed a considerable portion of responses. In addition, from earleir studies using combinations of Consensus, ancestral and Center-of-tree (COT) sequences based peptides sets and from the use of Toggle peptides, it is clear that long 18mer peptides, based on a monomorphic test sequence may likely miss half of the existing CTL responses.[71,72] In addition, this is in line with data from testing peptide sets matching the autologous viral sequence, which increased response rates by 30% compared ot consensus-based peptide sets. Importantly, our in our study, differences rates of responses in non-controllers were not due to a suboptimal match between test peptide sequences and autologous viral HIV-1 sequences in the non-controllers as their dominant autologous p24 sequence was in all cases clade B and mostly (99%) represented by the test peptides.

On the other hand, the lack of detecting responses that was seen with 18mer but not 10mer peptides may not be too dramatic, as the shorter peptides likely revealed more low-avidity responses, although as mentioned above, not reliable thresholds are yet stablished to rule out their potential physiological relevance. Still, basing vaccine development on only a fraction of our possible knowledge of HIV specific CTL responses can possibly severly impact the immunogen design. In addition, for immune monitoring assays, the most sensitive test peptides possible should be used to reliably assess the vaccine's in vivo immunogenicity.

Designing a T cell immunogen and putting it to the test.

The last and most translational work of this thesis is based on the preliminary results of the first in vivo immunogenicity tests of a novel T cell immunogen that has been designed incorporating all the information compiled from the previous studies. As described in Chapter 4, the identified beneficial OLP as well as the information from conserved regions in Gag-p24 that induced responses with the highest avidity and cross-reactivity potential were assembled into a total of 16 sequence segments which were linked to each other using triple alanine linkers.

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A final T cell immunogen sequence was translated into an expression-optimized nucleotide sequence and cloned into a CMV based plasmid harbouring a GM-CSF signal peptide for efficient secretion of the encoded protein sequence. The first immunogenicity studies were evaluated in 6-8-week old female C57BL/6 mice two weeks after two DNA vaccinations (weeks 0 and 4). Although still preliminary, the results show that the T-cell immunogen was able to elicit a balanced, broad T cell response to all protein components (Gag, Pol, Vif and Nef) contained in the immunogen. In contrast, mice immunized with plasmids encoding for the full-length Gag, Pol, Nef, Tat and Vif protein sequences showed a strong Gag dominance of the relatively narrow responses. For instance, in mice immunized with plasmids encoding for the entire proteins, only 26% of the total vaccine-induced response targeted the beneficial regions included in our immunogen sequence. Thus, the T-cell immunogen developed in the local HIVACAT program, is postulated as a novel, reductionist T-cell vaccine candidate whose design has been rationally based on the extensive ex-vivo immunogenicity data from HIV infected individuals and provides a broad HLA class I allele coverage. To better deconvolute the full immunogenicity potential in the context of different HLA genotypes, to rule out immunogenicity to juntional epitopes and to characterize the antiviral effect of the vaccine-induced responses the initial mice data are now being confirmed in humanized BLT mice.

DNA plasmid technology has been used as a first choice to assess immunogenicity of the T-cell immunogen as a proof-of-concept strategy, due to its versatility, and feasible scalability, as discussed in the Introduction section of this thesis. If a broad, functional and balanced vaccine-induced response is seen in the humanized BLT mice model for different HLA genotypes, the candidate could be considered to be moved into human clinical testing and used to express the immunogen in other live recombinant vectors, such as MVA or ChAd to allow for subsequent prime/boost heterologous regimens. Undoubtedly, any potential preventive vaccine regimen will have to incorporate a vaccine candidate that induces complementary humoral response as well as CD4 T cell help. We postulate that boosting with a VLP constructed using the above mentioned designer full length Gag sequence and expressing novel full length and/or MPER envelopes sequences could enhance cross-reactivity to our beneficial viral T cell targets in Gag without disrupting the dominance of the initial induced response and providing the helper cell responses needed for the induction and maintenance of a robust complementary B cell immune response.

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In conclusion, this compiled work was aimed at providing an overview of the T cells that play a major role in HIV control as well as the most important difficulties in the HIV vaccine development field. It included a review of the most significant immune, virological and host genetic factors involved in HIV control. We then identified potential viral targets, described new characteristics of CTL responses associated with better disease outcomes and ended by proposing a reductionist T cell vaccine candidate, that aims to overcome some of the major hurdles in vaccine development. Although a better understanding of the mechanisms of protection is still required and recent discoveries on broadly neutralizing antibodies have now started to be incorporated in new humoral vaccine candidates, novel rationalized T cell immunogens such as ours still have room to be screened for the induction of immune responses of improved quality that could potentially increase effectiveness in protection and disease control with respect to past vaccines.

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CONCLUSIONS

1) The most benefical CTL targets are predominantly located in HIV Gag, Pol and Vif, but rarely in more variable proteins, providing a strong rationale for vaccine approaches that focus on conserved viral regions to deal with viral diversity.

2) The presence of responses of high functional avidity with broad variant recognition ability is a hallmark of controlled HIV-1 infection and the induction of such responses by future HIV-1 vaccines may be crucial to constrict rapid viral escape.

3) Standard approaches using 15-20 mer overlapping peptides in ELISpot IFNγ assays significantly underestimate the breadth of CTL responses, especially in individuals who do not control HIV infection. This observation has important implications in the design of improved vaccine immune readouts.

4) The identification of beneficial viral T cell targets and new functional properties of CTL associated with relative viral control allowed us to design a reductionistic T cell immunogen that includes T cell targets restricted by a wide array of HLA class I alleles.

5) A DNA plasmid expressing our polypeptide immunogen induced a broad repertoire of CTL and was able to break immunodominance to potentially non-beneficial targets in C57BL/6 mice.

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FUTURE RESEARCH QUESTIONS

Based on the findings of this thesis and the currently ongoing work, the following research questions emerge:

1) In-vivo testing of the immunogenicity of the DNA plasmid expressing the T cell immunogen in the humanized BLT mice model. Address the breadth and immunodominance of vaccine-induced responses in the context of different HLA genotypes, and using a regular intramuscular and electroporation delivery system.

2) Address functional avidity, cross-reactivity potential and viral replicative inhibition on selected mapped responses from vaccinated animals.

3) Use a novel Gag sequence to cover common CTL escape variants in the identified beneficial targets to increase the depth of CTL responses in a prime/boost strategy introducing a humoral vaccine component.

4) In vivo assessment of immunogenicity in heterologous combined vaccines (DNA-MVA, or DNA-VLP) expressing the T-cell immunogen in B57BL/6 mice

5) Further our understanding of viral and immune factors mediating HIV control by studying the mechanisms involved in the loss of viral control in a group of HIV controller individuals subjected to longitudinal follow-up.

6) Implement new available and comprehensive immune readouts in the upcoming phase I proof-of-concept therapeutical vaccine trials.

ADDENDUM I

Published Manuscripts in Portable Document Format (PDF)

CHAPTER 1

Beatriz Mothe, Javier Ibarrondo, Anuska Llano and Christian Brander
Virological, immune and host genetics markers in the control of HIV infection.
Dis Markers. 2009;27(3):105-20. Review.

CHAPTER 2

Beatriz Mothe, Anuska Llano, Javier Ibarrondo, Marcus Daniels, Cristina Miranda, Jennifer Zamarreño, Vanessa Bach, Rosario Zuniga, Susana Pérez-Álvarez, Christoph T. Berger, Maria C. Puertas, Javier Martinez- Picado, Morgane Rolland, Marilu Farfan, James J. Szinger, William H. Hildebrand, Otto O. Yang, Victor Sanchez-Merino, Chanson J. Brumme, Zabrina L. Brumme, David Heckerman, Todd M. Allen, James I. Mullins, Guadalupe Gómez, Philip J. Goulder, Bruce D. Walker, Jose M. Gatell, Bonaventura Clotet, Bette T. Korber, Jorge Sanchez and Christian Brander
Definition of the viral targets of protective HIV-1-specific T cell responses.
J Transl Med. 2011 Dec 7;9(1):208.

CHAPTER 3

Beatriz Mothe, Anuska Llano, Javier Ibarrondo, Jennifer Zamarreño, Mattia Schiaulini, Cristina Miranda, Marta Ruiz-Riol, Christoph T. Berger, M. José Herrero, Eduard Palou, Montse Plana, Morgane Rolland, Ashok Khatri, David Heckerman, Florencia Pereyra, Bruce D. Walker, David Weiner, Roger Paredes, Bonaventura Clotet, Barbara K. Felber, George N. Pavlakis, James I. Mullins and Christian Brander.
CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control.
PLoS One. 2012;7(1):e29717. Epub 2012 Jan 4.

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Disease Markers 27 (2009) 105–120
DOI 10.3233/DMA-2009-0655
IOS Press

Virological, immune and host genetics markers in the control of HIV infection

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Abstract. HIV infection, if left untreated, leads in most cases to the development of wide immune deterioration, opportunistic infections and eventually AIDS and death. The identification of individuals who despite persisting infection show no or few signs of HIV disease progression has spurred hopes that an effective HIV vaccine could be attainable. The design of such a vaccine will greatly depend on the precise definition of disease markers, host genetic and immune characteristics that mediate relative *in vivo* control of this virus. Accordingly, a number of viral factors and host genetic characteristics have been shown to play a crucial role in the control of HIV disease by delaying progression to AIDS or even preventing infection. There is also an improved understanding of humoral and cellular immune responses in terms of specificity, functional repertoire, longevity and tissue distribution and their ability to contain HIV replication. However, the definition of good immune correlates unequivocally and causally associated with protection or disease progression remains elusive. Here we review work on viral factors, host genetic markers and immunological determinants that have been identified in individuals with superior control of HIV infection or in subjects who remain uninfected despite frequent exposure to the viral pathogen.

Keywords: HIV-1, long-term non-progressors (LTNP), Elite Controllers (EC), Highly exposed persistently seronegatives (HEPS), CCR5, CTL, Innate immunity, HIV control, immune correlates, HLA allele frequency

1. Introduction

The AIDS pandemic is one of the greatest global health crises of our time. Since HIV was identified 25 years ago, 23 million people have died and 33 million more are living with HIV [1]. Despite advances in education, HIV prevention and improvements in access to antiretroviral drugs, the pandemic continues to outpace global efforts at prevention and control. According to the United Nations Development Program (UNDP), HIV has inflicted the "single greatest reversal in human development" in modern history [2].

AIDS vaccine research and development is of highest priority today and, at the same time, one of the biggest scientific challenges the immunology field faces. The enormous viral variability together with human host genetic diversity throughout the world are primary factors hampering the development of efficient strategies to control and prevent HIV infection and the design of potentially effective immune-based therapeutic interventions and prophylactic vaccines. The recent failure in 2007 of a phase III trial of a prophylactic vaccine candidate further highlighted how far the field is still away from reliable markers of HIV control and how to best design an effective vaccine. Thus, an improved understanding of the immunopathogenesis of HIV infection and the role of host genetic markers and viral diversity in this control is urgently needed. However, it will likely not be sufficient to link certain more or less random markers with clinically well-established parameters of disease progression, such as plasma viral RNA levels and nadir CD4+ T-cell counts or CD4+ T-cell counts decline over time. Rather, specific markers that are directly mediating viral control need to be identified so that vaccine design is not misled by focusing on epiphenomena and functionally unlinked markers. This is certainly easier said than done as determinants of an effective viral control will likely need to be identified in multifactorial models that incorporate viral variability, environmental particularities (such as co-infections) and host genetics.

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Here we review the role of viral factors, host genetics markers and HIV specific immune responses in the control of HIV infection and their possibly underlying mechanisms. The determinants of viral attenuation, specific HLA class I and II alleles, certain polymorphisms in co-receptor genes and ligands, the specificity and functionality of virus-specific CD4+ and CD8+ T-cell responses, as well as new insights into factors of the innate immune response in HIV control are being discussed.

2. Insights from studies in individuals with self-controlled viral replication and HEPS cohorts

Infection with HIV leads to a devastating erosion of the immune system clinically characterized by a progressive rise in HIV viral load and decrease of the number of CD4+ T-cells. This decline in CD4+ T-cells heralds the progression to AIDS with its associated opportunistic infections and cancers and ultimately ends in death.

Among the HIV-infected population, several groups of individuals have been identified that remain clinically stable and free of any AIDS defining conditions for decades after infection in the absence of antiretroviral therapy [3]. These individuals (estimated to be 5–8% out of the total HIV-infected population) have been referred to as long term survivors (LTS) or long-term non-progressors (LTNP) and are generally able to control viral replication to low levels – plasma RNA levels < 2,000 copies/ml (or < 5,000-10,000 copies/ml, depending on arbitrary cut-offs decided upon by different investigators) and to maintain normal CD4+ T-cell counts over time with a reduced rate of CD4+ T-cell loss (18 cells/μl/year) compared to that in normal progressors (around 60 cells/μl/year) [4]. A subset of such LTNP is able to maintain undetectable plasma viral loads for extensive period of times and is known as elite controllers (EC). The clinical and scientific interest in such individuals is great as they may indeed hold the key of spontaneous control of HIV infection. Current efforts have allowed for the establishment of large international cohorts of controllers and elite-controllers and an intensive search for host genetic, virological as well as immunological markers of HIV control is ongoing in these subjects.

Some recent work on elite controllers (estimated to represent less than 1% of the HIV infected population) has shown wide heterogeneity in the immunological and clinical course of HIV infection despite certain similarities in genetic determinants [5], again suggesting that HIV control needs to be seen in a context that integrates host genetics, immune function as well as virological diversity. Nevertheless, these and earlier analyses have directly allowed to identify a number of properties of the immune response to HIV (specificity, T cell polyfunctionality), as well as host genetic markers (mainly HLA class I alleles) that are strongly associated with disease control and which will prove helpful in the quest of developing a broadly applicable HIV vaccine.

Similarly, highly Exposed Persistently Seronegatives (HEPS) individuals, such as some commercial sex workers or discordant couples who are multiply exposed to HIV yet remain uninfected, may also provide important clues into potential mechanisms of HIV control and even prevention of infection [6,7]. However, these studies need to always be well documented for real exposure to HIV, even more so now that studies in discordant couples sometimes include HAART treated subjects which put the seronegative partner at clearly reduced risk of HIV acquisition. Some recent studies have identified genetic polymorphisms in the SDF1 gene, specific CD8+ T cell responses and IgA production as factors associated with a reduced risk of HIV acquisition in these groups [8–12]. However, T cell response rates and antibody production have not emerged consistently in all studies as potentially protective [13, 14] and further studies are needed to clarify these findings. In addition, immune analyses conducted to identify potentially protective immune responses need to be based on sensitive but specific assays and stringent cut-offs [15]. Given these considerations, it can reasonably be expected that future analyses on HEPS may provide further valuable information.

3. Viral determinants in control of HIV-infection

While individuals with apparently effective control of HIV infection (LTNP, elite controllers) have been studied exhaustively for virus-specific immune responses, less is known about potential virological determinants that could be driving the observed control. A number of studies have identified single individuals or small cohorts (such as the Sydney Blood Bank Cohort –SBBC- a unique collection of individuals infected with an attenuated HIV-1 virus from a common donor) that harbor partially defective viruses that seem to have a decreased replication competency. Others have also

been able to isolate virus with impaired replicative capacity from PBMC cultures generated from LTNP, supporting the hypothesis that primary infection by 'attenuated virus strains' with slow replication kinetics may facilitate control of viremia [16]. This is not only of relevance to the replication fitness of potentially partly defective viruses as it could also reduce the kinetics of viral evolution and CD4+ T cell depletion over prolonged period of time.

A certain 'acquired' degree of attenuation in terms of impaired replicative capacity could be partially induced by antiretroviral therapy, as individuals who were efficiently treated for long periods of time showed a prolonged delay in restoration of pretreatment viral diversity after therapy interruption (STI). This suggested that punctuated antiretroviral therapy may cause a considerable evolutionary bottleneck leading to the emergence of viral populations with overall reduced viral fitness [17]. However, it could also reflect improved immune competence and at least partial immune restoration after prolonged treatment periods, which could on its own impact viral repopulation dynamics.

Aside from treatment-induced viral variants, several viral genetic defects and polymorphisms that impair replicative capacity have been implicated in mediating relative viral control. Viral genomes carrying deletions or inactivating variants in the nef gene or in the overlap of nef and the U3 region of the long terminal repeat (LTR) were among the very first viral defects associated with control. These cases were identified in 1995 in a group of 6 individuals from the SBBC cohort who had become infected after obtaining blood transfusions from the same HIV-infected blood donor but remained free of HIV-related disease [18]. Although still unclear, one potential underlying mechanism suggested for the beneficial effects of this nef deletion is that Nef is no longer available to down-regulate HLA class I molecules and infected cells thus would be more readily recognized by HIV-specific CD8+ T cells [19]. However, longitudinal analysis of viral evolution of the *nef*/LTR sequences over time in the SBBC cohort, were unable to fully explain mechanisms that could have contributed to slow progression of HIV disease in 2 (out of the 6) individuals of the cohort, suggesting that nef gene deletions are not necessarily mediating life-long protection from disease progression. Thus, other viral and/or host factors plus immune pressure was likely contributing to the long-term control in these individuals [20].

Aside from Nef-mediated effects, other mutations in structural proteins have been associated with slower disease progression as well. These include unusual, difficult-to-revert polymorphisms and 1–2 amino acids deletion in gp41 and Gag or a four amino acids insertion in Vpu among others [21]. In addition, replication defective strains have been identified when constructing viral clones with mutations at the rev activation domain, which were seen more frequently in controller individuals than in subjects with regular HIV disease progression [22]. In addition, to these mutations of unknown origin in terms of specific selective force (apart from reduced replication capacity), viral mutations evolving under strong immune selection pressure have been show to lead to reduced viral replication (see below).

Despite these reported cases of reduced replicative viruses and slower disease progression, the frequency and contribution of such defects on the maintenance of undetectable viremia is not well established. In particular, conflicting data exists partly due to the fact that the identification of such attenuated virus strains *in vivo* is limited to small numbers of subjects and that sequence analyses have often been based on analyzing cellular proviral DNA, thus possibly also including some level of grave-yard sequences. Today, with more sensitive assays, some elite controllers have been shown to have persistent viral replication detectable in plasma at levels below 50 copies/ml [23]. In addition, a recent report by Miura et al for instance did not reveal any gross genetic defects in HIV-1 coding gene sequences derived from plasma viral RNA from a large cohort of elite controllers [24]. This observation suggests that active viral suppression by the immune system rather than shared viral genetic defects or polymorphisms is driving viral control in HIV controllers. This conclusion is also in line with work on accessory genes from HIV controllers, where replication-competent viruses from CD4+ T-cell co-culture supernatants were analyzed and did not reveal any consistent defects in either vpr or vif genes [25].

4. Impact of host genetics on *in vivo* HIV control

An extensive number of host genetic markers have been identified over the last 20 years that are associated with either rapid or slow HIV disease progression or with protection from infection. Many older studies however were carried out using single gene approaches with small number of samples and the genetic associations found were not always confirmed in subsequent functional studies. Thanks to the advances of the Humane Genome Project, the use of whole-genome as-

sociation scans, and the establishment of international consortia such as CHAVI, EuroCHAVI or the HIV International Controllers consortium, it has been possible to uncover certain genetic factors that might play a relevant role in the control of HIV. This availability of large samples number and the possibility to sequence 650,000 single-nucleotide polymorphisms within the human genome should further enhance studies examining the contribution of multiple genetic factors [26]. However, unlike studies on genetic markers associated with for instance autoimmune diseases, the search for host genetic polymorphisms in HIV infection also needs to take into consideration the viral diversity in regions of different host genetics/ethnicities. An earlier report on the effect of a single genetic (HLA) marker and viral evolution has recently been confirmed in a massive international effort, demonstrating the inter-relationship between host genetics and viral evolution [27,28]. These findings highlight that viral diversity is likely be shaped by differences in the frequency of different host genetic markers and, based on viral evolution, can lead to opposite effects of a specific genetic marker on HIV disease control [27]. Thus, whole human genome approaches are severely complicated by viral diversity in different host ethnicities making comparisons across different clades of HIV and various geographically distinct human populations difficult. This consideration also points to the possibility that different clades of HIV may possess inherently different replication fitness and may drive disease development at variable levels, as recently considered as a possibly contributing factor in a case of severe acute HIV infection [29].

5. Association of HLA polymorphisms with HIV disease outcome

Many host genetic polymorphisms associated with levels of disease control involve genes encoding for receptors for viral entry and molecules expressed on the surface of cells of the innate or acquired immune system, such as HLA, CCR5 and KIR receptors. Moreover, it seems that in some cases their potential protective influence might have a cumulative effect as seen for the synergic effect of some KIR receptors and HLA-B complexes [30]. Likely the most robust analyses have focused on the HLA genes and their polymorphisms. The HLA class genes form highly polymorphic loci in the Major Histocompatibility Complex (MHC) located in the short arm of chromosome 6 and encode for cellular surface molecules that present foreign antigenic epitopes to T lymphocytes. There are two groups of HLA molecules including HLA class I and HLA class II antigens. The HLA class I molecules are divided into HLA-A, HLA-B, HLA-C all of which bind peptides derived from intracellularly processed proteins and present them to CD8+ cytotoxic T-cell lymphocytes (CTL). Among these, the HLA-B alleles, while most diverse (more than 1,000 HLA-B alleles have been identified to date) have also been shown to carry the bulk of the anti-viral T cell immune response in HIV infection [31]. Accordingly, the number of well-defined HLA-B restricted epitopes exceeds the number of defined epitopes restricted by HLA-A and, particularly, HLA-C alleles. However, especially the HLA-C alleles are currently under more intensive investigation as larger HIV infected cohorts with more complete and high-resolution HLA-C typing have become available.

