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Analysis of antibody neutralization specificities in Human Immunodeficiency Virus Type 2 infection

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“It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change”

— Charles Darwin

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PREFACE

The research described in this thesis was performed from January of 2008 to December of 2012 under the supervision of Prof. Doutor Nuno Taveira and the co-supervision of Prof. Doutora Helena Barroso and Prof. Doutor José Moniz Pereira.

The studies described in this thesis were performed at the Unidade de Retrovírus e Infecções Associadas – Centro de Patogénese Molecular in Faculdade de Farmácia de Lisboa. The results obtained were described in manuscripts submitted for publication or in preparation:

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Rocha C, Duarte J, Calado R, Marcelino JM, Borrego P, Palladino C, Tendeiro R, Foxall RB, Valadas E, Sousa AE and Taveira N. 2013. **Potent and broadly neutralizing antibodies are produced in chronic HIV-2 patients with marked memory B cell depletion**. (Manuscript submitted to AIDS)

Rocha C, Borrego P, Calado R, Maltez F, Barroso H and Taveira N. 2013. **X4 primary isolates of HIV-2 are less susceptible to antibody neutralization than R5 isolates**. (Manuscript in preparation)

Other Publications

Published manuscripts:

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RESUMO

O Vírus da Imunodeficiência Humana do tipo 2 (HIV-2) foi identificado pela primeira vez como agente causal da Síndrome de Imunodeficiência Adquirida (SIDA) em 1986 numa colaboração entre cientistas e clínicos portugueses (Professora Maria Odette Santos Ferreira da Faculdade de Farmácia de Lisboa e Professor José Luís Champalimaud do Hospital Egas Moniz) e franceses (equipe de investigadores liderada pelo Professor Luc Montagnier do Instituto Pasteur em Paris). Embora este vírus seja semelhante ao HIV-1 em termos de organização estrutural e genómica, muitos aspectos distinguem as infecções provocadas por estes dois vírus. O HIV-2 é apenas responsável por pequenas epidemias nos países onde teve origem e em países vizinhos ou com relações históricas com estas regiões, em oposição ao HIV-1 que é responsável pela pandemia mundial. A maioria dos indivíduos infectados com HIV-2 apresenta cargas virais indetectáveis, contagem normais de células T $CD4^+$ e ausência de progressão clínica. A resposta imunitária do hospedeiro ao HIV-2 também parece ser melhor comparativamente ao HIV-1, uma vez que a maioria dos indivíduos mantém fortes respostas celulares e humorais contra o vírus durante a fase crónica da infecção por HIV-2. Conhecer e compreender as interacções vírus-hospedeiro na infecção por HIV-2 poderá ser importante no desenho de vacinas, uma vez que as respostas imunitárias geradas contra o HIV-2 poderão ser mimetizadas na vacinação não só contra este vírus mas também contra o HIV-1.

Uma das maiores diferenças entre as duas infecções é a produção de anticorpos neutralizantes de elevado espectro e potência nos indivíduos infectados por HIV-2. Estes anticorpos e outras respostas imunitárias poderão ser fundamentais no controlo da infecção e a base para a progressão mais lenta para SIDA observada nestes doentes. Contudo, muitos aspectos da infecção por HIV-2 permanecem por esclarecer, nomeadamente como é que os anticorpos neutralizantes controlam o vírus e como o vírus reage a estes anticorpos, em particular na infecção aguda. Perante este cenário, o objectivo geral desta tese foi conhecer melhor a resposta dos anticorpos neutralizantes na infecção por HIV-2 e explorar a forma como eles influenciam a evolução genética e fenotípica do vírus.