HLA alleles are grouped into 9 supertypes based on their structure, peptide-binding motif, epitope representation and sequence similarity [32,33]. Particularly alleles included in the HLA-B7 (B*5101, B81), HLA-B27 (HLA-B27, B*1503) and HLA-B58 supertypes (HLA-B57, B*5801, B*1516, B*1517) have been associated with improved or impaired levels of HIV control. Of note, almost all the alleles in the HLA-B58 supertype appear to mediate superior control of HIV infection [34]; with the exception being the HLA-B*5802 allele, which is highly prevalent in South Africa and which is associated with elevated median viral loads [35]. The reasons how subtle changes in the HLA sequence (HLA-B*5802 only differs in three amino acids from the "good" HLA-B*5801 allele) can so profoundly affect HIV disease outcome are still unclear and are not in all cases simply attributable to different CTL epitope repertoires presented on these alleles [35,36]. The fact that this allele, as well as the HLA-B*1503 allele are present at high frequency and are both associated with higher viral loads in HIV infected individuals (HLA-B*1503 is a "good" allele in the North American population where it is rare; ref [27]) is in line with earlier reports that found an advantage of expressing rare HLA supertype alleles in controlling HIV [37].

In addition to HLA allele frequency, the homocygous expression of individual HLA alleles has been associated with reduced viral control. This heterozygote advantage has been widely observed in several cohorts, including Caucasian and non-Caucasian populations [38, 39] and has been reproduced in Hepatitis C infection for which HLA-associated markers of viral clearance

and virus control have been identified as well [40]. Furthermore, the effects of particular HLA supertypes or of individual alleles have also been reported to provide the basis for immunologically mediated resistance to infection [41,42]. It will be interesting to confirm the potential protective effects of such alleles in additional cohorts with variable allele frequencies and to assess other mechanisms and markers present in genetic linkage to these alleles that may possibly be involved to at least some levels in protection from HIV infection [43].

Associations between HIV control and specific polymorphisms in the HLA class II loci have been less well-defined, maybe reflecting a possibly only indirect antiviral effect of HLA class II restricted CD4+ T cells. Nevertheless, some cohort-based studies have reported that DRB1*13 allele expression is associated with partial protection from HIV disease progression, although this has not consistently been observed [44]. The DRB1*13/DRQ1*06 haplotype has also been found at increased frequency in individuals who were treated early in HIV infection and who maintained virus suppression after treatment interruption [45]. Furthermore, a protective role of DQB1*06 alleles, irrespective of their DR haplotype co-expression, has been identified [46]. While the HLA class II associations have not produced as strong markers as HLA class I analyses, the representative studies given above highlight the importance to further explore the contribution of the specific CD4+ T cell responses and their genetic basis in the control of HIV.

6. Specific HLA class I B alleles associated with variable levels of HIV control

HLA-B*5701 and, to a lesser extent HLA-B27 are HLA-B alleles overrepresented in North American and European cohorts of LTNP and EC individuals [26,38,39,47,48] and reviewed in [49]). Similarly, the HLA-B*5703 allele (which is the prevalent B57 subtype in Africans) is also significantly enriched among African subjects that control HIV replication [31,50]. These associations are further supported by survival analyses of HLA-B57 and HLA-B27 expressing individuals [51]. How these alleles mediate their beneficial effect has however not been entirely clear, although in the case of HLA-B27 and HLA-B57 compelling evidence suggests it may be due to their presentation of multiple immunodominant epitopes located in HIV Gag and reverse transcriptase [5,52,53]. The broad epitope repertoire and the wide cross-recognition of epitope variants presented by HLA-B57 suggest that effective viral escape from HLA-B57 restricted CTL responses may be difficult to achieve. Of note, HLA-B*5701 is in strong linkage disequilibrium with the polymorphism located in the HLA complex P5 gene (HCP5), located 100 kb centromeric from the HLA-B locus and who has been identified as a protective marker of HIV infection, independent HLA-B57 expression [26]. However, the mechanistic explanation of this protective effect remains elusive and probably is a result of a combined haplotypic effect with HLA-B*5701. This is also in agreement with recent studies showing significant fitness cost of HIV escape variants that affect HLA-B57 restricted CTL epitopes [54]. Similar results have recently also been reported for CTL responses targeting HLA-B13 restricted epitopes, which support the notion that the nature of the presented epitope, more than the restricting HLA molecule allele may determine the beneficial effects of a specific HLA allele [27,55]. However, evolutionarily closely related alleles with subtle sequence differences and comparable binding motifs but associated with opposite rates of HIV disease progression also indicate that the presenting HLA molecule may have a modulating effect on the effectiveness of the restricted T cell response.

Analogous to the HLA-B58 and -B13 alleles above, the protective effect of HLA-B27 allele is also thought to be due to its restriction of an immunodominant CTL response to a conserved HIV epitope located in p24 Gag. This 10mer epitope sequence contains the arginine residue at position 264 of HIV Gag which, once mutated weakens the epitope binding to HLA-B27 [56]. However, the classical mutation to lysine has detrimental effects on viral replication capacity and requires a complex series of compensatory mutations in partly distant sites in Gag to restore viral fitness [57–59]. Thus, the mutation is less likely to revert and to restore full replication fitness and individuals with the escape mutation may still present with lower viral loads than the rest of the population.

Apart from HLA alleles that mediate relative protection from HIV disease progression, a number of alleles have been identified that are linked to accelerated disease courses. Among these, the HLA-B35/Cw04 haplotype has been consistently found at increased levels in individuals with rapid progression to AIDS [38]. Subsequent studies revealed different peptide-binding specificities for the various HLA-B35 subtypes, which prompted their discrimination into Py and Px alleles, respectively [36]. Accelerated HIV disease progression has been associated with the HLA-B35 Px (HLA-

B*3502/3503/3504) alleles but not Py alleles (B*3501). This could explain why HLA-B35 associations were not observed in African-Americans as this ethnic group often expresses HLA-B35-Py alleles. It has also been suggested that the HLA-Cw04 association with rapid disease progression was due to its linkage disequilibrium with HLA-B35-Px alleles rather than exerting a deleterious effect by itself [36].

Aside from HLA linkage disequilibrium and other polymorphisms in the MHC region, the potentially synergistic effects of specific HLA type and NK inhibitory receptor (KIR) have recently obtained much attention. KIR receptors are polymorphic receptors that interact with HLA class I molecules and regulate the NK activity, either by mediating activating or inhibitory signals. A number of studies have associated the expression of specific HLA and KIR combinations with different diseases, such as cervical neoplasia and infectious diseases, including HIV [30,60–62]. The insight into potential mechanisms of these favorable combinations is most advanced in the case of HLA-B/KIR allele combinations. HLA-B molecules contain one of two mutually exclusive serological epitopes, Bw4 and Bw6, which differ by five amino acids spanning positions 77-83 of the HLA-B heavy chain, including the crucial Isoleucine residue at position 80 [63]. HLA-Bw4- but not HLA-Bw6-molecules have been considered ligands for KIR3DL1 and possibly KIR3DS1 [62, 64,65]. In a proportional hazard model that included all known genetic predictors of HIV progression, co-expression of KIR3DS1 and HLA-Bw4 was found to be an independent predictor of decreased time to AIDS. Interestingly, this beneficial effected was observed despite the fact that KIR3DS1 alone in the absence of its HLA ligand was associated with more rapid disease progression. Thus, the findings highlight the potential for KIR/HLA interactions to be important independent predictors of HIV progression and may help shed light on the relative contribution of these interactions compared to HLA-restricted CTL activity [62,66].

7. Non-MHC encoded genetic markers of HIV control

Although the CD4 antigen is the main receptor for HIV entry into susceptible cells, effective viral infection requires the presence of one of two major co-receptors, referred to as CCR5 and CXCR4, respectively. These two co-receptors belong to the superfamily of 7-transmembrane G-protein–coupled chemokine receptors and determine viral tropism, allowing for the differentiation of R5, X4 or R5/X4 viruses that can use either one or both of these receptors [67,68].

Chemokines are a superfamily of small molecules (8–15 kDa) that exert many roles in inflammatory and in homeostatic immune processes [69,70]. They are divided into four subfamilies based on the structural cysteine motif located in the amino-terminus of the mature protein (CXC, CC, CX_3C and C chemokines) and their receptor usage shows a considerable level of redundancy [71]. The chemokine Regulated on Activation Normal T-cell Expressed and Secreted (RANTES or CCL5), Macrophage Inflammatory Protein-1α (MIP-1α or CCL3) and 1β (MIP-1β or CCL4), were first identified as natural ligands for the CCR5 receptor and were subsequently shown to be potent inhibitors of R5 viruses *in vitro*. The natural ligand of CXCR4 is Stromal Cell Derived Factor-1 (SDF-1 or CXCL4), which also possesses potent inhibitor function of X4 viruses *in vitro* [72–74]. Other chemokine receptors that can act as HIV co-receptors have been described, including CCR3, CCR2b and CCR8 [75].

Shortly after their identification as crucial HIV co-receptors, genetic polymorphisms in the various chemokine receptors were reported. In particular, a 32 base pair deletion in the CCR5 gene (CCR5-Δ32) was identified that generates a non-functional protein that is not expressed on the cell surface. The homocygous expression of the CCR5-Δ32 variant provides *in vivo* resistance to infection by R5 HIV isolates. These observations were made in different cohorts of men who have sex with men and hemophiliacs with documented exposure to HIV. When present in a single copy only, the heterocygous expression of the wildtype CCR5 receptor was found to be sufficient to enable infection, although the lower levels of CCR5 on the cell surface were associated with reduced viral replication and a delayed onset of AIDS [76–79]. Spurred by the effects of heterocygous expression of CCR5-Δ32, mutations in the promoter region of CCR5 were identified that are also associated with altered transmission or delayed disease progression, although to a lesser extent than the CCR5-Δ32 mutation [80,81].

In addition to the CCR5-Δ32 mutation, the V64I substitution in the CCR2A protein sequence (CCR2-V64I) has also been found to delay HIV disease progression; however without preventing HIV transmission. Intriguingly, CCR2 is rarely used as a co-receptor in HIV infection and its impact on global epidemic is unclear. Furthermore, it has been demonstrated that the CCR2-V64I allele is in strong linkage disequilibri-

um with a point mutation in the CCR5 regulatory region [82]. Together with CCR5 mutations, approximately 29% of LTNP phenotypes in large cohorts have been estimated to be due to a mutant genotype for CCR2 or CCR5 [83]. Interestingly though, relative protection against AIDS provided by CCR5-Δ32 heterocygosity appears to be continuous during HIV disease over time, whereas the protective effects of the CCR2-64I variant was most pronounced in early infection [84].

The identification of CCR5 and CCR2 as crucial molecules for HIV-infection has also offered new treatment targets to inhibit viral replication and CCR5-based HIV entry-inhibitors have been developed are now part of effective rescue treatment strategies [85,86]. More recently, a single case of stem-cell transplantation from an homocygous CCR5-Δ32 donor to an HIV-infected individual with acute myeloid leukemia showed no signs of rebounding viremia in plasma, bone marrow or rectal mucosa 20 months post-transplantation and in the absence of antiretroviral treatment. This is even more surprising given that minor X4-variants were identified in the pre-transplantation viral population, giving rise to a number of questions on how viral populations with different cell tropism are controlled *in vivo* [87].

Polymorphisms involving other chemokine receptors and/or other chemokine receptor ligands have been identified as well. Especially, plasma levels of RANTES, which can significantly vary among healthy individuals, were found to be modulated by two single nucleotide polymorphisms in the RANTES gene promoter region. These changes have also been associated with delayed progression of HIV disease and experimental over-expression and increased promotor activity of RANTES functionally link the polymorphisms with reduced HIV replication capacity as a consequence of increased RANTES production [88–90]. Similarly, a genetic variant consisting of a transition (G-A) in the 3'untranslated region of the SDF-1 gene (SDF-1 3'A) has also been associated with a delay in AIDS onset when present as homocygous variant. Its underlying mechanism and effect on X4 viral populations *in vivo* is however not well understood [91–94]. Noteworthy, no mutations in the CXCR4 receptor gene have been proposed as markers of relative HIV control, probably because CXCR4 and SDF-1 are essential at embryonic development stages and, consequently, such mutations might be potentially lethal [95].

A genetic determinant that has recently been associated with rate of HIV disease progression is the copy number of the chemokine gene CCL3L1 (MIP-1α). Individuals with higher CCL3L1 copy numbers than the population race-adjusted average showed lower steady-state viral load; suggesting an increased rate of HIV disease progression in subjects with lower CCL3L1 copy number [96].

Finally, several polymorphisms in the DC-SIGN (dendritic cell specific intracellular adhesion molecule -3-grabbing nonintegrin, i.e. CD209) promoter have been found to be linked to an increase or decrease in susceptibility to HIV infection, particularly also in parenterally acquired HIV infection [97]. As DC-SIGN has also been associated with tuberculosis infection and disease outcome, studies such as a recent one in an Indian population, may help employing DC-SIGN based strategies for the combined fight against these two major human pathogens [98].

8. Immune markers in the relative control of HIV infection

An extensive amount of data indicates a potentially crucial role of HIV-specific cellular and humoral immune responses in the *in vivo* control of viral replication and HIV disease progression. As discussed above, a number of host genetic markers have been identified, particularly specific HLA class I alleles, suggesting an important contribution of the HLA class I restricted CD8+ T-cell immunity in virus control. Despite an increasingly more detailed understanding of the interplay between host immunity, viral evolution and the impact on viral control, clearly defined immune correlates of controlled HIV infection remain elusive. As a consequence, effective vaccine design is still hampered by the availability of well-defined immune parameters that actively mediate *in vivo* viral control. While much of the current investigations focus on the detailed characterization of individuals with exceptional ability to control their virus, it is important to notice that the immune markers associated with this control can often be biased towards subjects expressing specific host genetics and are thus not necessarily translatable to the general population. Nevertheless, a number of specific characteristics of the host immunity against HIV that have been identified clearly extend beyond these limitations and will provide important guidance to vaccine development and offer new immune-based therapeutic treatment options.

9. HIV disease markers associated with virus-specific cellular and humoral immunity

It is generally believed that the cytotoxic T cell (CTL)

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immune response contributes strongly to the *in vivo* control of viral control. Virus-specific CTL responses have been temporally associated with the initial decline in plasma viremia after acute HIV infection and are thought to determine viral set point in chronic stages of infection [99,100]. This is supported by studies in the SIV macaque model, where transient depletion of the total CD8+ T-cell population in controller animals resulted in 100- to 10,000-fold increases in viremia and where the re-establishment of the $CD8^+$ T-cell populations restored the ability of these animals to control SIV replication [101,102]. Further support for an important role of virus-specific CTL in HIV control stems from older studies conducted when tetramer technology became first available in the late 1990ties [103]. The use of such tetramer complexes allowed for the direct *ex vivo* visualization of epitope-specific CTL populations and analysis of specific responses against defined epitopes, without prior *in vitro* expansion and modulation of epitope-specific T cells [104]. Initial analyses using SL9 (SLYNTVATL, HIV Gag p17) specific tetramers, revealed a significant inverse correlation between SL9-specific CTL frequency and plasma RNA viral loads [105]. These analyses were however only based on a limited number of SL9-responding subjects and did not take into consideration the possibly impaired functionality of tetramer-specific T cells. Not surprisingly, the SL9 association with HIV control were not confirmed in studies that used *in vitro* expanded T cells, possibly due to the differential ability of such cells to expand *in vitro* [106]. Since these earlier studies, novel assays, including the IFN-γ based ELISpot assay and *in vitro* inhibition assays first developed by Yang et al. [107,108] provide additional tools to assess direct *ex vivo* T cell activity and functionality [109,110]. These analyses further support the relevance of HIV specific T cells in HIV control, although in many studies, the precise phenotypic and functional markers of these virus-specific T-cell responses attributed to viral control may reflect the effects rather then the cause of otherwise controlled HIV infection. Dissecting these two possibilities and assigning unambiguously causality to specific immune markers and T cell specificities remains one of the currently biggest challenges in defining functionally relevant immune correlates of HIV control.

Over the years, an number of studies have correlated strong and broad HIV-specific T-cell responses with the delayed progression to AIDS and vaccine success is oftentimes subjected to a quantification of the total breadth and magnitude of induced responses. While a detail characterization of vaccine-induced responses will always need to be conducted, it is also clear from a growing number of reports [111] that total virus specific immunity is not necessarily the best measure of *in vivo* immune control of HIV and that more detailed analyses of these responses are needed. Indeed, reanalyses of earlier total-virus specific CTL data suggest that T-cell responses preferentially targeting Gag or other highly conserved epitopes are most relevant specificities for the enhanced antiviral efficacy of T-cells seen in those individuals [109,112]. On the other hand, CTL responses against Env or accessory and regulatory proteins have been shown to have the opposite effect, and are directly correlated with elevated viral load [50]. These findings are in line with more recent studies in clade B as well as clade C infection and analyses that either assessed total viral immunity in peptide pools rather than individual peptide preparations or that focused on responses restricted by specific individual HLA alleles only [50,52,113–117].

However and despite strong associations between Gag-specific T cell immunity and relative HIV control, the causative relationship between the observed response patterns and viral control is still outstanding. Plausible explanations for how dominant Gag specific cytotoxic T-cells could mediate relative virus control stem from the ability of certain HLA class I molecules such as HLA-B57 to present a broad number of HIV Gag peptides and to induce high-magnitude CD8+ T-cell responses in early infection [52,113]. In addition, rapid re-presentation of epitopes derived from the Gag proteins contained in the incoming, infecting virus particles (possibly within less than 2hr after infection) may provide Gag-specific T cells with a decisive advantage to eliminate infected cells before massive virus production has been initiated [118]. However, not all dominant Gag-protein specific responses may be equally effective and comparative studies in clade C and B infection have identified subdominant Gag responses as well as responses outside of Gag as crucial components in relative virus control [27]. The important role of subdominant responses has also been confirmed in studies in the SIV macaque model and is also supported by the detection of subdominant CTL responses in groups of HEPS [11,101].

Apart from CTL specificity, the *in vivo* antiviral efficacy of HIV-specific CD8+ T-cell immunity has also been tightly linked to the functional competence of these responses. In particular, proliferative capacity with high perforin expression and secretion of multiple cytokines such as IFN-γ, IL-2, TNF-α, MIP-1b and/or

CD107a surface expression after antigen contact characterize the responses seen in LTNP [119,120]. In addition to polyfunctionality, the avidity of virus-specific T-cell responses is also considered a potentially important measure of an effective immune response and has been shown in HCV infection, to be associated with viral clearance and higher levels of cross-variant recognition [121,122]. Thus, the quality of the CD8+ T-cell response to HIV serves as a better marker of controlled infection than the quantity (i.e. breadth and magnitude) of these responses. In addition, a HLA-DR+, CD38- activation CD8+ T-cell phenotype was more frequently found among virus-specific T-cells in HIV controllers than in non-controllers and may represent a T cell population with superior ability to expand upon exposure to antigen and capacity to exert effector functions [110]. Whether full-differentiation into CCR7-/CD45- effector cells and broad functional CTL is only a hallmark of controlled HIV infection in the peripheral PBMC compartment or also extends to CTL in gut-associated lymphoid tissue -where massive initial depletion of CD4+ T-cells occurs- remains an open question. Nevertheless, a rapidly growing set of reports dealing with the emergence and accumulation of CTL escape mutations under appropriate CTL pressure, the transmission of "escaped" variants, implications of fitness costs incurred by CTL escape mutations and the global adaptation of HIV to HLA class I polymorphisms further document the crucial role that HIV-specific CTL overall play in the control of HIV infection [28,54,58,123–130].

An additional factor for an effective CD8+ T-cell response includes the presence and function of CD4+ 'helper' T-cells. HIV-specific CD4+ T-cell population have been shown to be required for long-term maintenance of antigen-specific CD8+ memory T-cells, both in the human setting as well as in the monkey model [101,131–133]. The relevance for functional CD4+ T-cell help in the maintenance of effective CTL populations has recently also been reported for viral infections other than HIV, including EBV and CMV infections [134,135]. The potential importance of virus-specific T helper cell activity is further highlighted by studies that have associated the presence of gp41-specific antibodies with CD4+ T-cell responses to Gag-p24 [136,137]. Regardless of the well-documented anti-viral effects of neutralizing Ab responses [138] general antibody-responses have not emerged as strong markers of HIV control. Some of the existing data have been inconsistent as some studies have associated higher titers of heterologous nAb in LTNP whereas more recent studies indicate the presence of lower Nab activity among elite controllers [5,16,139]. It will be interesting to investigate whether possible residual viral replication in the former group of patients may drive additional Ab production or whether additional, unaccounted factor and assay differences are responsible for the observed differences.

10. Innate immunity

During primary HIV infection, there is a massive destruction of the CD4+ T-cell population in the gut-associated lymphoid tissue (GALT) impairing local cellular immunity at mucosal sites and causing translocation of microbial products which in turn contributes to a deleterious persistent inflammation [140,141,143]. The potential damaging effects of chronic inflammation by continuous bacterial translocation are also highlighted by similar studies in HCV infection, where it has been implicated in the progression to advanced stages of cirrhosis [144]. Most importantly however, the massive depletion of CD4+ T-cells, general inflammation and immune activation occur at times when the adaptive immune system has not mounted an effective immune response yet. As mentioned above, some markers associated with the innate immune system, particularly KIR and Toll-like receptors (TLR) have been associated with variable levels of HIV control in these early stages of infection. As such, recent host genetic studies indicate that individuals co-expressing KIR3DS1 and HLA-Bw4-80I (family of HLA alleles that presumably bind to KIR3DS1 and activate NK cells) have lower viral loads and show a reduced risk of progression to AIDS [62,145]. In addition, polymorphisms in toll-like receptor 9, which mediates innate immune response against DNA motifs common in bacteria and viruses, have recently been shown to impact clinical outcomes as well [146]. However, significant functional data supporting the innate immunity and its linkage to disease pathogenesis is still scarce and needs to be further explored. In addition, extensive cohorts of individuals captured in earliest period of acute HIV infection will need to be comprehensively studied to assess the impact of these markers on initial peak viremia and the level of CD4+ T-cell depletion. However, the recent identification of immune memory mediated by NK cell populations may offer novel approaches for preventative and therapeutic interventions in HIV infection [147].