A forma como o hospedeiro reage ao primeiro contacto com um agente infeccioso poderá ser determinante para o controlo e evolução da infecção. Desta forma, estudar a infecção primária por HIV-2 poderá elucidar sobre quais os mecanismos envolvidos no melhor controlo deste vírus comparativamente ao HIV-1. Uma vez que a maioria dos doentes infectados com HIV-2 são apenas diagnosticados na fase crónica da infecção, muito depois da seroconversão, torna-se extremamente difícil estudar a infecção aguda. Devido a este facto, ao contrário do que se passa na infecção por HIV-1, nada se sabe sobre a resposta imunitária e a evolução viral na infecção aguda por HIV-2. Esta lacuna no conhecimento sobre a infecção por HIV-2 levou ao primeiro objectivo desta dissertação: caracterizar a resposta em anticorpos neutralizantes e a evolução molecular e fenotípica do HIV-2 desde as fases iniciais e ao longo da infecção (capítulo 3). Estudar crianças infectadas por transmissão vertical constitui uma oportunidade única para conhecer a infecção aguda por HIV-2. Apesar de ser um evento raro, existem casos documentados de transmissão mãe-filho de HIV-2. Foram colhidas à nascença e ao longo de vários anos amostras de sangue de duas crianças nascidas de mães infectadas com HIV-2. A criança 1, nascida em 1998, foi diagnosticada com HIV-2, por PCR, aos 39 dias de vida e a criança 2, nascida em 1992, aos 27 dias de vida. A criança 1 nasceu com contagens de células T $CD4^+$ normais e ausência de viremia, mas aos cinco anos de idade ocorreu um aumento drástico na carga viral em paralelo com o declínio das células T $CD4^+$. Iniciou-se o tratamento, o que levou à recuperação virológica e imunológica. A criança 2 nasceu com encefalopatia apesar ter contagens de células T $CD4^+$ normais e ausência de viremia. O tratamento foi iniciado de imediato, no entanto as opções terapêuticas existentes nessa altura (1992) eram muito limitadas. A carga viral aumentou e as células T $CD4^+$ diminuíram, acabando a criança por falecer aos 9 anos. Foi extraído DNA de células de final de cultura de ambas as crianças de vários anos de infecção. O gene *env* foi amplificado, clonado e sequenciado. As sequências geradas foram analisadas em termos de diversidade genética, pressão selectiva, taxa de evolução nucleotídica e locais de glicosilação. Igualmente a partir das sequências geraram-se modelos estruturais da glicoproteina Env por *homology modelling*. Determinou-se o tropismo viral dos vírus de ambas as crianças à nascença e ao longo da infecção. Efectuaram-se ensaios de

neutralização com os plasmas das crianças dos vários anos de infecção contra os seus vírus autólogos e, no caso da criança 1, também contra 5 vírus heterólogos, três R5 e dois X4. A diversidade nucleotídica e aminoacídica e os locais sobre selecção positiva foram significativamente maiores na criança 1 comparativamente à criança 2. Da mesma forma, a taxa de evolução viral na criança 1 foi quase o dobro da criança 2 e semelhante à taxa de evolução viral em indivíduos com infecção crónica por HIV-2 a fazerem tratamento antirretroviral, de onde se conclui que a rápida evolução molecular do HIV-2 começa logo no início da infecção. Ambas as crianças foram infectadas com vírus que utilizavam o co-receptor CCR5 (vírus R5) mas aos 5 anos de idade já possuíam vírus com tropismo para células exprimindo o co-receptor CXCR4 (vírus X4). Este foi o primeiro estudo em que se observou efectivamente uma alteração de tropismo R5 para X4 na infecção por HIV-2. De salientar que a transição de tropismo observada nestas crianças foi extremamente rápida, uma vez que vírus HIV-2 que utilizam o co-receptor X4 são geralmente encontrados em indivíduos infectados há vários anos e em fases avançadas da infecção. Desde o início de vida, a criança 1 apresentou uma forte resposta em anticorpos neutralizantes, tanto autóloga como heteróloga, particularmente contra vírus R5. A resposta contra vírus X4 foi significativamente mais fraca e diminuiu ao longo da infecção concomitantemente com o aparecimento das estirpes utilizadoras do co-receptor X4. A criança 2 desenvolveu uma resposta muito fraca contra vírus X4 autólogos, que decresceu rapidamente com a progressão da doença. Em ambas as crianças, a alteração no tropismo de R5 para X4, a diversificação das regiões V1 e V3 e a conversão da estrutura secundária da região V3 para conformação em β-*hairpin* foram associadas ao escape aos anticorpos neutralizantes. Os resultados indicam que na presença de uma forte resposta dos anticorpos neutralizantes (criança 1) a diversidade e a taxa de evolução viral são muito elevadas, ao passo que quando a pressão imposta pelos anticorpos neutralizantes é menor (criança 2) estes marcadores de evolução são semelhantes à infecção aguda por HIV-1 (tanto adultos como crianças). Concluindo, estes dados apoiam a hipótese de os anticorpos neutralizantes serem responsáveis pela rápida evolução molecular e fenotípica do HIV-2 logo no início da infecção.

A produção de anticorpos neutralizantes está a cargo das células B. Após encontro com um antigénio, as células B naïve sofrem maturação e diferenciação em plasmócitos e células B de memória, capazes de produzir anticorpos específicos contra esse antigénio. Na infecção por HIV-1 e HIV-2 há diminuição acentuada do número de células B de memória. Esta depleção é irreversível mesmo com terapia antirretroviral. No entanto, e contrariamente à infecção por HIV-1, a maioria dos indivíduos infectados com HIV-2 produzem anticorpos neutralizantes durante a fase crónica da infecção. Qual será a relação entre as células B de memória e a produção de anticorpos neutralizantes na infecção por HIV-2? Conhecer, nesta infecção, qual a população de células B responsável pela produção dos elevados níveis de anticorpos neutralizantes poderá ser importante para a produção de uma vacina contra o HIV-1 e o HIV-2. Neste contexto, o segundo objectivo desta tese foi investigar a associação entre a resposta em anticorpos neutralizantes e as células B de memória na infecção por HIV-2 (capítulo 4). Foram estudados 37 indivíduos infectados com HIV-2, 73% dos quais nunca tinham recebido tratamento antiretroviral, 76% tinham carga viral indetectável e 59% tinham contagem de células T $CD4^+$ igual ou superior a 350 células/µl. Os doentes apresentavam níveis variados de depleção das células B de memória sem alteração de classe ($CD19^+CD27^+IgD^+$) e com alteração de classe ($CD19^+CD27^+IgD^-$) directamente associada à diminuição do número de células T $CD4^+$. Conhecia-se igualmente a resposta em anticorpos de ligação contra as regiões C2V3C3 e gp36 do invólucro. Os ensaios de neutralização foram efectuados com os plasmas dos indivíduos em estudo contra quatro vírus R5 heterólogos. Verificou-se que todos os doentes produziam anticorpos neutralizantes de elevada potência. Estes anticorpos eram também de largo espectro uma vez que, com duas excepções, todos os plasmas neutralizavam pelo menos dois vírus (55% neutralizavam três ou quatro vírus). O título de anticorpos neutralizantes não estava associado à contagem de células T $CD4^+$, à carga viral ou ao tratamento antirretroviral. Contudo, o título de anticorpos neutralizantes estava associado ao nível de anticorpos de ligação contra a região C2V3C3 nos doentes com contagem de células T $CD4^+ \geq$ a 350 células/µl, e aos anticorpos de ligação contra a região gp36 em doentes em estados mais avançados da doença (células T $CD4^+ <$ 350 células/ µl). Além disso, o título de anticorpos neutralizantes estava inversamente associado à depleção das células B de memória (sem e com alteração de classe) no grupo de doentes não tratados.

Os resultados obtidos sugerem que, apesar da diminuição das células B de memória com a progressão da doença, continuam a ser produzidos anticorpos neutralizantes de elevado espectro e potência ao longo da infecção por HIV-2. Estudos recentes com o HIV-1 mostraram que, apesar de serem raros os doentes que produzem anticorpos neutralizantes, a produção de anticorpos não específicos é mantida pelos plasmócitos, que não necessitam de constante exposição ao antigénio para produzirem anticorpos. Os resultados apresentados nesta tese apontam para que outra população de células B (provavelmente os plasmócitos) seja responsável pela produção e perpetuação dos anticorpos na infecção por HIV-2, com a principal diferença, face ao HIV-1, destes serem neutralizantes. Estes anticorpos são maioritariamente dirigidos contra a região C2V3C3 e, em fases mais avançadas da infecção, contra a gp36.

Estudos recentes verificaram que vírus HIV-2 com tropismo para células que expressam o coreceptor CXCR4, isolados de doentes a fases avançadas da infecção, são resistentes aos anticorpos neutralizantes. Foi também descrito no Capítulo 3 que vírus X4 isolados de crianças recentemente infectados são mais resistentes à neutralização. Estes resultados sugerem uma associação entre escape aos anticorpos neutralizantes e alteração de tropismo de R5 para X4. No entanto, permanece a dúvida se os vírus com tropismo para X4 são intrinsecamente resistentes à resposta em anticorpos neutralizantes. O terceiro objective deste trabalho foi caracterizar o fenótipo de neutralização de isolados primários utilizadores do coreceptor X4 isolados de doentes infectados com HIV-2 em várias fases da infecção de forma a determinar se todas as estirpes X4 são resistentes aos anticorpos neutralizantes independentemente do estadio da doença. Foram estudados cinco vírus X4 isolados de doentes em estados avançados de doença (mediana de células T $CD4^+$=78 células/µl) e dois vírus X4 isolados de crianças infectadas por transmissão vertical (ver capítulo 3) em fase inicial da infecção (5 anos) (mediana de células T $CD4^+$=320 células/µl). A sensibilidade dos vírus X4 à neutralização foi comparada com três vírus R5 usados como controlo (mediana de células T $CD4^+$=275 células/µl). Os ensaios de neutralização foram efectuados contra 16 isolados clínicos heterólogos. Observou-se que os vírus X4 eram significativamente mais resistentes à neutralização que os vírus R5, independentemente da fase da infecção em que