11. Conclusions and implications for vaccine design

Control of viral replication in HIV infection is a mul-

tifactorial process. Polyfunctional CD8+ T-cell immunity against particular viral proteins along with virus-reactive CD4+ T-cell help have been most consistently implicated in modulating HIV infection *in vivo*. Viral factors such as specific mutations often emerging as a consequence of immune selection pressure and entire gene segment deletions have also been associated with reduced viral burden and slower progression of HIV disease. In addition, specific host genetic markers, particularly HLA, has been most compellingly linked to relative control of HIV replication. While such host genetic markers may provide great help in understanding the (immune)-pathology of HIV, they will likely not be directly informative for HIV vaccine development. However, they can guide vaccine immunogen design, although care needs to be taken that such immunogen design is not overly guided by observations made in individuals with favourable host genetics. To avoid the resulting vaccine product to be tailored unreasonably strongly towards individuals with beneficial genetics, subjects who do not express these markers yet control HIV on their own will be most informative. In addition, while immune parameters that could mediate sterilizing immunity, i.e. resistance to infection, still need to be defined, the development of vaccines that are able to induce partial *in vivo* control, albeit not prevent infection, would have significant impact on individual health by slowing HIV disease progression and would help to contain the HIV pandemic by reducing transmission rates.

In this regard, the early assessments of vaccine success in phase I and phase II trials will be based on immune read-outs, rather than prevention of infection (which would be the central end-point in a phase IIb/III trial). Thus, the definition of precise immune correlates of controlled HIV infection is crucial since vaccine induced responses will be compared to these parameters. If their definition is flawed or represents epiphenomena of otherwise controlled HIV infection, valuable vaccine candidates may be discarded prematurely. Together with immune parameters of controlled infection, the identification of host genetic markers may in the future facilitate the design of gene therapy approaches that would try to either block expression of unfavourable genes or introduce beneficial components. Although not based on gene-therapy, the case of the CCR5-Δ32 stem-cell transplanted individual referred to above, points towards the potential feasibility of such approaches.

Acknowledgments

Financial support: Supported in part by the Red de Investigación de Sida (RIS) (RD06/04). Instituto de Salud Carlos III, Madrid, Spain and HIVACAT.

Potential conflict of interest: None.

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JOURNAL OF TRANSLATIONAL MEDICINE

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Definition of the viral targets of protective HIV-1-specific T cell responses

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Abstract

Background: The efficacy of the CTL component of a future HIV-1 vaccine will depend on the induction of responses with the most potent antiviral activity and broad HLA class I restriction. However, current HIV vaccine designs are largely based on viral sequence alignments only, not incorporating experimental data on T cell function and specificity.

Methods: Here, 950 untreated HIV-1 clade B or -C infected individuals were tested for responses to sets of 410 overlapping peptides (OLP) spanning the entire HIV-1 proteome. For each OLP, a "protective ratio" (PR) was calculated as the ratio of median viral loads (VL) between OLP non-responders and responders.

Results: For both clades, there was a negative relationship between the PR and the entropy of the OLP sequence. There was also a significant additive effect of multiple responses to beneficial OLP. Responses to beneficial OLP were of significantly higher functional avidity than responses to non-beneficial OLP. They also had superior in-vitro antiviral activities and, importantly, were at least as predictive of individuals' viral loads than their HLA class I genotypes.

Conclusions: The data thus identify immunogen sequence candidates for HIV and provide an approach for T cell immunogen design applicable to other viral infections.

Keywords: HIV specific CTL, clade B, clade C, HLA, vaccine immunogen design, functional avidity, epitope, entropy, immune correlate

Background

HIV-1 infection induces strong and broadly directed HLA class I restricted T cell responses for which specific epitopes and restricting HLA class I alleles have been associated with relative in vivo viral control [1]. The bulk of the anti-viral CTL response appears to be disproportionately HLA-B restricted, but the relative contribution of targeted viral regions and restricting HLA molecules on the effectiveness of these responses remains unclear [2-5]. In addition, the impact of HIV-1 sequence diversity on the effectiveness of virus-specific T cell immunity in vivo is unclear, as functional constraints of escape variants, codon-usage at individual protein positions, T cell receptor (TCR) plasticity and functional avidity and cross-reactivity potential may all contribute to the overall antiviral activity of a specific T cell response [6-13]. Of note, T cell responses to Gag have most consistently been associated with reduced viral loads in both clade B and clade C infected cohorts [14-16]; however, the specific regions in Gag responsible for this effective control remain poorly defined. In addition, it is unclear whether the relative benefit of Gag is due to any other specific characteristic of this protein, such as rapid antigen-representation upon infection,

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protein expression levels, amino acid composition and/or inherently greater processability and immunogenicity, particularly in the context of selected HLA class I alleles [17,18]. Thus, concerns remain that a purely Gag-based vaccine might mainly benefit those people with a particular HLA genotype and will not take advantage of potentially beneficial targets outside of Gag [4,16,17,19]. In addition, CTL escape and viral fitness studies have focused largely on Gag-derived epitopes presented in the context of protective HLA class I alleles such as HLA-B27 and -B57 [7,20,21], yielding results that may not be generalizable to the genetically diverse majority of the human population. Furthermore, many studies have focused on immunodominant targets only, despite some studies in HIV-1 and SIV infection demonstrating a crucial contribution of sub-dominant responses to targets outside of Gag to the effective in-vivo viral control [4,22]. Thus, the current view on what may constitute a protective cellular immune response to HIV-1 is likely biased towards a immunodominant responses and those restricted by frequent HLA class I alleles and HLA alleles associated with superior disease outcome.

To overcome these potential limitations, the design of an effective and broadly applicable HIV-1 vaccine should to be based on information gained through comprehensive analyses that extend across large portions of the population's HLA class I heterogeneity. Here we focus on three cohorts totaling more than 950 untreated, chronically HIV-1 infected individuals with clade B and C infections, from which responses to certain regions of the viral genome and specific T cell response patterns emerge as correlates of viral control. Importantly, the analyses identify functional properties unique to these responses and control for the impact of HLA class I alleles known to be associated with superior control of HIV-1 infection, thus providing vaccine immunogen sequence candidates with potential usefulness in a broadly applicable HIV-1 vaccine.

Methods

Cohorts

A HIV clade B infected cohort of 223 chronically infected, treatment naïve individuals was recruited and tested at IMPACTA in Lima, Peru. The majority (78%) of enrollees were male and all recruited individuals considered themselves to be of a mixed Amerindian ethnicity [14]. The cohort had a median viral load 37,237 copies/ml (range < 50- > 750,000) and a median CD4 count of 385 cell/ul (range170-1151). A second clade B infected cohort was established at the HIV-1 outpatient clinic "Lluita contra la SIDA" at Hospital Germans Trias i Pujol in Badalona (Barcelona, Spain) consisting of 48 treatment-naive subjects with viral loads below 10,000 and CD4 cell counts > 350 cells/mm^3 ("controllers", n = 24) or above 50,000 copies/ml and CD4 cell counts < 350 cells/mm^3 ("non-controllers", n = 24). The HIV-1 clade C infected cohort has been described in the past and consisted of 631 treatment naive South African with a median viral load of 37,900 copies/ml (range < 50-> 750,000) and a median CD4 count of 393 cells/ul (range 1-1378) [16]. An additional 78 from a recently published cohort in Boston were included in the analyses of functional avidities [23-29]. HLA typing was performed as previously described using SSP-PCR [30]. For Hepitope and FASS analyses, 4digit typing was used for the Lima cohort and 2-digit typing for the Durban cohort. Protocols were approved in Lima by the IMPACTA Human Research Committee, in Durban by the Ethical Committee of the Nelson R. Mandela School of Medicine at the University of KwaZulu-Natal and in Barcelona by the Human Research Committee at Hospital Germans Trias i Pujol. All subjects provided written informed consent.

Peptide test set and ELISpot assay: Previously described peptide sets matching HLA-clade B and C consensus sequences were used in all experiments for which the OLP-specific entropies have been calculated in the past, based on available sequence datasets [31-33] and http://www.hiv.lanl.gov/content/immunology/hlatem/index.html. The peptides were clade-specific sets of adapted 18mers, overlapping by 11 residues designed using the PeptGen tool available at the Los Alamos HIV database http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html. The individual OLP in the peptide sets for clade B and clade C had all the same starting and ending position relative to the source protein and follow the same numbering across the entire viral proteome for both clades. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation and used directly to test for $CD8^+$ T cell responses in vitro. IFN-γ ELISpot assays were performed as described previously, using Mabtech antibodies (Mabtech, Stockholm, Sweden) and a matrix format that allowed simultaneous testing of all 410 overlapping (OLP) peptides in the respective test set [14]. Thresholds for positive responses were defined as: exceeding 5 spots (50 SFC/10^6) per well and exceeding the mean of negative wells plus 3 standard deviation or three times the mean of negative wells, whichever was higher. Stimulation with PHA was used as a positive control in all ELISpot assays.

Definition of functional avidity

Responses targeting 18 mer OLP in HIV-1 Gag p24 were assessed for their functional avidity using OLP-specific sets of 10 mer peptides overlapping by 9 residues that span the 18 mer peptide sequence. Functional avidity was defined as the peptide concentration needed to elicit half maximal response rates in the ELISpot assay

and was calculated as a sigmoidal dose response curve fit using GraphPad Prism software [13].

In vitro viral replication inhibition assay

A double mutant virus containing a Nef M20A and Integrase G140S/Q148H Raltegravir (integrase inhibitor) resistance mutations was tested for replication in CD4 T cells in the presence or absence of autologous T cell lines targeting protective or non-protective OLP. Use of the Raltegravir-resistant virus allows to prevent potential replication of autologous virus in the inhibition assays [28], excludes potential negative impacts on antigen processing or CTL functions attributed to protease inhibitors [34] and avoids overlap between the resistance mutations sites (i.e. G140S/Q148H) and location of beneficial and non-beneficial OLP sequences. In brief, the p83-10 plasmid containing mutations for a methionine to alanine substitution at position 20 of the Nef protein and the p83-2 plasmid engineered to contain the G140S and Q148H mutations in the integrase were combined to produce a virus that is replication competent, highly resistant to Raltegravir and does not downregulate HLA class I in infected cells [35,36]. Although not entirely physiological, this approach was chosen to potentially increase the signal in the in vitro inhibition assay, even when responses were restricted by Nef-sensitive HLA class I alleles. Plasmids were co-transfected into MT4 cells and virus was harvested after 7 days [35,37,38]. Autologous CD4 cells were enriched by magnetic beads isolation (Miltenyi) and expanded for 3 days using a bi-specific anti-CD3/8 antibody and IL-2 containing medium (50 IU r-IL2) before infecting them at multiplicities of infection (MOI) between 0.01 and 1. Effector cells were obtained by stimulating PBMC with either beneficial or non-beneficial OLP for 12 days before isolating specific OLP-reactive cells by IFN-γ capture assay according to manufacturers' instructions (Miltenyi, Bergisch Gladbach, Germany). The effector T cells were analyzed by flow cytometry for the specificity to their respective targets after capture assay and quantified to adjust effector-to-target ratios. Since the NL4-3 backbone sequence differed in several positions in beneficial and non-beneficial OLP, the epitope specificity was predicted based on the HLA class I genotype of the tested individual and responses confirmed to efficiently recognize variant sequences in the NL4-3 backbone sequence. Culture supernatant was harvested and replaced by Raltegravir containing medium 0.05 μg/ml after 72 h. Levels of Gagp24 in the culture supernatant were determined by ELISA as described [39].

Statistical Analyses

Statistical analyses were performed using Prism Version 5 and R Statistical Language [40]. Results are presented as median values unless otherwise stated. Tests included ANOVA, non-parametric Mann-Whitney test (two-tailed) and Spearman rank test. The significance of differences in viral load distribution between OLP-responders and OLP-non-responders was assessed by a two-sided Student's T Test with multiple tests addressed using, instead of a Bonferroni correction, a q-value approach to compensate for multiple comparisons [39]. The multivariate analysis was based on a novel multivariate combined regression method known as FASS, a forward selection method combined with all-subsets regression [41-43]. Briefly, the FASS approach works by iteratively performing the following procedure: Let 'V' be the set of all variables and 'M' be the set of variables included in a model. In the first step, those variables that are not already in the model are divided into equal-sized blocks of variables (the last block may have less than 'g' variables). Then, for each block of variables, 'm' is a new estimated and evaluated model using the Bayesian Information Criterion (BIC). The best model 'm' according to its BIC is retained and the procedure starts all over again until in one step or more the model is not improved.

Results

HIV-1-specific T cell responses targeting conserved regions are associated with lower viral loads

In a first analysis, HIV-1-specific T cell responses were assessed in a cohort of 223 HIV-1 clade B infected individuals recruited in Lima, Peru using IFNg ELISpot assays and a previously described set of 410 clade B overlapping peptides (OLP) [14,31]. For each OLP, a protective ratio (PR) was calculated as the ratio of the median viral loads between OLP non-responders and OLP responders, such that OLP with $PR > 1$ were reflective of OLP predominantly targeted by individuals with reduced viral loads. OLP-specific PR were a) compared between OLP spanning the different viral proteins and b) correlated with the viral sequence heterogeneity in the region covered by the OLP. The data showed highest median PR values for OLP spanning the Gag protein sequence, whereas Nef, Env and Tat had the lowest median PR values (Figure 1A, $p < 0.0001$, ANOVA). A protein-subunit-breakdown of PR values showed the p15 subunit of Gag and RT in Pol to score less favorable than the remainder of the respective proteins (Figure 1B, $p = 0.0032$ and $p = 0.0025$, respectively). While these data confirm the association between HIV-1 Gag-specific responses and lower viral loads, it is important to note that all proteins contained OLP with $PR > 1$, suggesting that some beneficial responses can be located outside of Gag; data that has not emerged from any of the previous studies linking Gag responses to relative viral control. At the same

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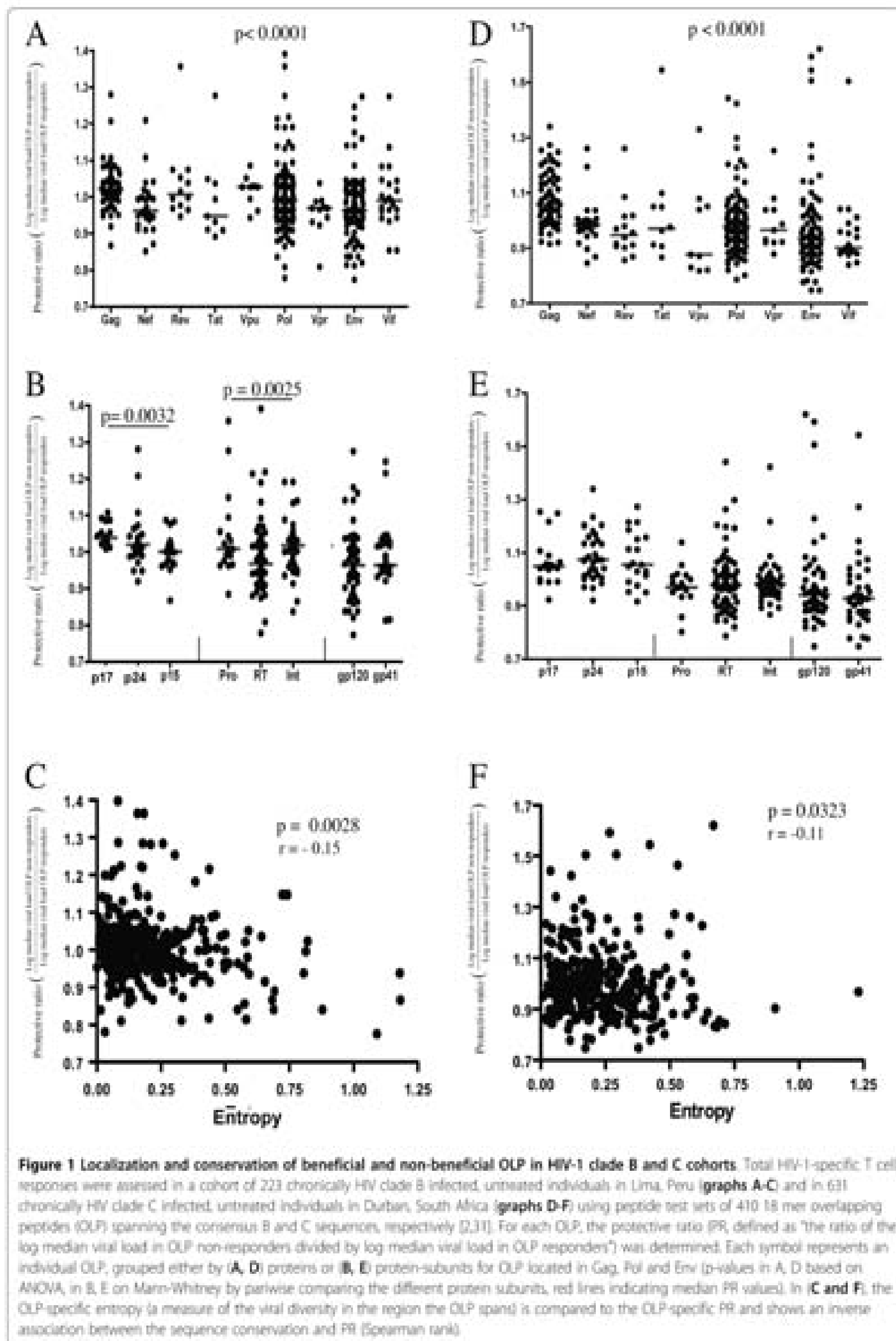

Figure 1 Localization and conservation of beneficial and non-beneficial OLP in HIV-1 clade B and C cohorts. Total HIV-1-specific T cell responses were assessed in a cohort of 223 chronically HIV clade B infected, untreated individuals in Lima, Peru (**graphs A-C**) and in 631 chronically HIV clade C infected, untreated individuals in Durban, South Africa (**graphs D-F**) using peptide test sets of 410 18 mer overlapping peptides (OLP) spanning the consensus B and C sequences, respectively [2,31]. For each OLP, the protective ratio (PR, defined as "the ratio of the log median viral load in OLP non-responders divided by log median viral load in OLP responders") was determined. Each symbol represents an individual OLP, grouped either by (**A, D**) proteins or (**B, E**) protein-subunits for OLP located in Gag, Pol and Env (p-values in A, D based on ANOVA, in B, E on Mann-Whitney by pairwise comparing the different protein subunits, red lines indicating median PR values). In (**C and F**), the OLP-specific entropy (a measure of the viral diversity in the region the OLP spans) is compared to the OLP-specific PR and shows an inverse association between the sequence conservation and PR (Spearman rank).

time, all proteins contained OLP with PR < 1, indicating that proteins considered overall beneficial may contain non-beneficial regions as well. In addition, when the OLP-specific PR was compared to the sequence entropy of the region spanned by the individual OLP, a significant negative correlation between PR and entropy was observed ($p = 0.0028$, $r = -0.15$; Figure 1C). Although rarely targeted OLP may have introduced statistically less robust data points in this comparison and caused a wide scatter of data points, the results show a relative absence of OLP with high entropy and high PR values, suggesting that responses to more variable regions are less effective in mediating in vivo viral control.

To assess whether the above observations would also hold true outside of clade B infection, the same analyses were conducted in a cohort of 631 clade C HIV-1 infected subjects enrolled in Durban, South Africa and tested for responses against a clade C consensus OLP sequence as described previously [33]. As in clade B infection, the OLP specific PR values were highest for OLP spanning Gag without any significant differences between the Gag and Pol protein subunits (Figure 1D and 1E). As in the clade B cohort, the PR values were negatively correlated with the OLP-specific entropy ($p = 0.0323$, Figure 1F), confirming the findings in the clade B cohort and further pointing towards the importance of targeting conserved segments of the viral proteome for effective in vivo viral control.

Identification of individual beneficial OLP sequences in clade B and C infection

In order to identify individual OLP that were significantly more frequently targeted in individuals with relative viral control and to compare the beneficial OLP in clade B and C infection, the viral load distribution in OLP-responders and non-responders was analyzed individually for each OLP. For the clade B cohort in Peru, the analyses yielded 43 OLP sequences for which the median viral load differed between the two groups with an uncorrected p-value of < 0.05. Of these 43 OLP, 26 were OLP with a PR > 1 (referred to as "beneficial" OLP), and 17 OLP with a PR < 1 ("non-beneficial" OLP, Table 1). The distribution of OLP with PR > 1 among viral proteins was biased towards Gag and Pol, while Env produced exclusively OLP with PR < 1 (Figure 2A).

The same analyses were repeated for the clade C cohort in Durban, which due to its larger size allowed to apply more stringent statistical criteria to identify beneficial and non-beneficial OLP. To compensate for multiple statistical comparisons, we employed a previously described false-discovery rate approach [39], resulting in the identification of 33 clade C OLP with q-values of < 0.2 (i.e. OLP with significantly different viral load distributions between OLP-responders and non-responders with a false positive discovery rate (q-value) of 20%). The 33 OLP identified were comprised of 22 beneficial OLP and 11 non-beneficial OLP, with the beneficial OLP being again located in Gag, Pol and Vif, similar to what was seen in the clade B cohort (Figure 2B).

In both cohorts, the total breadth and magnitude of responses did not correlate with viral loads as reported for parts of these cohorts in the past [14,16]. The OLP with significant differences in median viral loads (43 OLP in clade B and 33 OLP in clade C, Tables 1 and 2, respectively, i.e. "scoring OLP"), were more often targeted in their respective cohort than OLP that did not score with a significant difference in viral loads ($p = 0.0015$ Lima; $p < 0.0001$ Durban). However, beneficial and non-beneficial OLP were equally frequently targeted in either cohort. Also, there was no difference in the median magnitude of the OLP-specific responses, regardless whether it was a beneficial, non-beneficial or not-scoring OLP (all $p > 0.7$, data not shown). Finally, there was no correlation between the number of total OLP responses (against all 410 OLP) and the magnitude of responses to beneficial OLP in either cohort, indicating that the strength of beneficial OLP responses was not diminished by other responses to the rest of the viral proteome.

In the clade B cohort, the 26 beneficial and 17 non-beneficial OLP showed a significant difference in their median entropy ($p = 0.0327$, Figure 2C), in line with the overall negative association between higher PR and lower sequence entropy seen in the comprehensive screening including the entire 410 OLP set (Figure 1C). While this comparison was not significant in clade C infection, a detailed look at Gag showed that beneficial Gag clade C OLP had a lower entropy values than the rest of the Gag OLP, suggesting that targeting of the most conserved regions even in Gag provided particular benefits for viral control (Figure 2D, $p = 0.0172$). These beneficial OLP were also more frequently targeted (median of 36 responders) compared to the rest of Gag OLP (median 12 responders, $p = 0.0099$), likely reflecting the high epitope density in these regions [33,44].

Finally, the two cohorts showed a partial overlap in the targeted beneficial and non-beneficial OLP, despite the vastly different HLA genetics in these two populations [4,31,45,46]. As Gag was enriched in beneficial OLP scattered throughout the entire protein sequence, we used the available reverse transcriptase (RT) protein structure to assess whether beneficial responses were targeting structurally related regions of the protein, even though the linear position of beneficial OLP did not precisely match between the two clades. Indeed, superimposing the locations of beneficial OLP in the RT protein indicates that in both clades, beneficial OLP fell in structurally related domains of the RT protein (Figure

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Table 1 Beneficial and non-beneficial OLP identified in Lima clade B cohort ($p < 0.05$)

OLP #	Protein	Sub-unit	OLP clade B consensus sequence	Median viral load in OLP responders	Median viral load in OLP non-responders	Protective Ratio (PR)*	p-value
3	Gag	p17	EKIRLRPGGKKKYKLKHI	22947	39014	**1.053**	0.037
6	Gag	p17	ASRELERFAVNPGLL	15380	43189	**1.107**	0.001
7	Gag	p17	ERFAVNPGLLETSEGCR	25939	38974	**1.040**	0.049
10	Gag	p17	QLQPSLQTGSEELRSLY	16285	37237	**1.085**	0.031
12	Gag	p17	SLYNTVATLYCVHQRIEV	23855	37113	**1.044**	0.037
23	Gag	p24	AFSPEVIPMFSALSEGA	22947	37113	**1.048**	0.036
31	Gag	p24	IAPGQMREPRGSDIA	3563	35483	**1.281**	0.028
34	Gag	p24	STLQEQIGWMTNNPPIPV	6127	37360	**1.207**	0.002
48	Gag	p24	ACQGVGGPGHKARVLAEA	12975	35755	**1.107**	0.041
60	Gag	p15	GKIWPSHKGRPGNFLQSR	16266	36434	**1.083**	0.044
75	Nef	-	WLEAQEEEEVGFPVRPQV	13407	37360	**1.108**	0.026
76	Nef	-	EVGFPVRPQVPLRPMTYK	59618	29855	0.937	0.001
84	Nef	-	NYTPGPGIRYPLTFGWCF	55402	30538	0.945	0.006
85	Nef	-	RYPLTFGWCFKLVPV	69890	29903	0.924	0.002
90	Nef	-	SLHGMDDPEKEVLVWKF	89687	32650	0.911	0.042
159	Pol	Pro	KMIGGIGGFIKVRQYDQI	14736	36434	**1.094**	0.020
160	Pol	Pro	FIKVRQYDQILIEICGHK	3682	35755	**1.277**	0.031
161	Pol	Pro	QILIEICGHKAIGTVLV	9117	35483	**1.149**	0.050
163	Pol	Pro	LVGPTPVNIIGRNLLTQI	25965	45637	**1.055**	0.007
171	Pol	RT	LVEICTEMEKEGKISKI	1865	35483	**1.391**	0.014
181	Pol	RT	LDVGDAYFSVPLDKDFRK	65858	32871	0.937	0.041
195	Pol	RT	LRWGFTTPDKKHQKEPPF	5624	37113	**1.219**	0.006
196	Pol	RT	DKKHQKEPPFLWMGYELH	10103	35483	**1.136**	0.044
210	Pol	RT	EIQKQGQGQWTYQIY	18155	35483	**1.068**	0.045
222	Pol	RT	PPLVKLWYQLEKEPIVGA	412599	34640	0.808	0.030
230	Pol	RT	IHLALQDSGLEVNIV	85102	34117	0.919	0.030
237	Pol	RT	VYLAWVPAHKGIGGNEQV	85102	34117	0.919	0.029
240	Pol	RT	SAGIRKVLFLDGIDKA	116902	32761	0.891	0.019
269	Pol	Int	TKELQKQITKIQNFRVYY	6629	35755	**1.192**	0.030
270	Pol	Int	TKIQNFRVYYRDSRDPLW	18171	37360	**1.073**	0.019
271	Pol	Int	YYRDSRDPLWKGPAKLLW	25939	35755	**1.032**	0.043
276	Pol	Int	KIRDYGKQMAGDDCVA	6629	35755	**1.192**	0.021
279	Vpr	-	GPQREPYNEWTLELLEEL	60222	32650	0.944	0.042
307	Env	Gp120	DLNNNTNTTSSSGEKMEK	179419	34117	0.863	0.044
311	Env	Gp120	IRDKVQKEYALFYKLDVV	179419	32871	0.860	0.008
314	Env	Gp120	YRLISCNTSVITQACPKV	58206	31273	0.943	0.008
315	Env	Gp120	SVITQACPKVSFEPIPIH	61011	32871	0.944	0.034
320	Env	Gp120	TNVSTVQCTHGIRPVV	341587	34640	0.820	0.034
355	Env	Gp120	VAPTKAKRRVVQREKRAV	161602	34117	0.870	0.042
399	Env	Gp41	VIEVVQRACRAILHIPRR	388089	34640	0.812	0.026
405	Vif	-	VKHHMYISGKAKGWFYRH	16458	37237	**1.084**	0.021
406	Vif	-	GKAKGWFYRHHYESTHPR	16458	37237	**1.084**	0.022
424	Vif	-	TKLTEDRWNKPQKTKGHR	10319	36434	**1.137**	0.014

* PR values in bold indicate PR > 1, i.e. OLP-responses seen more frequently in individuals with reduced viral loads

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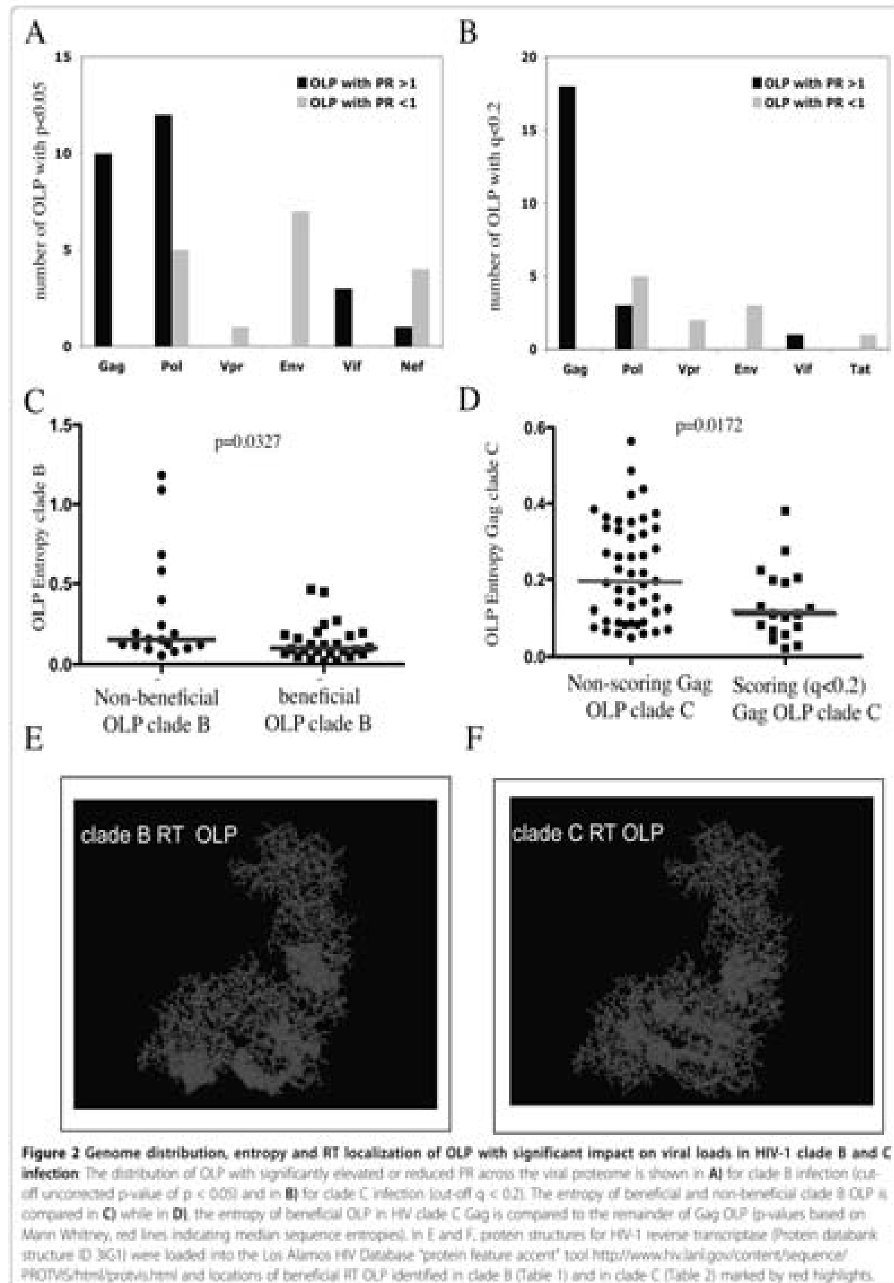

Figure 2 Genome distribution, entropy and RT localization of OLP with significant impact on viral loads in HIV-1 clade B and C infection. The distribution of OLP with significantly elevated or reduced PR across the viral proteome is shown in **A)** for clade B infection (cut-off uncorrected p-value of $p < 0.05$) and in **B)** for clade C infection (cut-off $q < 0.2$). The entropy of beneficial and non-beneficial clade B OLP is compared in **C)** while in **D)**, the entropy of beneficial OLP in HIV clade C Gag is compared to the remainder of Gag OLP (p-values based on Mann Whitney, red lines indicating median sequence entropies). In E and F, protein structures for HIV-1 reverse transcriptase (Protein databank structure ID 3IG1) were loaded into the Los Alamos HIV Database "protein feature accent" tool http://www.hiv.lanl.gov/content/sequence/PROTVIS/html/protvis.html and locations of beneficial RT OLP identified in clade B (Table 1) and in clade C (Table 2) marked by red highlights.

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Table 2 Beneficial and non-beneficial OLP identified in Durban clade C cohort (q < 0.2)

OLP #	Protein	Sub-unit	OLP clade C consensus sequence	Median viral load in OLP responders	Median viral load in OLP non-responders	Protective Ratio (PR)*	p-value	Q-value
3	Gag	p17	EKIRLRPGGKKHYMLKHL	18,700	45,100	**1.09**	0.0002	0.0006
6	Gag	p17	ASRELERFALNPGLL	6,570	44,100	**1.22**	0.0000	0.0000
7	Gag	p17	ERFALNPGLLETSEGCK	5,270	43,900	**1.25**	0.0000	0.0000
22	Gag	p24	WVKVIEEKAFSPEVIPMF	8,360	42,850	**1.18**	0.0000	0.0000
25	Gag	p24	GATPQDLNTMLNTVGGH	24,450	45,200	**1.06**	0.0021	0.0263
26	Gag	p24	NTMLNTVGGHQAAMQMLK	5,310	39,600	**1.23**	0.0061	0.0766
27	Gag	p24	GGHQAAMQMLKDTINEEA	9,715	42,100	**1.16**	0.0015	0.0170
29	Gag	p24	AAEWDRLHPVHAGPIA	19,700	40,900	**1.07**	0.0045	0.0544
31	Gag	p24	IAPGQMREPRGSDIA	6,480	38,950	**1.20**	0.0146	0.1478
33	Gag	p24	SDIAGTTSTLQEQIAWM	11,650	40,900	**1.13**	0.0025	0.0318
37	Gag	p24	WIILGLNKIVRMYSPVSI	9,360	44,100	**1.17**	0.0004	0.0018
39	Gag	p24	SILDIKQGPKEPFRDYV	2,630	38,250	**1.34**	0.0182	0.1838
41	Gag	p24	YVDRFFKTLRAEQATQDV	22,150	44,100	**1.07**	0.0020	0.0263
42	Gag	p24	LRAEQATQDVKNWMTDTL	16,480	40,900	**1.09**	0.0078	0.0935
55	Gag	p15	HIARNCRAPRKKGCWK	7,550	39,700	**1.19**	0.0092	0.1047
59	Gag	p15	RQANFLGKIWPSHKGR	9,840	42,200	**1.16**	0.0046	0.0539
60	Gag	p15	GKIWPSHKGRPGNFLQSR	6,130	39,700	**1.21**	0.0066	0.0799
63	Gag	p15	TAPPAESFRFEETTPAPK	6,040	38,950	**1.21**	0.0093	0.1020
116	Tat	Tat	TKGLGISYGRKKRRQRRS	109,000	36,700	0.91	0.0033	0.0410
178	Pol	RT	FWEVQLGIPHPAGLKKKK	258,000	37,300	0.84	0.0033	0.0384
181	Pol	RT	LDVGDAYFSVPLDEDFRK	7,100	38,950	**1.19**	0.0186	0.1832
190	Pol	RT	RAQNPEIVIYQYMDDLYV	84,900	34,700	0.92	0.0043	0.0555
199	Pol	RT	TVQPIQLPEKDSWTVNDI	6,700	38,300	**1.20**	0.0198	0.1926
216	Pol	RT	QKIAMESIVIWGKTPKFR	18,150	43,000	**1.09**	0.0026	0.0317
239	Pol	RT	QVDKLVSSGIRKVLFL	373,200	37,700	0.82	0.0205	0.1937
253	Pol	Int	PAETGQETAYFILKLAGR	92,800	35,400	0.92	0.0082	0.0954
265	Pol	Int	AVFIHNFKRKGGIGGYSA	63,650	33,800	0.94	0.0178	0.1826
283	Vpr	-	GLGQYIYETYGDTWTGV	78,000	35,600	0.93	0.0126	0.1302
284	Vpr	-	ETYGDTWTGVEALIRIL	85,050	35,200	0.92	0.0099	0.1034
312	Env	Gp120	YALFYRLDIVPLNENNSSEY	270,000	37,700	0.84	0.0208	0.1915
365	Env	Gp41	GIKQLQTRVLAIERYLK	151,000	34,700	0.88	0.0001	0.0002
393	Env	Gp41	LLGRSSLRGLQRGWEALKYL	750,000	37,450	0.78	0.0007	0.0041
417	Vif	-	CFADSAIRKAILGHIV	1,110	38,200	**1.50**	0.0178	0.1891

* PR values in bold indicate PR > 1, i.e. OLP-responses seen more frequently in individuals with reduced viral loads

2E and 2F). This suggests that despite differences in response patterns between ethnicities and clades, viruses from both clades may be vulnerable to responses targeting the same structural regions of at least some of their viral proteins.

Increased breadth of responses against beneficial OLP is associated with decreasing viral loads, independent of Gag-specificity or the presence of protective HLA class I alleles

To assess whether individuals targeting more than one beneficial OLP profit from a greater breadth of responses to these targets, subjects in both cohorts were stratified by the number of responses to beneficial OLP and their viral loads compared. In both cohorts, negative correlations between the number of responses to beneficial OLP and viral loads were observed ($p < 0.0001$, $r = -0.33$ for Lima; $p < 0.0001$, $r = 0.-25$ for Durban; data not shown), suggesting that there is a cumulative benefit of responses to these particularly effective targets. Similarly, when individuals in the clade C cohort were grouped based on mounting 1-2, 3-4 or five and more beneficial OLP responses, a gradual reduction in median viral loads was seen. This reduction was close to 20-fold when 5 or more of the 22 beneficial OLP were targeted (median viral load 5,210 copies/ml) compared to individuals without a response (98,800 copies/ml, Figure 3A). Importantly, this observation was not driven only by individuals expressing HLA class I alleles associated with relative control of viral replication (including HLA-

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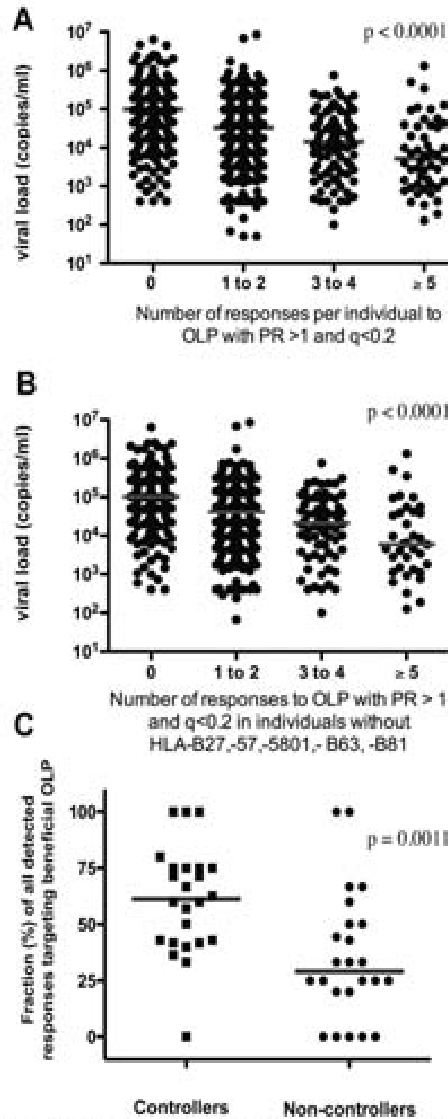

Figure 3 Increased breadth of responses to beneficial OLP results in gradually reduced viral loads and is independent of cohort and HLA-B27, -57, -B58, -B81 and -B63. (**A**) The number of responses to beneficial OLP in the clade C cohort in Durban was determined for each individual and compared to viral loads. An increased breadth of responses to the 22 beneficial OLP was associated with reduced viral loads (ANOVA, $p < 0.0001$). (**B**) This association remained equally stable after removing all individuals expressing known beneficial HLA allele (HLA-B27, -B57, -B5801, -B63, -B81) from the analysis (ANOVA, $p < 0.0001$). (**C**) The set of 26 beneficial and 17 non-beneficial OLP identified in the clade B infected cohort in Lima, Peru was tested in a second clade B infected cohort in Barcelona. HIV controllers showed a significantly higher focus of responses on the 22 beneficial OLP (61% of all responses to the 43 OLP) while non-controllers reacted predominantly with the non-beneficial OLP (only 29% of all responses targeting beneficial OLP). The Barcelona cohort did not included subject expressing any HLA allele previously associated with relative control of HIV-1 ($p = 0.0011$, Mann Whitney).

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B27, -B57, -B*5801, -B63 and -B81) as their exclusion still showed a strong association between increased breadth of responses to beneficial OLP and a gradual suppression of viremia (Figure 3B). This was further supported when translating the clade B data from Peru to a second clade B infected cohort in Barcelona, Spain where HIV-1 controllers also mounted a significantly greater proportion of their responses to the beneficial Peruvian OLP compared to the HIV-1 non-controllers (61% vs. 29%, p = 0.0011; Figure 3C); this despite the fact that the Barcelona cohort was genetically different and excluded individuals expressing HLA-B27, -B57, -B58 and B63. Thus, despite the frequent targeting of Gag and the inclusion of individuals expressing HLA alleles such as HLA-B*5701 and -B*5801 in the two larger clade B and C cohorts, the present data identify regions of the viral genome that serve as the targets of an effective host T cell response, largely independent of the presence of HLA alleles known to influence HIV-1 viral replication.

PR-values are mediated by individuals with broad HLA heterogeneity

To further assess the contribution of specific HLA class I alleles on the PR of individual OLP, the statistically significant OLP in the clade C cohort were further analyzed. In a first step, median viral loads in the OLP-responder and non-responder groups were compared after excluding individuals with specific HLA class I alleles. If the statistical significance of the comparison was lost, the excluded HLA class I allele was assumed to have significantly contributed to the initially observed elevated or reduced PR value and to restrict a potential CTL epitope in that OLP. In a second step, a "Hepitope" analyses http://www.hiv.lanl.gov/content/immunology/hepitopes was conducted to identify HLA class I alleles overrepresented in the OLP responder group; providing an alternative approach to identify specific epitopes that may contribute to relative viral control. Together, the two strategies permit to estimate the HLA diversity in the OLP responders and to identify the most likely alleles that restrict the epitope-specific responses to the OLP. Both are important measures when determining the relative usefulness of a selected beneficial OLP in a potential immunogen sequence as it should provide broad HLA coverage. The data from these analyses are summarized for beneficial and non-beneficial OLP in Table 3 and 3, respectively. The results demonstrate that with a few exceptions, for each OLP, several HLA alleles appeared to be mediating the observed effects as their removal caused the statistical significance to be lost. However, for the most frequent HLA class I alleles, the loss of significance may be due to a reduction in sample size rather than the actual allele, since the exclusion of many allele carriers could reduce the number of OLP responders (and non-responders) sufficiently to lose statistical power. The "Hepitope" analysis controlled for this effect and confirmed the obtained results, strongly indicating that responses to beneficial OLP were mediated by responder populations with heterogeneous HLA allele distributions.

Effects of T cell specificity on in vivo viral load are at least as strong as those associated with host HLA genetics

To assess whether specific response patterns and/or HLA combinations could be identified that mediated synergistic or superior control of viral infection in clades B and C, multivariate combined regression analysis was conducted on either OLP only, HLA only or the combination of OLP and HLA variables [41-43]. The OLP-only analysis for Lima identified 7 OLP of which 4 were associated with lower median viral loads and 3 with increases in viral loads, respectively (Table 4). Targeting at least one of these beneficial clade B OLP was associated with significantly reduced viral loads (median 11, 079 copies/ml) compared to the subjects who did not target any of these four OLP (median 52, 178 copies/ml; p < 0.0001, Figure 4A). As seen in the univariate analysis (Figure 2C), the four beneficial OLP emerging from the Lima FASS analysis were more conserved than the rest of the OLP (median entropy 0.0759 vs. 0.1649, p = 0.0267) or the three non-beneficial OLP (0.0759 vs. 0.1228, p = 0.0571, data not shown). In contrast to OLP-only FASS analysis, only one HLA allele (HLA-C04) emerged from the HLA-only multivariate analysis. The analysis for the combined variables (OLP and HLA) controlled for the potential bias in this result due to more OLP variables (n = 389) than HLA (n = 146) being included in the statistical tests; yet still identified more OLP variables (n = 9) than HLA class I alleles (n = 3). In addition, the relative co-efficients of these associations were stronger for the OLP than the HLA variables, suggesting that T cell specificity influenced viral loads to at least the same degree as host HLA class I genetics. Of note, the identified OLP and HLA variables did not reflect responses to known optimal CTL epitopes, as none of the OLP contained described epitope (s) restricted by any of the identified HLA alleles [44].