foram isolados. O facto de os vírus X4 das crianças serem também mais resistentes à neutralização que os vírus R5, demonstra que a resistência aos anticorpos neutralizantes pode surgir muito cedo após a transmissão. Além disso, os vírus X4 de indivíduos em estados avançados de doença eram significativamente mais resistentes aos anticorpos neutralizantes que os vírus X4 isolados das crianças (infecção recente), o que sugere que a resistência aos anticorpos neutralizantes é um processo gradual que vai ocorrendo ao longo da infecção. Ao analisar-se a região V3 dos vírus em estudo, detectou-se que todos os vírus X4 apresentavam as mutações nesta região anteriormente associadas a alteração de tropismo de R5 para X4 (uma mutação na posição 18, a mutação V19K/R, uma inserção na posição 24 e uma carga global igual ou superior a 7). A avaliação das estruturas secundárias desta região revelou igualmente diferenças importantes entre vírus R5 e X4. Os vírus R5 na infecção aguda e crónica são caracterizados por ausência de estrutura secundária regular, ao passo que os vírus X4 de infecção recente apresentam uma estrutura secundária em β-hairpin. Os vírus X4 de indivíduos em fase avançada caracterizam-se pelas conformações β-α-β ou helix-loop-helix. Estes dados sugerem um modelo de evolução da estrutura secundária da região V3 no qual, ao longo da infecção, a pressão exercida pelos anticorpos neutralizantes sobre a V3 força o vírus a escapar e a alterar esta região de forma que deixe de ser reconhecida pelos anticorpos neutralizantes. Estas alterações favorecem igualmente a mudança de tropismo para X4 o que por sua vez está associado ao decréscimo das células T $CD4^+$. Estes dados mostram que a resistência aos anticorpos neutralizantes é uma característica intrínseca dos vírus X4, provavelmente determinada por alterações na sequência e conformação da região V3, que dificultam o reconhecimento desta região pelos anticorpos.

Em conclusão, este trabalho permitiu demonstrar que a resposta dos anticorpos neutralizantes surge muito cedo na infecção aguda e persiste na fase crónica da infecção mesmo após significativa depleção das células B de memória, e ainda que os Nabs são responsáveis pela evolução viral, por alterações estruturais na região V3 e pela alteração do tropismo que leva à resistência à neutralização. Os resultados constituem também um novo e potencialmente relevante contributo na área das vacinas. Em primeira análise, o escape à acção dos anticorpos neutralizantes existe na infecção por HIV-2 e por conseguinte uma

vacina necessita de geral respostas contra estirpes R5 e X4. Os anticorpos são principalmente dirigidos contra a região V3 nas fases inicial e crónica da infecção. Em estádios mais avançados, a resposta contra esta região diminui mas surgem anticorpos cujo alvo é a gp36. Estes resultados confirmam que a V3 é um bom imunogénio a ser usado no desenho de vacinas mas salientam o facto de a gp36 poder conter epitopos importantes a serem incluídos numa vacina. Outra importante contribuição destes estudos foi a descoberta de que é possível manter uma forte resposta em anticorpos neutralizantes através de populações de células B que não as de memória, nomeadamente plasmócitos. Uma vacina contra o HIV-2 e o HIV-1 idealmente estimularia respostas deste tipo de células B. A dificuldade persiste em escolher o imunogénio ou grupo de imunogénios capazes de suscitar estas respostas. Destes estudos conclui-se que imunogénios baseados na região V3 e na gp36 do HIV-2 poderão ser bons candidatos para o desenho de vacinas.

ABSTRACT

Dynamics of the neutralizing antibody response and resulting HIV-2 escape during acute and chronic infections and their impact on viral evolution and disease progression remain unknown. The aims of this thesis were: characterize Nab response and molecular and phenotypic evolution of HIV-2 in early infection, investigate Nab responses in HIV-2 chronically infected patients with memory B cell imbalances and characterize the neutralization phenotype of HIV-2 X4-tropic isolates from diverse disease stages.