Results from the clade C cohort in Durban confirmed the clade B findings in Lima as the FASS analyses identified 16 OLP but only 8 HLA variables that had an impact on the individual viral loads. As in Lima, the impact of OLP specificity was at least as strong than HLA genotype (trend for higher coefficients for OLP than HLA; data not shown, p > 0.05). In addition, targeting at least one of the eight beneficial OLP in Durban was associated with strongly reduced viral loads (p <

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Table 3 Impact of HLA alleles on the statistical significance of observed PR values (clade C OLP)

OLP	Protein	PR	Removed HLA allele(s) abolishing statistical significance[1]	Alleles over-represented in the OLP responder group[2]
A) Beneficial OLP (PR > 1)				
3	Gag	1.09	A30, B42, C17	A30, B08, A03, A74, C17, A43, B42, B07
6	Gag	1.22	B15	B49, B82, C14
7	Gag	1.25	-	B42, C17, B49, A30
22	Gag	1.18	B57, C07	B57, A74, B45, C07, C16, B13
25	Gag	1.06	A30, B15, C04, C07	B42, C17, B81, B39, A01, C12, C18, A30, B67
26	Gag	1.23	A02, A23, A68, B07, B14, B58, C07, C08	C03, B15, A68
27	Gag	1.16	B15, C07	B15, A68, C03, C08
29	Gag	1.07	A68, B15, B58, C02, C03, C06, C12	B35, B39, C12, B40, B07, C04
31	Gag	1.2	A02, A11, A23, A29, A32, A34, A68, B07, B13, B15, B42, B44, B58, C04, C06, C07, C17	B13, A29, C06, A11
33	Gag	1.13	A02, A23, B44, B57, B58, C07	B58, B57, A02, C07, C03, A68
37	Gag	1.17	A30, B42, B58, C17	C18, B42, C17, A01, B81
39	Gag	1.34	A02, A03, A23, A29, A30, A68, A74, B08, B15, B18, B42, B45, B53, B57, B58, C02, C03, C06, C07, C08, C16, C17	A02
41	Gag	1.07	A23, C06	C03, B14, A68, C08, B15
42	Gag	1.09	A23, A30, B08, B15, B42, B53, B58, C03, C04, C07	B53, C03
55	Gag	1.19	A02, A24, A29, A30, B07, B15, B39, B42, B44, B58, C02, C06, C07, C17	B42, B08, C17
59	Gag	1.16	A02, A30, B08, B42, B44, B58, C04, C07, C17	A02, B13, A29
60	Gag	1.21	A02, A30, B42, B58, C06, C07, C17	A02, B41, C07, C17
63	Gag	1.21	A02, A23, A29, A30, A68, B08, B15, B44, B58, C02, C03, C06, C07	A23
181	Pol	1.19	A01, A23, A29, A30, A34, A68, A74, B14, B15, B18, B35, B44, B45, B57, B58, C02, C03, C04, C06, C07, C08, C16	B57, C18
199	Pol	1.2	A02, A03, A23, A24, A26, A30, A31, A34, A36, A66, A68, A80, B08, B13, B15, B18, B35, B40, B41, B42, B44, B45, B49, B50, B51, B53, B57, B58, B81, C01, C02, C03, C04, C05, C06, C07, C08, C15, C16, C17	B53, A23, C04
216	Pol	1.09	A02, A30, B58, C07, C17	B53, B58, C07, B57
417	Vif	1.5	A03, A23, A30, A34, A36, A68, B08, B14, B15, B44, B53, B58, C03, C04, C06, C08	B14, C08, A36
B) Non-beneficial OLP (PR < 1)				
116	Tat	0.91	A02, A34, B15, C04	B15, C02
178	Pol	0.84	A03, A68, B15, B58, C04, C06, C07	A68, C06, B58, B82, A03
190	Pol	0.92	A03, A30, A66, B18, B42, B45, B58, C06, C07	A02, B18, B35, C05, C16, B45, A80, C12, B67, B39
239	Pol	0.82	-	C05, A03
253	Pol	0.92	A03, A68, B15, B39, B42, B44, B58, C02, C04, C06, C08, C17, C18	A68, C03, B15, B07, C15, B41
265	Pol	0.94	A02, A03, A23, A24, A26, A29, A30, A31, A33, A34, A66, A68, B07, B08, B14, B15, B27, B40, B41, B42, B44, B53, B54, B55, B57, B81, C01, C02, C04, C06, C07, C12, C17, C18	B15, C02, A43, A74
283	Vpr	0.93	A02, A03, A23, A30, A66, A68, A74, B07, B14, B18, B39, B41, B42, B45, B57, C02, C04, C07, C08, C15, C17	A68, C03, B07, C17, B41
284	Vpr	0.92	A03, A23, A30, A66, A68, A74, B07, B14, B18, B39, B42, B45, B57, C07, C08, C15, C17	A68, C03

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Table 3 Impact of HLA alleles on the statistical significance of observed PR values (clade C OLP) *(Continued)*

312	Env	0.84	-	B08, C07
365	Env	0.88	B58, C06	C06, B58, A43, B45, C16, A66
393	Env	0.78	A30, B58, C06	A31, C06, B45

1) in italics HLA alleles that do not emerge from the Hepitope analysis 2) cut-off in Hepitope analyses for $p < 0.05$, alleles sorted according to strength of association

0.0001, Figure 4B). This effect was, as in the univariate analysis, additive for more than one response ($p < 0.0001$, Figure 4C) and included OLP that were, aside from Gag, located in Pol and Vif. Also, the combined (OLP and HLA) analysis suggests the effect of OLP specificity on viral loads to be at least as strong as HLA genetics as 8 OLP and 7 HLA variables were identified. This especially since among the 7 HLA alleles, two (HLA-B57 and HLA-A74) are expressed in linkage disequilibrium [47], further reducing the number of HLA variables with a significant impact on viral loads.

Responses to beneficial OLP are of higher functional avidity and suppress viral replication in vitro more effective than responses to non-beneficial OLP

Functional avidity and the ability to suppress in vitro viral replication have emerged as two potentially crucial parameters of an effective CTL response against HIV-1 [23-29]. To assess this potential functional characteristic of beneficial CTL populations, we determined the functional avidity of responses to the four beneficial OLP located in Gag p24, a region that has been most consistently associated with eliciting relatively protective CTL responses. As 18 mer peptides are suboptimal test peptides to determine functional avidity, 10 mer overlapping peptide sets were synthesized to cover the four beneficial OLP and all detected responses were titrated. The SD50% was determined for a comparable numbers of responses detected in controllers (n = 21 responses) and non-controllers (n = 24 responses) and showed a statistically significant difference between the two groups (median 3, 448 ng/ml vs. 25, 924 ng/ml, $p = 0.0051$, Figure 5A). This reduced avidity in HIV non-controllers to beneficial OLP could possibly explain why HIV-1 non-controllers did not control their in vivo viral replication despite targeting these regions in some instances and with responses of comparable magnitude as HIV controllers (278 SFC vs 305 SFC/10^6 PBMC, $p = 0.55$, data not shown).

To more directly assess whether responses to beneficial OLP were of particularly high functional avidity, regardless of HIV controller status, we determined SD50% of responses to 17 optimal epitopes from beneficial, neutral and non-beneficial OLP (Figure 5B). Median epitope-specific SD50% were determined from an average of 7 titrations per epitope and compared to the OLP specific PR. A strongly significant, negative association between the PR and the SD50% was noted ($p = 0.002$, $r = -0.69$), indicating that beneficial OLP are targeted by high-avidity responses. To control for inter-individual differences due to disease status and viral load, we identified 10 individuals who targeted optimal epitopes in beneficial and non-beneficial OLP and determined their functional avidity. As in the cross-sectional analysis before, this matched comparisons showed in all cases a higher functional avidity for the epitopes located in the beneficial OLP compared to the responses targeting non-beneficial OLP (Figure 5C, $p = 0.0020$). Lastly, to relate the higher functional avidity to potential superior anti-viral effects in vivo, the ability to inhibit in vitro viral replication was assessed in three individuals who mounted robust responses against both beneficial and non-beneficial OLP. The in vitro inhibition assay first developed by Yang et al [48], was modified so that the NL4-3 based test virus contained a single nucleotide mutation in Nef (M20A) that blocks the Nef-mediated down-regulation of HLA class I molecules as well as two mutations in the integrase gene that mediate Raltegravir-resistance to permit the suppression of potentially replicating autologous virus in the assay. Indeed, CTL specific for the beneficial OLP(s) were up to 2 logs more effective inhibiting viral replication than CTL targeting non-beneficial OLP (Figure 5D), in line with recent data demonstrating different suppressive ability of HIV-1 specific CTL populations targeting Gag and Env-derived epitopes [24]. Although the in vitro inhibition assays were limited to few individuals with suitable response patterns, these data together with the results from the extensive titration assays in Figure 5B and 5C indicate that responses to beneficial OLP are of particularly high functional avidity and inhibit in vitro viral replication more effectively than responses to non-beneficial OLP. Of note, higher avidity responses to beneficial OLP compared to non-beneficial OLP were seen in all 10 tested individuals, ruling out that inter-individual variability in viral loads, duration of infection and HIV disease status could have biased the analyses.

Conclusions

Defining functional correlates of HIV-1 immune control is critical to the design of effective immunogens. T cell responses to specific HIV-1 proteins and protein-

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Table 4 Multivariate analysis of OLP and HLA variables for clade B and C cohorts

	OLP variables only (Lima, clade B) change viral load (co-efficient) *	p-value		OLP variables only (Durban, clade C) change viral load (co-efficient) *	p-value
Beneficial			Beneficial		
OLP.6	-0.4591	0.0008	OLP.7	-0.6256	0
OLP.31	-1.4055	0.0002	OLP.21	-0.6663	0
OLP.171	-2.5981	0	OLP.22	-0.4926	0.0006
OLP.276	-1.127	0.0007	OLP.25	-0.2822	0.0002
			OLP.27	-0.4719	0.0053
Non-beneficial			OLP.33	-0.3396	0.0024
OLP.76	0.2486	0.0067	OLP.398	-1.8179	0.0027
OLP.306	3.2968	0.0001	OLP.417	-1.6535	0.0008
OLP.411	1.3329	0.012			
			Non-beneficial		
			OLP.38	0.9045	0.0022
			OLP.84	0.1947	0.0091
			OLP.116	0.6156	0.0039
			OLP.183	0.661	0.0013
			OLP.224	0.2508	0.0036
			OLP.265	0.5782	0
			OLP.365	0.4911	0.0009
			OLP.393	1.2624	0.0013
HLA variables only (Lima, clade B)			**HLA variables only (Durban, clade C)**		
Non-beneficial			Beneficial		
HLA-C0401	0.35652	0.00024	HLA-A74	-0.3553	0.0025
			HLA-B13	-0.6443	0.0004
			HLA-B57	-0.5195	0.0007
			HLA-B81	-0.3619	0.0015
			HLA-C12	-0.6544	0.0001
			Non-beneficial		
			HLA.B.15	0.2506	0.0012
			HLA.B.18	0.3521	0.0005
			HLA.C.6	0.3958	0
HLA and OLP variables together (Lima)			**HLA and OLP variables together (Durban)**		
Beneficial			Beneficial		
OLP.6	-0.5792	0	OLP.6	-0.4798	0.0023
OLP.31	-1.1607	0.0005	OLP.7	-0.4528	0.0015
OLP.171	-2.7948	0	OLP.27	-0.4676	0.0049
OLP.276	-0.9609	0.0011	OLP.59	-0.4196	0.0115
			OLP.417	-1.387	0.0041
Non-beneficial			Non-beneficial		
OLP.2	0.3945	0.018	OLP.148	2.5215	0.0029
OLP.237	0.7211	0.0035	OLP.183	0.6108	0.0023
OLP.288	1.5537	0.0016	OLP.393	1.1442	0.0023
OLP.311	0.7197	0.0091			
OLP.411	1.5306	0.0024	Beneficial		
			HLA-A74	-0.3744	0.0007
Beneficial			HLA-B57	-0.4887	0.0007
HLA-B1502	-1.4688	0.0164	HLA-B81	-0.3859	0.0004
			HLA-C12	-0.6003	0.0002
Non-beneficial			Non-beneficial		
HLA-B0801	0.66	0.0049	HLA-B15	0.2797	0.0001
HLA-C0401	0.2894	0.0006	HLA-B18	0.5316	0.0003
			HLA-B49	0.9713	0.0007

* negative co-efficient values indicate reduction in median viral loads

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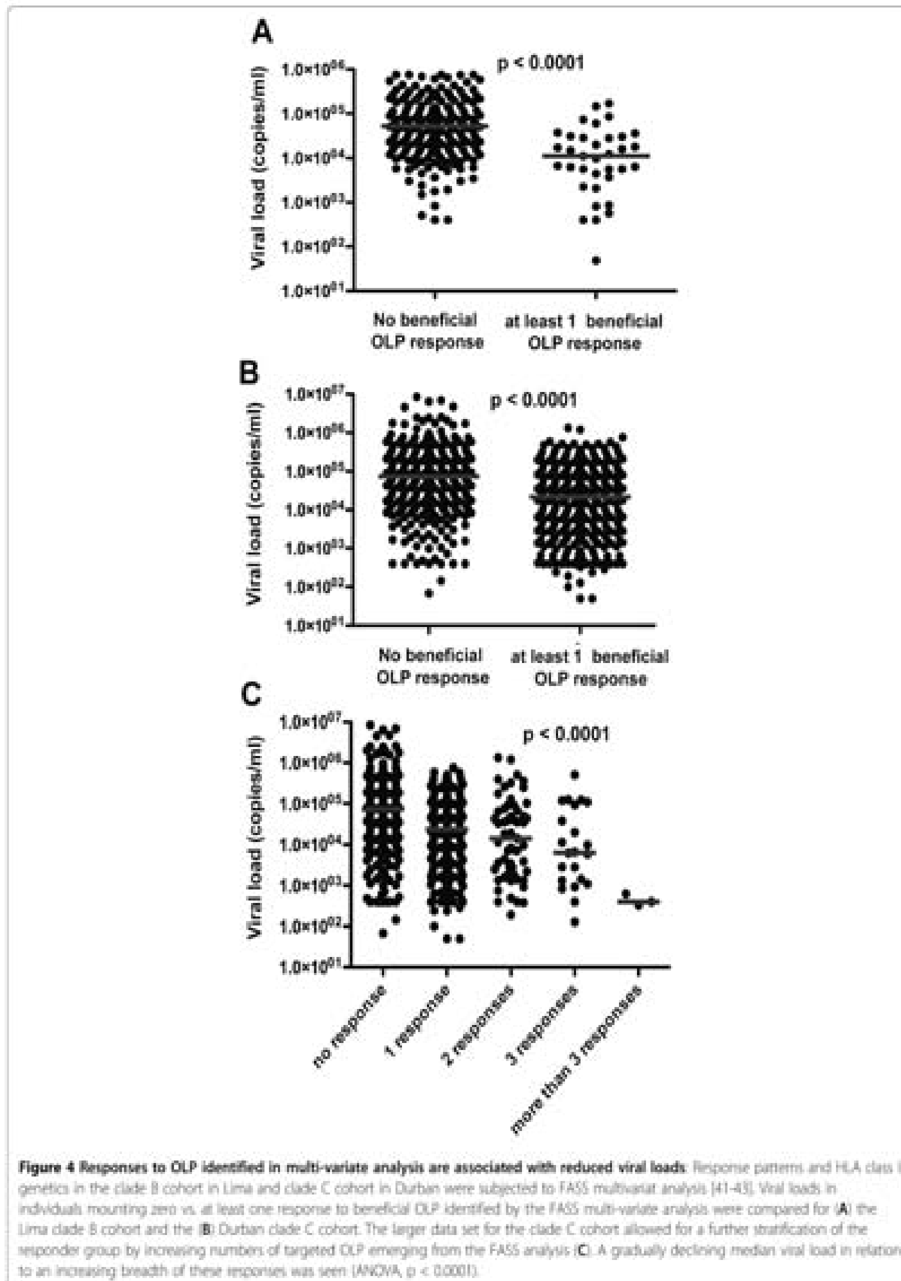

Figure 4 Responses to OLP identified in multi-variate analysis are associated with reduced viral loads. Response patterns and HLA class I genetics in the clade B cohort in Lima and clade C cohort in Durban were subjected to FASS multivariat analysis [41-43]. Viral loads in individuals mounting zero vs. at least one response to beneficial OLP identified by the FASS multi-variate analysis were compared for (**A**) the Lima clade B cohort and the (**B**) Durban clade C cohort. The larger data set for the clade C cohort allowed for a further stratification of the responder group by increasing numbers of targeted OLP emerging from the FASS analysis (**C**). A gradually declining median viral load in relation to an increasing breadth of these responses was seen (ANOVA, $p < 0.0001$).

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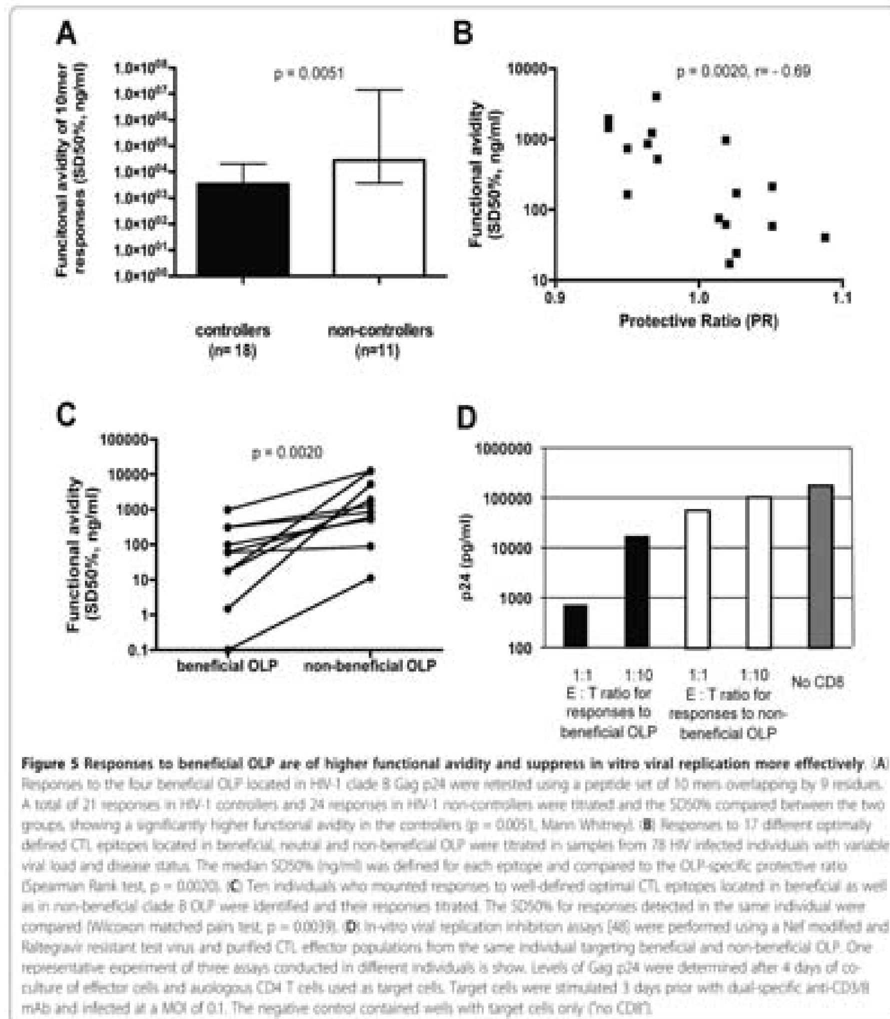

Figure 5 Responses to beneficial OLP are of higher functional avidity and suppress in vitro viral replication more effectively. (**A**) Responses to the four beneficial OLP located in HIV-1 clade B Gag p24 were retested using a peptide set of 10 mers overlapping by 9 residues. A total of 21 responses in HIV-1 controllers and 24 responses in HIV-1 non-controllers were titrated and the SD50% compared between the two groups, showing a significantly higher functional avidity in the controllers (p = 0.0051, Mann Whitney). (**B**) Responses to 17 different optimally defined CTL epitopes located in beneficial, neutral and non-beneficial OLP were titrated in samples from 78 HIV infected individuals with variable viral load and disease status. The median SD50% (ng/ml) was defined for each epitope and compared to the OLP-specific protective ratio (Spearman Rank test, p = 0.0020). (**C**) Ten individuals who mounted responses to well-defined optimal CTL epitopes located in beneficial as well as in non-beneficial clade B OLP were identified and their responses titrated. The SD50% for responses detected in the same individual were compared (Wilcoxon matched pairs test, p = 0.0039). (**D**) In-vitro viral replication inhibition assays [48] were performed using a Nef modified and Raltegravir resistant test virus and purified CTL effector populations from the same individual targeting beneficial and non-beneficial OLP. One representative experiment of three assays conducted in different individuals is show. Levels of Gag p24 were determined after 4 days of co-culture of effector cells and autologous CD4 T cells used as target cells. Target cells were stimulated 3 days prior with dual-specific anti-CD3/8 mAb and infected at a MOI of 0.1. The negative control contained wells with target cells only ("no CD8").

subunits have been associated before with relatively superior viral control in vivo [14,16,49], but evidence from recent clinical trials suggests that including maximal immunogen content into various vectors does not necessarily induce more effective CTL responses [50,51]. In fact, it has been argued that the existence of potential "decoy" epitopes may divert an effective CTL response towards variable and possibly less effective targets in the viral genome [52]. Thus, the definition of a minimal yet sufficient immunogen sequence that can elicit CTL responses in a broad HLA context is urgently needed. Thereby, focusing vaccine responses on conserved regions could help induce responses towards mutationally constrained targets and provide the basis for protection from heterologous viral challenge.

We present here the results of an extensive analysis that included more than 950 HIV-1 infected individuals with diverse HLA genotypes, from three different continents and including clade B and C infections. In both, the analysis in clade B in Lima and clade C in Durban,

individual OLP were identified that are predominantly targeted by individuals with reduced or elevated viral loads, although the different size of the cohorts required different statistical approaches for their identification. In general, most of these OLP were among the more frequent targets in the HIV proteome, possibly due to both, the need for sizable responder groups to achieve statistical significance in the viral loads comparison as well as the high epitope density in these OLP. The identified OLP were frequently located in HIV-1 Gag and Pol, but rarely in the more variable proteins such as Env and Nef. With one exception, Nef and Env featured only non-beneficial OLP, thus arguing against their inclusion, at least as full proteins, in a CTL immunogen sequence [16]. In addition, in both cohorts, the Vif protein yielded few, yet exclusively beneficial OLP, which may warrant a renewed look at the inclusion of regulatory proteins in vaccine design [53,54]. Also common to both clades, (and despite the wide scatter possibly due to the inclusion of less-frequently targeted OLP), an negative correlation between sequence entropy and PR was observed providing strong rationale for vaccine approaches that focus on conserved viral regions where T cell escape may be complicated by structural constrains [55]. This was particularly evident in the clade C cohort, where even within the relatively conserved Gag protein, a lower entropy was seen for the beneficial OLP compared to the remainder of the OLP spanning the protein. On the other hand, while beneficial and non-beneficial OLP showed a significant difference in their median entropy in the clade B cohort, this comparison was not significant in the clade C cohort. It is possible that the immunogen sequence, designed in 2001, did not optimally cover the circulating viral population in Durban throughout the enrollment period (until 2006), leading to missed responses particularly in the more variable segments of the virus [32,56]. The study may have thus failed to identify beneficial as well as non-beneficial OLP in the more variable genes of HIV. This should have preferentially affected highly variable OLP due to a more frequent mismatch between autologous viral sequence and in vitro test set in these regions. However, even if scoring as beneficial OLP, such high-entropy OLP may from an immunogen-design point of view be of less interest as they would possible contribute only little to protection from heterologous viral challenge. It needs however also to be considered that the OLP-specific entropy values are based on variable numbers of sequences in the Los Alamos HIV database covering the different OLP, introducing potential further bias into these analyses, particularly for less covered proteins such as Vpu and other viral protein products. Such differences between autologous viral sequences and in vitro test sets may also have impacted the assessment of functional avidities. These determinations included responses in the same individual towards epitopes located in beneficial and non-beneficial OLP; with the former overall being more conserved. Thus, the higher functional avidity towards epitopes located in beneficial OLP could be biased by the higher chance that these epitopes matched the autologous viral sequence compared to epitopes located in non-beneficial OLP and which may thus have induced a more robust, avid response. Apart from covering autologous sequences, future studies will ideally also include comparable analyses in individuals identified and tested in acute infection that go on to control the infection at undetectable levels of viral replication (i.e. elite-controllers) so that the selective early emergence of responses to beneficial OLP could be linked to relative control of viral replication in chronic infection. As is, the identified beneficial responses may be particularly important to maintain low viral replication in chronic stages of infection, which in theory could be different (for instance due to more accelerated intra-individual viral evolution in variable genes) from responses determining viral set point during acute infection. However, the existing HLA bias in such cohorts and the small number of responses identified during earliest stages of infection may make such analyses a formidable undertaking that will require large numbers of individuals to be tested longitudinally.

A broadly applicable T cell immunogen sequence should include T cell targets restricted by a wide array of HLA class I alleles. Although broad representation of HLA-B alleles may be particularly important in this regard, emerging data on the effects HLA-C alleles in these cohorts may warrant a broad HLA-C representation as well [2,47,57]. In the present study, the 26 beneficial OLP from Lima and the 22 beneficial OLP from Durban covered 26 described, optimally defined CTL epitopes restricted by 20 different HLA alleles for the clade B cohort and 33 epitopes presented by 34 alleles for the clade C cohort, respectively [44]. As this is likely to be an underestimate of the true diversity in HLA restriction (Table 2 and ref [58]), it is reasonable to predict that the inclusion of identified beneficial OLP, or even a subset thereof, could evoke potential responses in a widely diverse HLA context. This could also provide the basis for the induction of poly-specific T cell responses with increased breath, which the present data clearly associates with progressively lower viral loads and which emerge as a potentially important parameter from several recent vaccine studies showing superior protection from SIV challenge in animals with a broad vaccine induced responses to Gag p17 [59,60].

Recent studies have suggested a global adaptation of HIV-1 to its various host ethnicities [4,46]. The consequence of such adaptation has led in some cases to the

elimination of protective CTL targets, causing a profound absence of responses to these epitopes and detrimentally changing the association between HLA allele and HIV-1 disease outcome [4]. It is thus not surprising that the two main cohorts tested here yielded only partially overlapping sets of beneficial OLP as the impact of host genetics and viral evolution in the studied populations cannot readily be overcome. In fact, given studies by Frahm et al [4], the past and current adaptation of HIV-1 to common HLA class I alleles will likely still call for somewhat population tailored vaccine approaches, especially if the immunogen sequences should be kept short to avoid regions of potentially reduced immunological value [52]. Such approaches will also profit from more extensive structural analyses that may identify specific domains of viral proteins that are or are not enriched in valuable T cell targets; of which the latter could possibly be ignored for the design of T cell immunogen sequences. Additional analyses in other genetically unrelated cohorts of HIV-1 infected individuals and studies in SIV infection may further help to guide such selective immunogen design and to understand the factors defining the effectiveness of different epitopes in mediating relative HIV-1 control. Of note, the beneficial OLP identified here, 24 in clade B and 22 in clade C infection matched other immunogen design based on conserved elements in some parts as well, i.e. of the 14 conserved elements proposed by Hanke et al, eight (57%) overlapped at least partly with beneficial OLP identified here [61]. Similarly, among the highly conserved elements proposed by Rolland et al [52], 35% (5/14) were covered by our beneficial OLP in clade B infection. These differences possibly emerge because the present analysis is based on functional T cell data rather than viral sequence alignments, which may not take into consideration epitope density and processing preferences of certain regions. Nevertheless, the partial overlap with these other immunogen design support the focus on conserved regions and offers the opportunity for alternative or combined vaccine approach that elicit responses to regions where the virus is and possibly remains vulnerable [4,46,55,62].

Finally, we used the extensive data set available to approach the question of relative effects of host genetics (i.e. HLA) and CTL specificity on HIV-1 control. While the two factors cannot be entirely disentangled, our data suggest that CTL specificity has an at least equal if not stronger effect on viral control than HLA class I allele expression. These findings are also in line with data by Mothe et al [63] showing that targeting key regions in p24 surrounding the dominant epitopes restricted by known protective alleles (KK10 for HLA-B27 and TW10 for HLA-B57/58) in HLA-B27, -57 or B58 negative individuals is associated with significantly reduced viral loads. In addition, the presence of individuals not expressing known beneficial alleles in HIV-1 elite controller cohorts [64], further indicates that HIV-1 control is not necessarily bound to a few specific HLA class I alleles. A detailed study of the total HIV-1-specific CTL response of subjects not expressing these alleles yet effectively controlling HIV-1 can be expected to provide further and crucially needed insight into the importance of targeting specific (conserved) regions of the viral genome for HIV-1 control. Similarly, the characterization of functional attributes of these responses, including functional avidity and the ability to suppress *in vitro* viral replication will need to be further assessed in such individuals. Building on experimentally derived and potentially promising immunogen sequences as defined here may thus provide a suitable basis for further immunogen design and iterative clinical trials in the human setting.

Acknowledgements
This work was supported by NIH contracts N01-AI-30024 and N01-AI-15422 (CB, TA, BDW, BTK, JS), NIH-NIDCR R01 DE018925-04 (CB), grant MTM2008-06747-C02-00 (GG) from the Ministerio de Ciencia y Tecnologia, a grant from the Instituto de Salud Carlos III (FIS PS09/00283, AL) and a grant from the Fundacio para la Investigacion y Prevencion del SIDA en España (FIPSE) # 36073?/09, CB), Spain as well as a grant from the European Community FP7 ("CUTHIVAC"). BM holds a research fellowship grant from the FIS (Rio Hortega, CM08/00020), Madrid, Spain. ZB is supported by a New Investigator Award from the Canadian Institutes of Health Research (CIHR). CB and JMP are ICREA (Institució Catalana de Recerca i Estudis Avançats) Senior Research Professors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Authors' contributions
BM conducted cellular immune analyses, in vitro inhibition analyses and drafted the first version of the manuscript. AL, JZ, VB generated the recombinant test virus, performed viral inhibition analyses and did the OLP screening of the Barcelona patients. JI and MD conducted OLP data analyses and HLA-epitope predictions. CM recruited patients and provided samples. RZ conducted and coordinated the screening of the Lima cohort subjects. SFA conducted statistical analyses and developed the multivariat analysis approach. CTB performed functional avidity analyses. MCP, JMP, OOY provided semi-genome plasmids and helped in the construction of the

mutant test virus for inhibition analyses. MR, CJB, ZLB analyzed beneficial OLP sequences for HLA footprints associated with reduced viral fitness. MF conducted the screening of the Lima cohort subjects. JJS developed HEPITOPE tool and conducted HLA linkage analyses. WH performed HLA typing. VSM provided samples. DH performed initial multivariat analyses. TMA coordinated HLA typing and sequence analyses for the Lima cohort. JBM analyzed beneficial OLP sequences for HLA footprints and levels of sequence conservation. GG helped with the statistical analyses and the development of the multivariat analysis. PJG and BDW coordinated all OLP screenings, HLA typing and data collection in the Durban cohort. JMG and BC coordinated sample access and HLA typing in Barcelona. BTK helped with data analysis and writing of the manuscript. JS coordinated patient enrollment, ethical approval for the Lima cohort. CB conceived the study, conducted initial data analyses, and helped writing the manuscript. All authors were involved in the writing of the final manuscript and have given final approval of the version to be published.

Competing interests
The authors declare that they have no competing interests.

Received: 14 September 2011 Accepted: 7 December 2011
Published: 7 December 2011

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doi:10.1186/1479-5876-9-208
Cite this article as: *Mothe et al.*: **Definition of the viral targets of protective HIV-1-specific T cell responses.** *Journal of Translational Medicine* 2011, **9**:208.

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CTL Responses of High Functional Avidity and Broad Variant Cross-Reactivity Are Associated with HIV Control

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Abstract

Cytotoxic T lymphocyte (CTL) responses targeting specific HIV proteins, in particular Gag, have been associated with relative control of viral replication *in vivo*. However, Gag-specific CTL can also be detected in individuals who do not control the virus and it remains thus unclear how Gag-specific CTL may mediate the beneficial effects in some individuals but not in others. Here, we used a 10mer peptide set spanning HIV Gag-p24 to determine immunogen-specific T-cell responses and to assess functional properties including functional avidity and cross-reactivity in 25 HIV-1 controllers and 25 non-controllers without protective HLA class I alleles. Our data challenge the common belief that Gag-specific T cell responses dominate the virus-specific immunity exclusively in HIV-1 controllers as both groups mounted responses of comparable breadths and magnitudes against the p24 sequence. However, responses in controllers reacted to lower antigen concentrations and recognized more epitope variants than responses in non-controllers. These cross-sectional data, largely independent of particular HLA genetics and generated using direct ex-vivo samples thus identify T cell responses of high functional avidity and with broad variant reactivity as potential functional immune correlates of relative HIV control.

Citation: Mothe B, Llano A, Ibarrondo J, Zamarreño J, Schiaulini M, et al. (2012) CTL Responses of High Functional Avidity and Broad Variant Cross-Reactivity Are Associated with HIV Control. PLoS ONE 7(1): e29717. doi:10.1371/journal.pone.0029717

Editor: Rachel Louise Allen, University of London, St George's, United Kingdom

Received September 6, 2011; **Accepted** December 2, 2011; **Published** January 4, 2012

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Funding: This work was funded by Bill and Melinda Gates Foundation and was supported in part by the Red de Investigación de Sida (RIS) (RD06/04), Instituto de Salud Carlos III (ISCIII), Madrid, Spain and the Spanish FIPSE 36-0737-09. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DH is employed by Microsoft Research. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Several studies in cohorts of clade B and clade C-infected individuals have shown that cytotoxic T-cell (CTL) responses against HIV-1 Gag correlate with relative control of HIV-1 [1,2,3,4]. The rapid re-presentation of epitopes derived from the Gag proteins contained in the infecting viral particles and structural constraints of the Gag protein that complicate CTL escape have been suggested as possible mechanisms that lend Gag-specific CTL responses this superior effectiveness in controlling HIV-1 [5,6]. However, in all studies reporting beneficial effects of Gag-specific responses, some HIV-1-infected non-controllers mount detectable responses against Gag as well, raising the question as to why these individuals are unable to control their viral replication. A possible answer to this question is that functional characteristics [7,8,9], including functional avidity and variant cross-reactivity are distorted in the CTL population in HIV non-controllers. However, some of these characteristics may not be captured reliably when using some standard in vitro antigen test sets and assay systems [10,11].

In the present study, we analyzed HIV Gag-p24 specific T cell responses in HIV-1 controllers and non-controllers using 18mer and 10 mer peptide sets to compare relative response rates using either longer or shorter test peptides and to determine the functional avidity of these responses as well as their ability to react with naturally occurring sequence variants. Furthermore, the data also allowed to assess whether the most conserved regions within p24 are differentially targeted by HIV-1 controllers and non-controllers in order to provide in vitro relevance for vaccine approaches focusing on such conserved elements (CE) in the viral genome [12,13].

Although responses to Gag p24 were of comparable breadth and magnitude in HIV-1 controllers and non-controllers when using the 10 mer peptide set, significantly higher avidity responses were seen in controllers, who also showed broader epitope variant cross-reactivity than non-controllers. The data suggest that the maintenance of high avidity responses with broad variant recognition potential is a potential hallmark of controlled HIV-1 infection; a finding that may have important implications in the development of preventative as well as therapeutic vaccine strategies.

Results

Gag p24 specific T cell responses in controllers and non-controllers are significantly increased when using 10 mer peptides sets

Chronically HIV-1 infected individuals with controlled HIV infection (n = 25; median viral load 810 RNA copies/ml and median CD4 cell count 642 cells/mm^3) and non-controlled viral replication (n = 25; viral load median viral load 200,000 RNA copies/ml and median CD4 cell counts 98 cells/mm^3) were recruited from the HIV Unit in Hospital Germans Trias i Pujol, Badalona, Spain. The study was approved by the Institutional Review Board of the Hospital Germans Trias i Pujol and all individuals provided written informed consent. Median age of individuals was slightly higher for the non-controllers group compared to controllers (44 years-old (24–55) vs 38 years-old (26–56), p = 0.04) but individuals did not significantly differ in time since HIV diagnosis (p = 0.07) (Table 1). The participants were mostly of Caucasian ethnicity (79% Caucasian, 17% Hispanic, 2% African and 2% Asian) and the ethnic origin did not differ between the two groups. HLA diversity was heterogeneous in both groups and individuals expressing HLA-B27, HLA-B57, or HLA-B58 were intentionally excluded from the cohort to avoid bias due to the presence of dominant Gag p24 CTL epitopes restricted by these alleles and to overcome the limitations of past studies in which these alleles were highly over-represented (**Table S1**).

In a first step, the distribution of total HIV-1-specific T-cell responses using a 18 mer overlapping peptide (OLP) set covering the full HIV-1 proteome was assessed in the 50 individuals included. The majority of responses were directed against OLP located in the HIV-1 Gag, Pol and Nef proteins with a relative dominance of Gag/p24 in HIV-1 controllers (p = 0.0336 for Gag, p = 0.0486 for Gag p24). These data confirmed the expected distribution of responses from earlier reports in HIV-1 controllers even though the present cohort was smaller and did not include individuals expressing known protective HLA class I alleles [4,14]. Of note, the peptide concentrations used were relatively high (14 ug/ml), and in our hands saturating [15], to avoid missing responses due to suboptimal peptide concentrations.

To increase the sensitivity of the assay and to discern potential functional differences of Gag responses in non-controllers unable to mediate relative viral control in these subjects, all individuals were tested against a set of 223 10 mer peptides (overlapping by 9 residues) spanning the group M Center-of-Tree (COT-M) Gag p24 sequence (**Figure S1**). Significantly more responses were identified by using the 10 mers in both groups (**Figure 1A,B**; p = 0.0002 for controllers, p = 0.0006 non-controllers). 20 of the 25 individuals in each group showed an increase in the detected responses with the 10 mer test set; while only 3 had equivalent breadth and 2 individuals in each group had one response less compared to the 18 mer peptides. Controllers and non-controllers showed a 2–3-fold increase of their responses which abolished the broader response rates seen in controllers when using the 18 mer peptides (p = 0.4260). Responses detected with the 10 mer peptide set were of comparable magnitude in the two groups, both in terms of total magnitude (median 4,250 vs. 2,600 SFC/10^6 PBMC in controllers and non-controllers, respectively; p = 0.6004, **Figure 1C**) and the average magnitude of individual responses (median 614 vs. 657 SFC/10^6PBMC, p = 0.9178, **Figure 1D**).

Table 1. Demographic and main clinical characteristics of the 25 controllers and 25 non-controllers tested[a].

	C (n = 25)	NC (n = 25)	P value
Age, years	38 (26.2–55.7)	44.5 (24.3–54.8)	0.04
Time since HIV-1 diagnosis (years)	9.3 (3.5–26.3)	15.9 (1.5–23.3)	0.07
Gender (Female/Male)	F 40%/M 60%	F 40%/M 60%	
HIV risk group			
Heterosexual[b]	6 (24%)	10 (40%)	0.36
Men who have sex with men[b]	8 (32%)	4 (16%)	0.32
Injecting drug users[b]	7 (28%)	9 (36%)	0.76
Other[b]	4 (16%)	2 (8%)	0.66
Last CD4+ T cell counts (cells/mm^3)	642 (434–1114)	98 (11–361)	<0.001
% CD4 cells	32 (16–50)	9 (1–27)	<0.001
Last HIV-1 RNA levels (copies/ml)	810 (UD[c]–10,000)	200,000 (52,000–1,200,000)	<0.001
HLA alleles representation			
HLA-A (n = 24 alleles)	20 alleles	15 alleles	
HLA-B (n = 34 alleles)	27 alleles	17 alleles	
HLA-C (n = 20 alleles)	17 alleles	15 alleles	

[a]Data are expressed as median (min-max range).
[b]n, (%).
[c]UD: undetectable viremia (<49 copies/ml).
doi:10.1371/journal.pone.0029717.t001

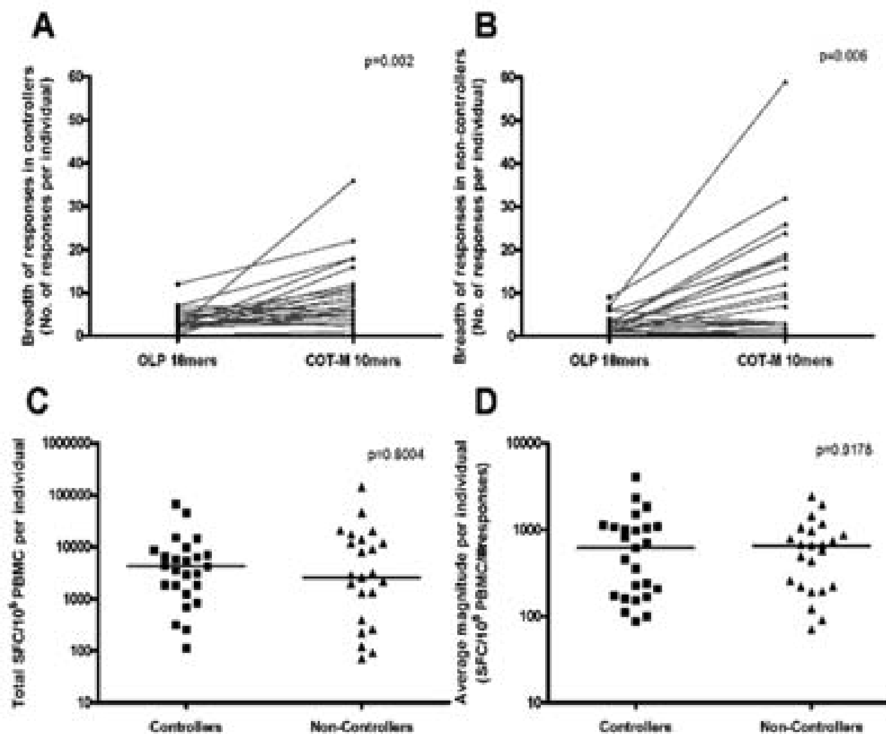

Figure 1. Increased detection of Gag p24 specific responses using a 10 mer peptide set. IFN-γ ELISpot responses against Gag p24 elicited either by consensus B overlapping 18 mer or COT-M 10 mer peptide sets in 25 HIV-1 controllers (**A**) and 25 HIV-1 non-controllers (**B**) P-values reflect the increase in median breadth of responses when using 10 mer peptide sets instead the 18 mer peptides (two-tailed Wilcoxon matched paired test). Total magnitude of responses (**C**) and average magnitude of responses (**D**) to COT-M Gag p24 10 mer peptides are shown for 25 controllers and 25 non-controllers, respectively. Lines represent median values and indicated p values are based on Mann-Whitney t-tests.
doi:10.1371/journal.pone.0029717.g001

These results demonstrate that Gag p24 specific responses are readily detectable in HIV-1 non-controllers when using a sensitive 10 mer peptide set and that they are unlikely to represent spurious, nonspecific reactivities. The data also show that using 18 mer peptides may potentially miss up to 2/3 of responses, al least in some of the HIV non-controllers and the antigen (i.e. p24) tested here.