Broad and potent Nabs are elicited very early in HIV-2 infection, the potency of this response being associated with high evolutionary rates. Nab escape was associated with R5-to-X4 switch, increased diversity in V1 and V3 regions and changes in V3 conformation. These findings show that Nabs are the main driver of the rapid molecular and phenotypic evolution of HIV-2 in early infection.

Despite the loss of memory B cells observed with disease progression, broad and potent Nabs were elicited throughout HIV-2 infection. Nabs were found to target the C2V3C3 envelope region and, in advanced disease stage, the gp36 ectodomain. These data suggest a role for other B cell subsets in the production and perpetuation of Nabs.

HIV-2 X4-tropic viruses were found to be significantly more Nab resistant than R5 viruses (independently of disease stage) and late infection X4 isolates were significantly more Nab resistant than early infection X4 viruses. X4-tropism was associated with sequence changes and significant gain in the V3 loop secondary structure. The results prove that Nab resistance is an intrinsic feature of X4-tropic HIV-2 isolates, acquired through infection period, and is associated with amino acid and conformational changes in the V3 loop that favour R5-to-X4 switch.

In conclusion, Nab responses emerge very early in infection, persist despite memory B cells imbalances and drive tropism switch, supporting a major role for Nabs in HIV-2 evolution.

ABBREVIATIONS

µl	Microliters
^{0}C	Celsius degree
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCMI	Antibody-dependent complement-mediated inactivation
ADCVI	Antibody-dependent cell-mediated viral inhibition
AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-retroviral therapy
BSA	Bovin serum albumin
CDC	Centers for Disease Control
CO_2	Carbon dioxide
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DMEM	Dulbecco's minimal essential medium
dN	Rate of nonsynonymous substitutions
DNA	Desoxiribonucleic acid
dS	Rate of synonymous substitutions
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
FcγRs	Fc receptor
GALT	Gut associated lymphoid tissue
GM	Growth medium
GTR	General time reversible model
h	hour
HAART	Highly active anti-retroviral therapy
HIV	Human Immunodeficiency Virus

HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
IC_{50}	50% inhibitory concentration
IDU	Injection drug users
IFN	Interferon
Ig	Imunoglobulin
IN	Integrase
KDa	Kilodalton
LTRs	Long terminal repeats
mRNA	messenger RNA
MSM	Men who have sex with men
MTCT	Mother to child transmission
Nabs	Neutralizing antibodies
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	Protease
PT	Portuguese patients
RNA	Ribonucleic acid
RT	Retrotranscriptase
SIDA	Síndrome de Imunodeficiência Adquirida
SIV	Simian Immunodeficiency Virus
SIV_{cpz}	SIV from *Pan troglodytes troglodytes* chimpazees
SIV_{gor}	SIV from Western lowland gorillas
SIV_{smm}	SIV from *Cercocebus torgnatus atys sooty* mangabeys
SU	Surface glycoprotein
$TCID_{50}$	50% tissue culture infectious dose
TLR	Toll-like receptor
TM	Transmembrane glycoprotein

TNF	Tumor necrosis factor
USA	United States of America
VSV	Vesicular Stomatitis Virus

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CHAPTER 1

General Introduction

The origin and discovery of the Human Immunodeficiency Virus type 2 (HIV-2)

Immunodeficiency viruses, belonging to the *Lentivirus* genus, can be found in several species including non-human primates [1, 2]. These simians are the natural reservoir of many different specific variants of Simian Immunodeficiency Virus (SIV). Several independent zoonotic transmission events lead to the introduction of these viruses to humans. Human Immunodeficiency Virus type 1 (HIV-1) was introduced to humans from SIV_{cpz} that infects West Central African chimpanzees (*Pan troglodytes troglodytes*) and from SIV_{gor} that infects Western lowland gorillas (*Gorilla gorilla gorilla*) [1-6]. HIV-2 is closely related to SIV_{sm} which is found in sooty mangabey monkeys (*Cercocebus atys atys*) [7, 8]. Phylogenetic analysis shows that there have been many cross-species transmissions to humans, with each successful event resulting in a specific form (group) of HIV [1, 9, 10]. In HIV-2 transmission events are thought to be at least eight, giving rise to groups A to H [8, 11], whereas in HIV-1 tree cross-species events occurred from chimpanzees to humans (groups M, N and O) [12, 13], and one from gorillas (group P) [5, 6]. Transmission from simians to man is estimated to have occurred in the late nineteenth through early twentieth century but its consequences have only be detected in 1981 for HIV-1 and 1985 for HIV-2 [14-16]. Indeed, the Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 in the United States of America (USA), after the observation of opportunistic infections along with immune suppression in young men who have sex with men (MSM) in New York City and California [17, 18]. Soon after similar observations were made in patients from Haiti, Africa and Europe [19-21] and it became clear that the new disease with unknown cause struck also haemophiliacs, injection drug users (IDU), women and infants (mother to child transmission - MTCT) [22-25]. In May 1983, at the Pasteur Institute (Paris), Luc Montagnier and Françoise Barré-Sinoussi isolated a new retrovirus from an AIDS patient [26] and in the following year a similar retrovirus was isolated by American investigators [27]. These findings proved that this retrovirus, later classified as HIV-1, was the causative agent of AIDS [28].

In the early 80's, patients from Guinea-Bissau and Cape Verde Islands were admitted in a Portuguese hospital in Lisbon. These patients presented clinical symptoms similar to AIDS

but with constant negative serologic tests. At the time Maria Odette Santos Ferreira from the Faculty of Pharmacy of Lisbon took the blood samples from these patients to Luc Montagnier at the Pasteur Institute (Paris). The collaboration between Portuguese and French researchers lead to the characterization of a second retrovirus, distinct from HIV-1, classified as HIV-2 [29-31].

The global spread of HIV-2

According to UNAIDS, globally there has been a decline the number of newly infected adults and children in the last 10 years [32]. With the scale up of antiretroviral therapy (ART) over the past few years, the number of AIDS-related deaths has also decreased, but with the significant reductions in mortality the number of people living with HIV worldwide has increased. By the end of 2011, there were an estimated 34 million people living with HIV, 2.5 million newly infected adults and children and 1.7 million AIDS-related deaths [32]. Sub-Saharan Africa still accounts for the highest HIV burden, with 69% of the total number of HIV infected individuals and an average prevalence among adults of 4.9%. However, in 23 Sub-Saharan countries, the incidence of HIV has decreased more than 25% [32].

Despite the existence of eight HIV-2 groups, only groups A (HIV-2A) and B (HIV-2B) are considered endemic, with group A frequently found in the western part of West Africa (Guinea-Bissau, Senegal, The Gambia, Ivory Coast and Cape Verde) and group B more restricted to Ivory Coast and Ghana [11, 29, 33-38]. The remaining groups have only been identified in a few individuals from Sierra Leone, Liberia and Ivory Coast [7, 39-45]. The first HIV-2 A/B recombinant was isolated from a patient from Ivory Coast and recently three other A/B recombinants have been described in Japan, which lead to the classification of the first circulating recombination form (CRF) of HIV-2, the HIV-2 CRF01_AB [43, 46, 47].

Besides its confined geography, recent reports indicate that HIV-2 prevalence is now decreasing even in countries where the number of cases used to be high [48-52]. For instance, in Guinea-Bissau the prevalence of HIV-2 decreased from 8.3% in 1990 to 4.7% in 2007, and in The Gambia it decreased from 7.0% in 1988-91 to 4.0% in 2001-03. The prevalence of HIV-1 increased in these regions in the same period of time [49, 51, 52].

The origins of HIV-2 groups A and B are estimated to be around 1938 and 1945, respectively [15, 16, 53]. The epicentre of the HIV-2B epidemic is most likely Ivory Coast, whereas some doubts remain about the region where the cross-species event took place for HIV-2A. There is strong phylogenetic evidence that trace both Ivory Coast and Guinea-Bissau as the epidemic centres: serologic data tends to favour Guinea-Bissau, but the recent discovery of SIVsmm strains closely related to HIV-2A in faecal samples from sooty mangabeys in Ivory Coast suggests that this region is also the epicentre of HIV-2A [8, 49, 53, 54]. Whether the cross-species event took place in Ivory Coast and Guinea-Bissau was where the epidemic was established very early after transmission or Guinea-Bissau is indeed the epicentre of HIV-2A remains to be clarified [8, 53, 55]. From these two countries, or because of commercial relations, sex trade and migration between them, HIV-2A spread to other countries in West Africa, like Senegal, The Gambia, Cape Verde, Nigeria and Burkina Faso [53]. The viral migration outside West Africa most likely happened through immigration and socio economic connections with high-prevalence countries. For instance, past relations between France and Ivory Coast and Senegal led to multiple viral introductions in France. Furthermore, HIV-2A is thought to have spread from Guinea-Bissau and Cape Verde to Portugal, mainly during the independence war [15, 53, 56]. Within Europe, strong evidences point to transmission from Portugal to the main immigration destinations, like Luxemburg and the United Kingdom (UK), but most likely to Switzerland, Belgium and Germany as well [53, 56]. Portugal is also thought to be responsible for transmitting HIV-2A outside of Europe to other countries with socio economically linkage such as India, Mozambique and Brazil [11, 50, 57, 58].