Responses in HIV-1 controllers are of higher functional avidity than in non-controllers and mediate better variant recognition

Data from animal studies and our own analyses in HCV infection suggest that T cell responses of high functional avidity are superior in mediating viral control [3,16,17,18]. We thus tested whether HIV-1 controllers and non-controllers differed in the overall functional avidity of their responses. Based on cell availability, the functional avidity was determined for a total of 474 individual positive responses (219 in controllers and 255 in non-controllers). Controllers indeed showed responses of higher functional avidity (median 6,110 ng/ml, range $0.05–7.6\times10^{7}$) compared to non-controllers (median of 13,548 ng/ml, range $0.64–4\times10^{5}$; $p = 0.0101$, **Figure 2A**). This difference was more pronounced when the analyses was limited to the 32 10 mer-specific responses that were titrated in both groups (6,998 ng/ml vs. 46,637 ng/ml, respectively; $p = 0.0173$, **Figure 2B**) While it is possible that even within one 10 mer peptide more than one epitope could be located (i.e. 9 mer optimal epitopes) and that 10 mer responses could be due to presentation of different epitopes on different HLA alleles, the HLA representation between controllers and non-controllers was similar (particularly because HLA-B57, 58, B27 expressing individuals were excluded). Therefore, it is likely that the same epitope in the same HLA context was being targeted in most of the cases included in the matched analysis and that differential allele-effects would not have impacted the comparison between the two groups.

As high avidity responses may be more prone to react with sequence variants in their cognate epitopes, they may provide a crucial advantage in the control of highly variable pathogens such as HIV and HCV [7,17]. To address whether high avidity responses in HIV controllers would indeed react with more epitope variants, naturally occurring sequence variants were tested for cross-recognition in all 50 subjects using a set of 88 additional

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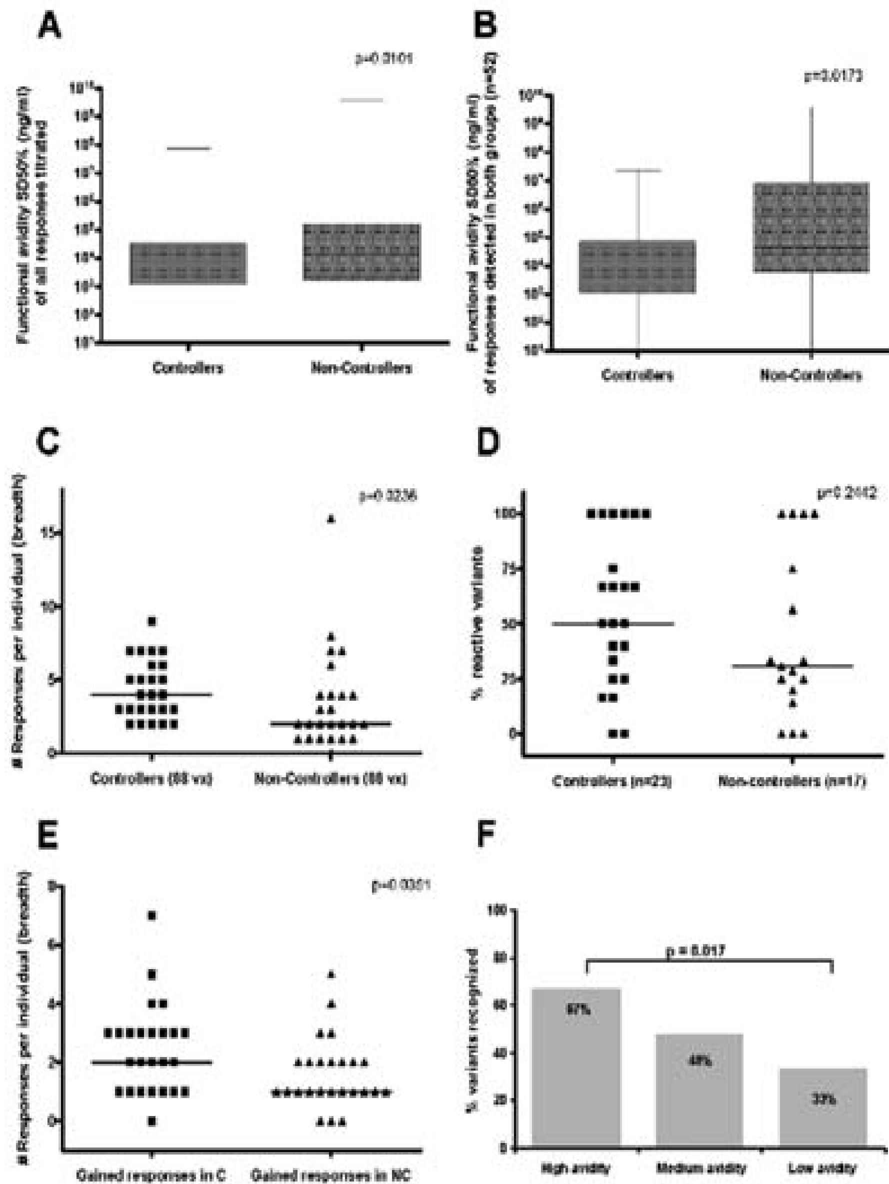

Figure 2. High avidity responses are enriched in HIV-1 controllers and mediate superior variant recognition. (**A**) Comparison of functional avidity of all COT-M Gag p24 responses titrated in controllers (n = 219 responses) vs. non-controllers (n = 255 responses) (**B**) Comparison of functional avidities limited to responses targeting the same 10 mer OLP in the two groups (n = 52 responses, Wilcoxon). In (**C**) the total breadth (number) of the response to the tested COT-M Gag p24 variant peptides (n = 88) is indicated for controllers and non-controllers. (**D**) Shows the percentage of variant peptides that were reactive when the COT-M sequence elicited a response ("cross-reactive responses") and (**E**) indicates responses to variant peptides for which the COT-M sequence did not elicit a response ("gained responses"). The association between functional avidity and cross-reactivity is shown in (**F**) where responses with functional avidities in either the first quartile of all titrated responses

(SD50%<1,401 ng/ml) or the second or third quartile (SD50% 1,401–71,594 ng/ml) or the fourth quartile (SD50%>71,594 ng/ml) were defined as "high", "intermediate" and low" avidity responses. The percentage of variants that elicited a response was compared between the three groups (Fishers Exact Test).
doi:10.1371/journal.pone.0029717.g002

10 mer peptide variants. The median number of responses to these 88 variants was 2-fold greater in controllers (median of 4 responses; range 2–9) than in HIV-1 non-controllers (median 2 responses, range 0–16, $p = 0.0236$, **Figure 2C**). In particular, controllers showed responses to both the wild-type and variant peptides in half of the cases where a COT-M and a variant peptide was tested (50%) while only 31% in HIV-1 non-controllers reacted to variants (**Figure 2D**). While this did not reach statistical significance, controllers reacted with significantly more variant peptides for which the COT-M sequence did not elicit a response (median of 2 additional responses by inclusion of variants, range 0–7) than the non-controllers (median 1, range 0–5; $p = 0.0351$; **Figure 2E**). The average magnitude of the variant-specific responses was comparable between controllers (median of 742 SFC/10^6PBMC, range 90–3,073) and non-controllers (median 473 SFC/10^6PBMC, range 60–2,707; $p = 0.5605$, data not shown) indicating that cross-reactive responses in HIV non-controllers were robust, when present.

In order to directly test whether functional avidity was related to the ability to recognize peptide variants, titrated responses were grouped into high, intermediate and low avidity responses and their variant recognition potentials were compared. Indeed, responses with functional avidities in the first quartile of all titrated responses (SD50%<1,401 ng/ml) showed cross-reactivity with their variants in 67% of all cases, whereas fewer (48% and 33%) responses of intermediate or low functional avidity were cross-reactive with their variants, respectively (**Figure 2F**). Collectively, the data demonstrate that high avidity responses were more prevalent in HIV-1 controllers and that these responses mediate superior variant recognition than responses of low functional avidity.

Conserved regions in Gag p24, containing HLA-B14, -B27 and B57 restricted, protective CTL epitopes are frequently targeted by HIV-1 controllers that do not express protective HLA alleles

The high degree of sequence conservation in HIV Gag p24 makes this protein an interesting vaccine component and many vaccine immunogen designs indeed include Gag p24 [12,19,20]. A recently developed immunogen sequence is based on a strong focus on the most conserved elements (CE) within Gag p24, excluding variable segments that could contain potential decoy epitopes that may divert the host T cell response towards less valuable targets [13]. These CE were defined as sequence stretches of at least 12 amino acids in length that contain only amino acids residues with at least 98% sequence conservation across all available independent group M sequences [13]. Gag p24 contains 7 of such CE segments, ranging from 12 to 24 amino acids in length and corresponding to a total of 124 residues (**Figure S1**). To validate this immunogen concept, we stratified the T cell data from the 50 controllers and non-controllers based on the location of the targeted 10 mer, i.e. whether they were located within or outside of these conserved elements. Both groups showed comparable breadth and magnitudes of total CE-specific responses (**Figure 3A**). Also, controllers reacted with significantly more epitope variants located in CE regions than non-controllers (median of 2 responses in controllers vs. 1 response in non-controllers, $p = 0.0145$, data not shown). Of note, these differences were not due to a suboptimal match between test peptide sequences and autologous viral HIV-1 sequences in the non-controllers as their dominant autologous p24 sequence was in all cases clade B and mostly (99%) represented by the test peptides (**Figure S2**).

Of interest, there were three CE that were recognized by at least 50% more controllers compared to non-controllers (referred to as CE #4, #5, #6), suggesting that these may be preferential targets in HIV-1 controllers (**Figure 3B**). In fact, HIV-1 controllers mounted significantly more responses to at least one of these three CE than did non-controllers ($p = 0.0006$; **Figure 3C**) and showed a trend towards these being responses of higher functional avidity, even though only a fraction of the overall data points were included in this comparison (median 7,189 ng/ml, range 0.99–2.5×10^7 vs. median of 17,058 ng/ml, range 16.28–2.62×10^7; $p = 0.0666$, data not shown). In addition, the total magnitude of the responses to CE #4+5+6 showed a statistically significant correlation with HIV-1 viral load ($r = -0.5$, $p = 0.0002$ by Spearman's rank, **Figure 3D**) across all 50 subjects, suggesting that stronger responses to these three regions may mediate better control of viral replication.

Interestingly, these 3 regions included the well-characterized HLA-B57 restricted TW10 epitope (in CE #4), the HLA-B27 restricted KK10 epitope (in CE #5), and the HLA-B14 restricted DA9 epitope (in CE #6), all of which have been previously associated with containment of in vivo HIV-1 replication [21,22,23,24]. However, as the cohort did not contain any HLA-B57+ and -B27+ individuals and only 2 subjects expressed HLA-B14, the data indicate that mounting responses to these regions is effective even if these protective responses are being restricted by HLA class I molecules different from the originally described restricting HLA molecules [25,26]. Indeed, the HLA class I allele representation of CE #4, #5 or #6 responders was heterogeneous and not limited to individuals with a few shared HLA alleles (**Table S1**) indicating that the CE regions represent a very rich set of epitopes that are not being blocked from presentation in natural chronic infection and that are able to be recognized in a wide HLA class I context.

Discussion

Together, our data strongly suggest that the presence of responses of high functional avidity and with broad variant recognition ability is a potential hallmark of controlled HIV-1 infection. To the best of our knowledge, this is the first demonstration of a direct link between high avidity T cell responses, broad variant recognition and in vivo HIV-1 control using ex-vivo blood samples from a cohort with largely unbiased HLA genotypes. Our data also support that standard approaches using 15–20 mer overlapping peptides underestimate the breadth of responses to HIV Gag p24 significantly. While similar findings have been reported in earlier studies, none has addressed this in a systematic way and including a comparably extensive avidity determination as in the present study. More importantly though, since HIV non-controllers profited the most from using a more sensitive peptide set, the data have also important implication for our understanding of HIV immunopathogenesis and vaccine immunogen design: It is not that HIV-1 non-controllers would not mount Gag-specific T-cell responses; rather, they may have either

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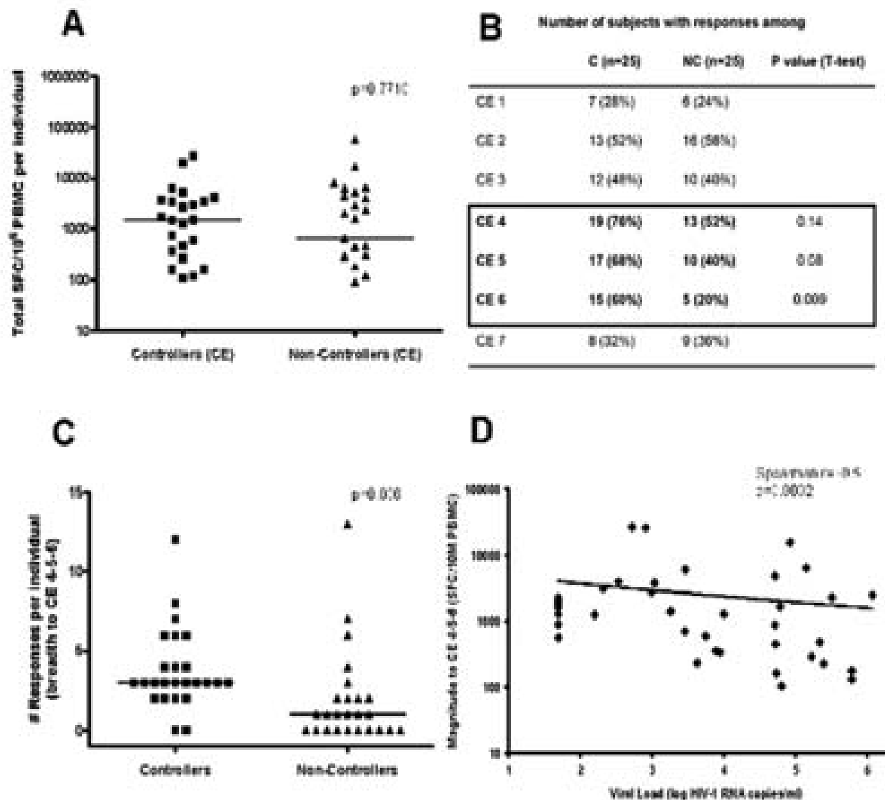

	C (n=25)	NC (n=25)	P value (T-test)
CE 1	7 (28%)	6 (24%)	
CE 2	13 (52%)	16 (58%)	
CE 3	12 (48%)	10 (40%)	
CE 4	19 (76%)	13 (52%)	0.14
CE 5	17 (68%)	10 (40%)	0.08
CE 6	15 (60%)	5 (20%)	0.009
CE 7	8 (32%)	9 (36%)	

Figure 3. CE containing HLA-B14, -B27 and B57 restricted, protective CTL epitopes are predominantly targeted by HIV-1 controllers. (**A**) The total magnitude of responses to CE regions is compared between HIV controllers and non-controllers. (**B**) The frequency of recognition of the 7 different CE is shown for 25 HIV-1 controllers (C) and 25 non-controllers (NC), respectively. CE regions targeted by at least 50% more controllers than non-controllers are boxed and p-values indicated (T test). (**C**) Breadth of responses to the combination of CE 4+5+6 regions in controllers vs. non-controllers is shown. Horizontal lines represent median values and Mann-Whitney t-test p value is shown. (**D**) Correlation between the cumulative magnitude of responses to CE 4+5+6 and HIV viral loads in all 50 tested individuals is shown (Spearman's rank test).
doi:10.1371/journal.pone.0029717.g003

induced low-avidity responses during acute infection or induced originally high functional avidity responses that were lost over the course of HIV-1 infection. This latter interpretation would be in line with results from longitudinal analyses in individuals followed from acute infection time points [27,28]. Although these analyses were based on fewer individuals and included many donors expressing HLA-B27 and -B57 alleles, clonal exhaustion of high avidity cells in the course of chronic HIV replication is certainly a possible explanation why the HIV non-controllers in our study showed responses of reduced functional avidity. On the other hand, it is interesting to note that in a recent report by Berger et al, high avidity responses were not only not restored upon HAART initiation but were actually further diminished [7], suggesting that possibly other factors than duration and extend of antigenimia impact the measurable avidity of an epitope-specific T cell population.

The wide spread and overlap in the avidity measurements between responses among the two groups is probably the biggest challenge for this kind of study to conclusively demonstrate its potential biological significance. Quite likely, inter-epitope and inter-individual differences hamper a clearer observation. In addition, given that different effector functions are subject to variable activation thresholds [29], the inclusion of additional in vitro read-outs could possibly provide a larger discrimination in the minimal antigen amounts required for responses in HIV controllers and non-controllers. Indeed, as limited effector functions have been previously described for HIV-1 non-controllers, one would expect additional reactivities to occur preferentially in the controllers group. Despite the limitation of assessing a single effector function (IFNγ release) though, our findings are supported by a number of previous studies in animal models and in HIV and HCV infection that have assessed the

relationship between virus control/clearance, functional avidity and variant recognition [17,30,31,32,33,34,35,36,37]. Moreover, none of earlier studies in humans has been based on the extensive number responses analyzed here (close to 500 titrated responses) and most used either *in vitro* expanded short-term T cell lines or T cell clones [17,32]. While analyzing the functional avidity in clonal T cell populations allows eliminating some of the inherent heterogeneity faced in cross-sectional, directly ex-vivo studies, the *in vitro* selection and expansion of epitope-specific clones may be biasing results too, as shown for HCV specific responses where short-term culture consistently increased the functional avidity compared to directly ex-vivo isolated cells [17]. On the other hand, our data may be limited by the use of 10 mers instead of optimal epitopes for the determination of functional avidity. However, many optimally defined epitopes in Gag are 10 mers and, it has frequently been shown that 9 and 10 mers can have similar SD50%. In fact, in many cases, the definition of optimal epitopes is more driven by the shorter length of 9 mers rather than a substantially lower SD50% [26], suggesting that the 10 mer approach used here is an acceptable approximation to avoid biases or missing responses by alternative optimal epitope or 9-mer approaches.

In order to control for some of the heterogeneity in our data set, we also compared SD50% between matched responses targeting the same 10 mer peptide in the controller and non-controller group. This indeed enhanced the otherwise modest differences in SD50% between the two groups considerably and was statistically significant despite the much smaller number of responses analyzed. The limited difference in SD50% for the overall analysis may also be due to variable activation thresholds for specific CTL effector functions [29]. As such, the observed difference in the SD50% necessary for IFNγ release may not be directly relevant for the improved viral control in vivo but may still reflect a more avid and thus more effective interaction between the CTL and the antigen-presenting cell, regardless of the ensuing cascade of effector function(s).

As mentioned above, high functional avidity may also render CTL more prone to immune senescence or clonal exhaustion, particularly in individuals with suboptimal control of viral replication. The data from cleared and chronic HCV infection support this idea as only HCV clearers seem to maintain responses of high functional avidity in the absence of possible sources of residual antigen [17]. Thus, while high avidity responses may win out during the induction phase of the virus-specific T cell response, these cells may be preferentially lost over time if viral antigenemia cannot be controlled sufficiently well [28,38]. Whether such losses of high avidity responses correspond to changes in the clonal composition of the CD8 T cell response or to a gradual decrease in their functional avidity due to altered cell reactivity/signaling needs to be further addressed in different clinical settings, as the existing data discussing the 'cause/effect' quandary are conflicting and generally limited to responses restricted by few selected HLA alleles [28,32,39,40,41,42].

Further studies will ideally also include other highly conserved regions in the viral genome outside Gag p24, which may serve as additional components of vaccine immunogens. Such extended analyses would also increase the number of responses per individual, which in the present study is relatively small given that only a short segment of the entire viral proteome was analyzed. Despite this focus on p24, our comparisons reached statistical significance and compared well to the breadth of responses reported in earlier studies looking at responses to the entire HIV proteome or optimally defined HIV CTL epitopes [4,14,15,25]. Together with the results here, these earlier analyses also provide support that the detected responses are generally CD8 T-cell mediated, particularly when testing short 10 mer peptides and that they are HIV-specific since testing with even optimally defined short HIV-derived CTL epitopes did not readily elicit responses in HIV negative individuals [15,25].

Given these considerations, our study suggests that HIV-1 controllers mount ex-vivo responses of significantly higher functional avidity than HIV-1 non-controllers. Since the high avidity responses were also more apt to react with epitope variants, their induction by a future HIV-1 vaccine may be crucial to prevent rapid viral escape from the vaccine induced immune response. Finally, as the data presented here confirm findings in HCV infection, they strongly suggest that the ability to maintain T cell responses of high functional avidity is a more general hallmark of effective immune control of infections with highly variable pathogens.

Materials and Methods

Synthetic peptides set

An overlapping peptide set of 223 peptides of 10 amino acids in length (overlapping by 9 residues) spanning the entire group M Center-of-Tree (COT-M) Gag p24 sequence was synthesized using 9-Fluorenylmethyloxycarbonyl (Fmoc)- chemistry. Additional 88 10-mer peptides were generated to cover the most frequently occurring variants in the 7 most conserved (CE) regions. We also included a previously described overlapping peptide (410 18 mers OLP) set spanning the entire viral proteome [4,14] based on the 2001 consensus-B sequence (http://hiv-web.lanl.gov/content/hiv-db/CONSENSUS/M_GROUP/Consensus.html). Peptides were 18 mers varying from 15–20 amino acids in length and overlapping by 10 amino acids, designed using the PeptGen algorithm at the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html).

IFN-γ ELISpot assay

PBMC were separated from whole blood within 4 h of venopuncture and used directly for the IFN-γ ELISpot. Each COT-M Gag p24 peptide was tested individually and added at a final concentration of 14 μg/ml. For all assays, between 75,000–100,000 PBMC per well were added in 140 ul of R10 96-well polyvinylidene plates (Millipore, Bedford, MA). The IFN-γ Mabtech kit was used following manufacturer instructions. In parallel, CTL responses to the clade B full proteome were assessed using the 18 mer peptide set in a previously described optimized peptide matrix, followed by deconvolution of reactive pools and reconfirmation of each response at a single peptide level on the following day and tested at the same concentration of 14 μg/ml [14]. The number of spots was counted using a "CTL ELISpot Reader Unit" and the magnitude of responses was expressed as spot forming cells (SFC) per million input cells. The threshold for positive responses was defined as at least 5 spots per well and responses exceeding the "mean number of spots in negative control wells plus 3 standard deviations of the negative control wells" and "three times the mean of negative control wells", whichever was higher. As a conservative approach and not to overestimate the breadth of responses, positive responses to 3 consecutive 10 mers in the COT-M Gag p24 peptide set were counted as 1 unique response. Similarly, reactivity to 2 consecutive 18 mer OLP was counted as 1 response. The highest magnitude of the sequential responses was taken as the magnitude for each identified response.