Portugal is one of the few countries outside West Africa with a significant number of HIV-2 cases. In December 2011 the total number of AIDS cases associated with HIV-2 was 527, which represents 3.1% of the total number of AIDS cases [59]. Most of the cases (73.1%) were associated with heterosexual transmission and were in individuals with ages between 35 and 54 years (60.4%). Parenteral transmission through blood transfusions or surgical procedures during the independence ward against Guinea-Bissau (between 1960 and 1974) might have been an important transmission route since sexual transmission of HIV-2 is less efficient than for HIV-1 [60], and multiple exposures to HIV-2 might be necessary to facilitate

infection [61, 62]. The number of new diagnosed infections has been decreasing in the last ten years (from 23 new infections in 2001 to 8 in 2011) [59]. Several studies addressed HIV-2 epidemic in Portugal over the past few years. Gomes *et al* found that in Lisbon and the Southern part of the country, between 1997 and 2002, 57% of infected patients were from either Guinea-Bissau or Cape Verde and the incidence was similar between genders [61]. A few months later, Mota-Miranda and collaborators performed a similar study in the North region of Portugal, with data from 1985 to 2003, and concluded that 95% of infections were among Portuguese individuals but in 51% of the cases a connection with West Africa was established [63]. Another study performed in a Lisbon hospital between 1987 and 2006 showed a majority of infections in patients from West Africa (67.5%) and predominantly in women (66.9%) [64]. More recently, a large study involving several hospitals across the country, with data from 1985 to 2007, detected a mobility pattern of the epidemic before and after the year 2000. In the beginning most infected patients were Portuguese men, probably due to the return of Portuguese soldiers after the independence war in the late 70's. After 2000 a change was observed towards women of West African origin, most likely because of the increase in migration from West Africa to Portugal in the late 90's [62].

Biology of HIV-2

Genome and structure

HIV-2 is a spherical enveloped virus with a diameter of approximately 110 nm (Figure 1.1) [65-67]. The envelope comprises a lipid bilayer derived from the host cell plasma membrane at budding, and therefore may also contain some host cell proteins from the human leukocyte antigen (HLA) system class I and II. Embedded in the lipid bilayer is the transmembrane glycoprotein (TM) bound non covalently to the outer surface glycoprotein (SU), this complex is arranged in trimers. Internally the viral particle is coated by the matrix proteins, essential to stabilize the spherical structure. Within the matrix resides the cone shaped capsid which contains two copies of a positive sense single stranded RNA associated with the nucleocapsid proteins. Inside the capsid there are also all the necessary enzymes to

viral maturation and early phases of replication, such as protease (PR), reverse transcriptase (RT) and integrase (IN) and the accessory proteins Nef, Vif, Vpr and Vpx [65-67].

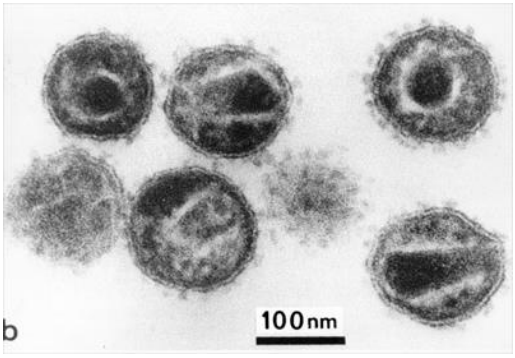

Figure 1.1 - Electron Micrograph of HIV. The virus is more about 110 nm wide. (Adapted from http://www.histology.leeds.ac.uk)