Determination of functional avidity

The functional avidity of responses was determined by performing serial 10-fold limiting peptide dilutions ranging from 100 µg/ml to 10 pg/ml using the 10 mer peptide set; in duplicate whenever enough PBMC were available. Half-maximal stimulatory antigen doses (SD50%) were calculated as the peptide concentration needed to achieve a half-maximal number of spots in the ELISpot assay calculated by a sigmoidal dose response curve fit using GraphPad Prism4.

Gag p24 sequencing

Viral RNA was extracted from 1 millilitre of plasma spun at 25000 rpm for 1 hour (QIAamp Viral RNA Kit™, QIAGEN, Valencia, CA). The whole Gag region was reverse-transcribed and amplified in a One-Step reaction (SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen, Carlsbad, CA) under the following conditions: 30 min at 52°C for the reverse transcription step; 2 min at 94°C; followed by 35 cycles at 94°C during 30 sec, 58°C during 30 sec and 68°C during 2 min; followed by a final extension step at 68°C during 5 min. Primers used for the RT-PCR were: Gag U761 (HXB2: 761–778) 5'-TTT GAC TAG CGG AGG CTA G-3' and Gag D2397 (HXB2: 2397–2376) 5'-CCC CTA TCA TTT TTG GTT TCC A-3'. One microliter of the RT-PCR product was subsequently used as a template for a nested PCR (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen, Carlsbad, CA), using primers p24 U1070 (HXB2: 1070–1088) 5'-TAA AAG ACA CCA AGG AAG CT and p24 D2063 (HXB2: 2063–2044) 5'-TCT TTC ATT TGG TGT CCT TC-3'. PCR cycling conditions were: 2 min at 94°C; followed by 35 cycles at 94°C during 30 sec, 54°C during 30 sec and 68°C during 2 min; followed by a final extension step at 68°C during 5 min. The final PCR products were column-purified (QIAquick PCR Purification Kit, QIAGEN, Valencia, CA) and sequenced bidirectionally. Sequences were assembled using Sequencher® 4.10.1 (Genecodes Corp. MI). Assembled sequences were codon-aligned using the Hidden Markov Model implemented in the HIValign tool (www.lanl.hiv.gov). Autologous Gag p24 bulk sequences were obtained for 22 of the 25 HIV-1 non-controllers included in our study. Sequences were submitted to Genbank; accession numbers BCN-NC-1.seqs BCN-NC-1 JQ246370-246391.

Statistical analyses

All values are presented as median values unless otherwise stated. GraphPad Prism version 4.0 for Windows (San Diego, CA) was used to compare response rates in both groups and subgroup analyses. Mann-Whitney test and Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. Spearman rank correlation was used to assess association.

Supporting Information

Figure S1 COT-M Gag-p24 sequence and location of CE segments. The Center-of-tree (COT) M sequence is indicated for entire Gag p24. The location of known optimally-defined CTL epitopes listed at the Los Alamos HIV database, are indicated above the protein sequence while the shaded boxes beneath indicate the 7 CE segments and variant (down) residues included in this study.
(TIF)

Figure S2 Autologous Gag-p24 CE sequences in 21 HIV-1 non-controllers. Shaded boxes indicate the 7 CE sequences located within in p24 with variant residues included (separated by "/"). The amino acid sequences of autologous Gag p24 bulk sequences obtained from 22 HIV non-controllers are shown.
(TIF)

Table S1 HLA genotypes of the 25 controllers and 25 non-controllers tested.
(DOC)

Author Contributions

Performed the experiments: BM AL JI JZ MS MRR CTB. Analyzed the data: BM CM CB. Contributed reagents/materials/analysis tools: MJH EP AK DH MR FP BW DW BF GP. Wrote the paper: BM JM BC RP MP CB. Reviewed the final manuscript: BC.

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cell minimal epitope peptides applying a novel cationic adjuvant CAF01. Vaccine.

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Table S1. HLA genotypes of the 25 controllers and 25 non-controllers tested

Gates ID	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
C-1	3001	3601	4201	4201	17MN	17MN
C-2	0201	0205	0702	1402	0702	0802
C-3	0101	3001	1302	3503	0602	1203
C-4	0205	3201	5001	5201	0602	1202
C-5	1101	2402	4403	5201	1202	1601
C-6	2402	3201	3503	4002	202	1203
C-7	3002	6802	1501	5301	0202	0401
C-8	0201	2402	1401	1402	0802	0802
C-9	0201	3301	1402	3801	0802	1203
C-10	0101	2402	1517	5101	0102	0701
C-11	0201	0201	1402	3924	0701	0802
C-12	1101	2402	3501	5201	0401	1202
C-13	0101	6802	1402	3502	0401	0802
C-14	0101	6901	0801	5501	0102	0701
C-15	0202	3002	1516	4403	0401	1402
C-16	3201	3303	1510	4002	0202	0304
C-17	2402	6601	1801	5107	0701	1402
C-18	0201	6801	1501	5101	0304	1502
C-19	1101	3303	3501	5301	0401	0401
C-20	0201	3201	1302	1501	0303	0602
C-21	0211	2601	4402	5202	1502	1502
C-22	0301	1101	0702	5201	0702	1202
C-23	1101	2402	0702	5101	0702	1502
C-24	0301	1101	1301	5501	0102	0403
C-25	0201	2501	3901	4402	0501	1203
NC-1	0201	0301	0702	1801	0702	1205
NC-2	0201	0301	1801	3501	0401	0701
NC-3	0201	0201	3502	4101	0401	17MN
NC-4	2402	2402	4501	5101	1402	1601
NC-5	2301	2402	4402	4405	0202	0501
NC-6	2902	6801	1801	4501	0501	0602
NC-7	1101	2601	0801	4403	0202	0701
NC-8	0101	2601	3503	3701	0602	1203
NC-9	2601	2902	3801	4403	1203	1601
NC-10	1101	3002	1801	3503	0401	0501
NC-11	0201	0205	1801	4402	0202	0501
NC-12	2601	3201	1401	3503	0401	0802
NC-13	3201	74AB	1401	5001	0602	0802
NC-14	0201	2902	3503	4403	1203	1601
NC-15	0201	0201	1503	4402	0202	0501
NC-16	0301	2501	1801	3801	1203	1203
NC-17	0101	0201	1801	4403	0701	1601
NC-18	0205	3002	1801	3502	0401	0501
NC-19	0201	0301	0702	4402	0501	0702
NC-20	0201	0301	0702	3801	1203	1203
NC-21	0201	0301	0702	1801	0501	0702
NC-22	0201	2402	1801	3701	0501	0602
NC-23	2301	2402	1503	3543	0102	0210
NC-24	0201	2902	0702	4403	0702	1601
NC-25	2402	2901	3503	3503	0401	0401

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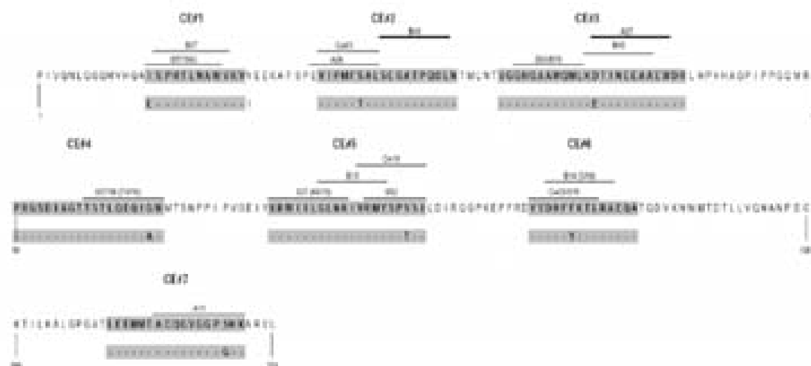
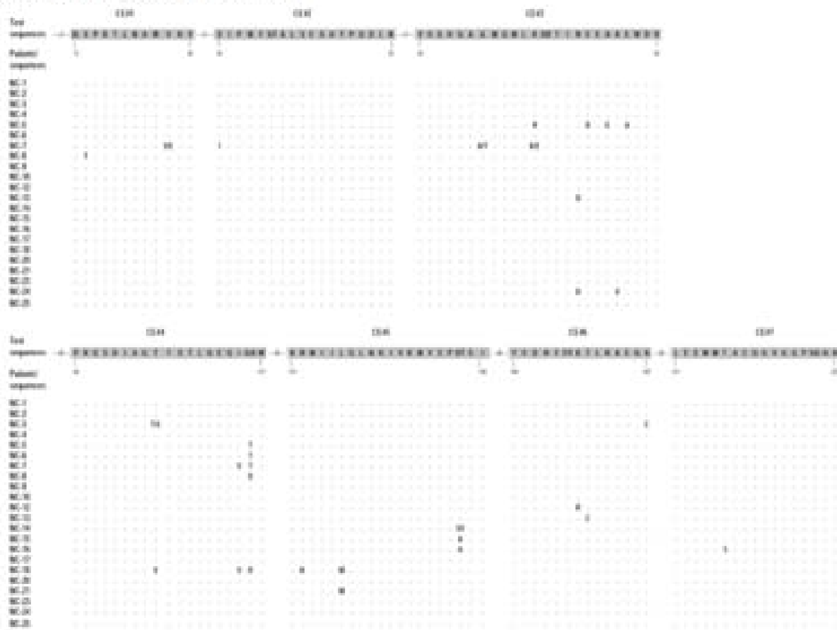
Supplementary Figure 1



Supplementary Figure 2



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ADDENDUM II

Pubmed Indexed Publications by the Author as for March 2012.

2012

-Pernas M, Casado C, Arcones C, Llano A, Sánchez-Merino V, **Mothe B**, Vicario JL, Grau E, Ruiz L, Sánchez J, Telenti A, Yuste E, Brander C, Galíndez CL. Low-Replicating Viruses and Strong Anti-Viral Immune Response Associated with Prolonged Disease Control in a Superinfected HIV-1 LTNP Elite Controller. PLoS One. 2012;7(2):e31928. Epub 2012 Feb 24. IF: 4.351 Q: 1 D: -

-Ballana E, Ruiz-de Andres A, **Mothe B**, Ramirez de Arellano E, Aguilar F, Badia R, Grau E, Clotet B, Del Val M, Brander C, Esté JA. Differential prevalence of the HLA-C -35 CC genotype among viremic long term non-progressor and elite controller HIV+ individuals. Immunobiology. 2012 Jan 8. [Epub ahead of print] IF: 3.586 Q: 2 D: -

-Fumaz CR, Gonzalez-Garcia M, Borras X, Muñoz-Moreno JA, Perez-Alvarez N, **Mothe B**, Brander C, Ferrer MJ, Puig J, Llano A, Fernandez-Castro J, Clotet B. Psychological stress is associated with high levels of IL-6 in HIV-1 infected individuals on effective combined antiretroviral treatment.
Brain Behav Immun. 2012 Jan 25. [Epub ahead of print] IF: 5.061 Q: 1 D: -

-**Mothe B**, Llano A, Ibarrondo J, Zamarreño J, Schiaulini M, Miranda C, Ruiz-Riol M, Berger C, Herrero MJ, Palou E, Plana M, Rolland M, Khatri A, Heckerman D, Pereyra F, Walker B, Weiner D, Paredes R, Clotet B, Felber B, Pavlakis G, Mullins J, Brander C. CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PLoS One. 2012;7(1):e29717. Epub 2012 Jan 4. IF: 4.351 Q: 1 D: -

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2011

-**Mothe B,** Llano A, Ibarrondo J, Daniels M, Miranda C, Zamarreno J, Bach V, Zuniga R, Perez-Alvarez S, Berger CT, Puertas MC, Martinez-Picado J, Rolland M, Farfan M, Szinger JJ, Hildebrand WH, Yang OO, Sanchez-Merino V, Brumme CJ, Brumme ZL, Heckerman D, Allen TM, Mullins JI, Gomez G, Goulder PJ, Walker BD, Gatell JM, Clotet B, Korber BT, Sanchez J, Brander C. Definition of the viral targets of protective HIV-1-specific T cell responses. J Transl Med. 2011 Dec 7;9(1):208. [Epub ahead of print] IF: 3.407 Q: 1 D: -

-Berger CT, Frahm N, Price DA, **Mothe B**, Ghebremichael M, Hartman KL, Henry LM, Brenchley JM, Ruff LE, Venturi V, Pereyra F, Sidney J, Sette A, Douek DC, Walker BD, Kaufmann DE, Brander C. High-functional-avidity cytotoxic T lymphocyte responses to HLA-B-restricted Gag-derived epitopes associated with relative HIV control. J Virol. 2011 Sep;85(18):9334-45. Epub 2011 Jul 13. IF: 5.150 Q: 1 D: -

2010

-Joseph J, Fernández-Lloris R, Pezzat E, Saubi N, Cardona PJ, **Mothe B**, Gatell JM. Molecular characterization of heterologous HIV-1gp120 gene expression disruption in mycobacterium bovis BCG host strain: a critical issue for engineering mycobacterial based-vaccine vectors. J Biomed Biotechnol. 2010;2010:357370. IF: 1.750 Q: 3 D: -

-Moltó J, Valle M, Santos JR, **Mothe B**, Miranda C, Cedeño S, Negredo E, Yritia M, Videla S, Barbanoj MJ, Clotet B. Treatment simplification to once daily darunavir/ritonavir guided by the darunavir inhibitory quotient in heavily pretreated HIV-infected patients. Antivir Ther. 2010;15(2):219-25. IF: 4.322 Q: 1 D: -

2009

-**Mothe B**, Perez I, Domingo P, Podzamczer D, Ribera E, Curran A, Viladés C, Vidal F, Dalmau D, Pedrol E, Negredo E, Moltó J, Paredes R, Perez-Alvarez N, Gatell JM, Clotet B. HIV-1 infection in subjects older than 70: a multicenter cross-sectional assessment in Catalonia, Spain. Curr HIV Res. 2009 Nov;7(6):597-600. IF: 1.978 Q: 3 D: -

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-**Mothe B**, Ibarrondo J, Llano A, Brander C. Virological, immune and host genetics markers in the control of HIV infection. Dis Markers. 2009;27(3):105-20. Review. IF: 2.026 Q: 2 D: -

-Dalmau J, Puertas MC, Azuara M, Mariño A, Frahm N, **Mothe B**, Izquierdo-Useros N, Buzón MJ, Paredes R, Matas L, Allen TM, Brander C, Rodrigo C, Clotet B, Martinez-Picado J. Contribution of immunological and virological factors to extremely severe primary HIV type 1 infection. Clin Infect Dis. 2009 Jan 15;48(2):229-38. IF: 8.195 Q: 1 D: 1

2008

-**Mothe B,** Lopez-Contreras J, Torres OH, Muñoz C, Domingo P, Gurgui M. A case of hyper-reactive malarial splenomegaly. The role of rapid antigen-detecting and PCR-based tests. Infection. 2008 Mar;36(2):167-9. IF: 2.051 Q: 3 D: -

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Inventions, Patents and Utility Models Related wiht this Thesis as of March 2012

AUTHORS: Christian Brander, Beatriz Mothe Pujadas, Anuska Llano
TITLE: Immunogens for HIV vaccination
APPLICATION NUMBER: EP12382031.8
FILING DATE: January 23rd, 2012
RESEARCH INSTITUTION: Institut Recerca de la sida IrsiCaixa, Hospital Universitari Germns Trias i Pujol.
COUNTRY: ES

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ACKNOWLEDGEMENTS/
AGRAÏMENTS/AGRADECIMIENTOS

Una amiga me aconsejó empezar a escribir la tesis por la sección de agradecimientos...

(y menos mal!)

A mi familia, por serlo TODO. Mis padres, un ejemplo de vida, de lucha, de principios, de amor. Constantes consejeros. Mi motor y mi motivación. A mis hermanos, Sebas, Salva y nuestro 'medio hermano' Víctor, tan diferentes, tan incondicional su apoyo. A mis abuelos, cuya huella es inconfundible en mis padres, tíos y primos; argentinos y catalanes. A todas las mujeres Badell, 3 generaciones de genio, tenacidad y perseverancia. A Clara, Helena, Montse y Marilia, por no poder ser mejores las 'elegidas' incorporaciones. A Ricardo y Paula. A todos los nuevos 'enanos' de la familia.

A mis mosqueperros: Ann, Pedro y Sonia. Por aguantarme, en mis días buenos y en mis días de esos tontos. Por escucharme, por dejarme escucharos, por haber compartido y seguir compartiendo tanto. Por seguir a Pastora. Porque no se puede ser mejor amigo. Ann vuelve ya, que estás mayorcita para seguir de Erasmus.

Nayra, *estel que brillava massa*… Gràcies per enlluernar-nos a tots els que et vam conèixer. No deixo d'enyorar-te.

A todos mis amigos/as y a esos 'más que amigos', viejos y nuevos, que hacen que el día a día esté lleno de nuevos descubrimientos, ilusiones y emociones. Las risas con 'la Laa', y los cines y conciertos con la Dooley (sustituta en funciones de Ann como guía del ocio de Barcelona!) Por el buen sushi y el mejor jamón, que te ayudan a recordar que la vida no es más que lo que ocurre entre buenas comidas.

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A Raquel, pon una loquera en tu vida!! Por serenarme, porque a veces no sólo puedes tirar de copas entre amigos los sábados ;) Miss you!

A tots els que van aconseguir motivar-me durant els estudis de medicina i a aquells que em van fer un primer lloc entre pipetes a la facultat en dies emboirats. A tot l'equip del Dr Ros.

To all the friends I did during my canadian clerkship.

Als companys de la 'mili' a Oviedo, 10 anys del MIR!

A tots els residents de Sant Pau; 5 anys de planta, d'urgències, d'interminables jornades i de guàrdies, en especial a les meves CoR, els més grans i els petits que vingueren darrera. A la 'Puig', a l'Esther, la 'Laiz', lo Pere i el 'Contreras'. Tot plegat, no em podia haver destetat millor en el món de la medicina.

To my first PI in Boston, Manish. To my second fellows at the Brander lab. To all the 'Harvard bunch', Esti, Judy, Christoph, Nicole, Kari, Nancy and Leah who helped me incredibly on those tough and cold days.

A la gent de Lluita, un exemple d'unitat multidisciplinar, feina ben feta, però sobretot companyerisme. Especialment a la Ginger, la Mirindi, la Pat i el Puig, senzillament uns cracks; però també a tot l'excel·lent equip de metges, estadísticA, dietistA, psicòlegs, infermeres i administratives. A l'Euge, la Pérez i el Pepe, uns sols ! Per molts més sopars de 'nenes' i divendres de sultanitos.

A Irsi, aquest territori inicialment extrany i hostil pels metges, competitiu però 'pagès' alhora, un lloc que t'atrapa i del qual no vols sortir-ne … serà la pressió negativa del p3?!?! Serà l'olor del Clotet que impregna tot espai per on passa?? Impossible nombrar a tots els que han estat aprop durant aquests darrers 4 anys, però en especial, el més sincer agraïment a tots els 'Brander': ANUSKA ! se lo merece todo y más; Javi i Jenni, Alex, Martita, Vanessa, Sandra, 立川　愛, Susana, Pep i Mireia. Al Julià, el Javier, el Paredes i tots els seus respectius equips, amb els que sempre és un plaer 'col·laborar', gracias Jorge! Al Noguera, per aguantarme uns quants bufidos i treure'm a ventilar quan percep que els cables estan aprop de fer-me un curtcircuit amb el seu sisè sentit de bioinformàtic ;) A l'equip de comunicació, al final aconseguireu que em facin pubilla a Lleida!

To all the NCI fellows at Frederick, where I had a totally unexpected wonderful experience. Cloning under napolitanean guidance could not have been greater. Thanks to Margha, Cri, Candido, Terry, Viraj and Smita. To Barbara and George to let me be part of their lab. To Amaya, Fede, Fra, Laura, Chawa, Tobi,

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Bojan, Taisuke and Luca, all charming to burn. To the NIH library, thankfully accessible for hundreds of papers these last months of tedious writing from a very good NIH-friend ;) 'no una buena amiga, lo siguiente!'

A la biblioteca del barri de St Antoni, Joan Oliver, simplement genial. Per què les retallades no afectin aquests valuosos espais. Al Calders i tots els qui el fan, per deixar-me clavar el cul uns quants vespres quan no vull tornar a pujar a casa. A la Maya i la Brenda –les Punset girls- i la Sara, membres d'honor del club de la feina a contrarellotge.

Thanks to all the coauthors of my papers and the funding agencies cited throughout this thesis; to Albert and Lourdes and their common 'lluita vs irsi-belonguing' arguments; and very specially, to the innovative HIVACAT program.

Als pacients, sense els quals, res de tot això, tindria sentit.

And specially, to my 'three men':

> To Christian, my '*just a PhD*' mentor, the smartest, most positive, enthusiastic and excellent scientist I will ever meet. A more relaxed and closer version of a Harvard-boy. I could not have been luckiest. Thanks for 'taking me' even when you had promised yourself that you would never hire an MD again. Thanks for your constant and unlimited support, teaching, guidance and stand of my too common frowning and mood swings. Larga vida!

Gràcies Ventura, per deixar-me formar part de tot això. Merci pel teu instint, per haver confiat cegament en mi des d'un principi, pel dia que et vaig dir que igual podies 'oferir-me al suís que venia per lo de la vacuna'.
Pel teu carisma, les teves ilimitades idees, canes i '*saboir faire*',
per haver creat i seguir dirigint aquest gran equip,
per inspirar-nos a tots,
per ser molt més que el 'boss', Love

I sobretot, a en Roger, etern R gran. Confident i confessor. Dels qui es riu de mi i després m'abraça quan m'atabalo perque perdré 'TRES!' dies si l'experiment no em surt. Per introduïr-me al món Clotet i presentar-me a Doña Flor.
Un humanista del segle XXI, dels que avui manquen.

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Dels pocs que creuen en el ser bona persona davant que res.
Dels qui es fascinen per Darwin quan descriu sorprès un pop al 1832,
s'emocionen amb una edició antiga de 'La Rayuela'
i creu en quelcom molt 'pitit, pitit, pitit' com les resistències minoritàries.
Thanks Mr Big Stuff!

Gràcies, gràcies per tot als tres.

Barcelona, gener de 2012.

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FUNDING AGENCIES

Rio Hortega Fellowship grant (2009-2011)

and all t

partners :

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