Each RNA molecule is about 9800 nucleotides long and is flanked by long terminal repeats (LTRs) at both ends (5' and 3'). Nine genes are encoded in the compact genome, tree of them encode for structural or enzymatic proteins (*gag*, *pol* and *env*), two for regulatory proteins (*tat* and *rev*) and four for the mentioned accessory proteins (*nef*, *vif*, *vpr* and *vpx*) (Figure 1.2). The three open reading frames are used to translate all the proteins [65, 66, 68]. The *gag* encodes the polyprotein precursor $Pr55^{Gag}$ that is then cleaved into the proteins p26 (capsid), p16 (matrix), p6 (nucleocapsid) and p6 (C-terminal protein) by the viral PR. The Gag proteins are essential for virion assembly and release. The *gag* and *pol* genes produce a $Pr160^{GagPol}$ precursor polyprotein, which is then processed by the viral PR. The *pol* encodes for tree enzymes necessary for replication: the RT (p53), the PR (p11) and the IN (p34). The *env* gene encodes for the polyprotein precursor $Pr140^{Env}$, cleaved by PR in the glycoproteins SU (gp125) and TM (gp36). These glycoproteins are essential for viral attachment and fusion to the host cell membrane [65, 66, 68].

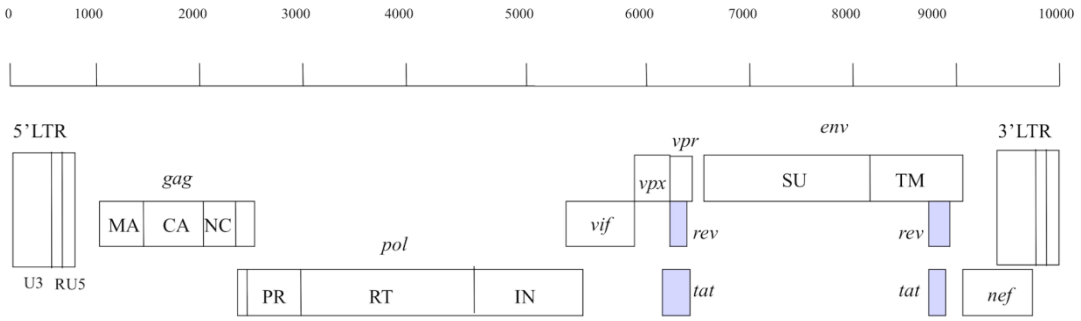

Figure 1.2 – Genomic organization of HIV-2. (Adapted from Taveira *et al*, Manual sobre SIDA 2011 [65])

Life cycle

HIV-2's life cycle usually begins with the binding of the virus to a specific receptor, the CD4, present in the cellular surface of the host cell [T-lymphocytes, monocytes, macrophages, dendritic cells (DC) and brain microglia] (Figure 1.3) [65, 68]. Besides CD4, other molecules expressed in the cell surface are essential to the interaction between virus and host cell. These are the chemokine receptors and work as coreceptors for HIV entry in the host cell [65, 68].

After entry and uncoating of the virus in the cytoplasm, RT starts reverse transcription of the viral RNA into double stranded DNA, that together with the proteins IN, RT, matrix and Vpr form the pre-integration complex. The translocation of this complex to the nucleus is mediated by IN and Vpx [69]. Once in the nucleus the IN integrates the viral DNA into an open region of the host chromosomal genome. The proviral DNA can remain latent (transcriptionally silent) in the host cell or be immediately transcribed by the cellular machinery continuing the virus life cycle [65, 69, 70].

The transcription of the proviral DNA is mediated by the promoter region within the 5' LTR and originates tree classes of messenger RNA (mRNA): completely spliced mRNA or early transcripts (Rev, Tat, Nef), incompletely spliced mRNA or late transcripts (Env, Vif, Vpr and Vpu/Vpx) and unspliced mRNA or late transcripts (precursor polyproteins Gag and Gag-Pol). All these mRNA are later incorporated in the viral particles as genomic RNA. Early transcripts are transported outside the nucleus like any cellular RNAm and are needed to complete the expression of late transcripts. Rev is responsible for the transport of late transcripts. This

protein binds to the Rev responsive Element (RRE) in the RNA *env* region and carries the late transcripts to the cytoplasm to be translated [65, 70].

The Env precursor polyproteins are translated and glycosylated before they oligomerize in trimers. Then the polyproteins are cleaved in SU and TM glycoproteins and transported to the cytoplasmic membrane. The viral RNA and proteins are directed to the cellular surface, where new immature viral particles are formed and released by gemmulation of the cytoplasmic membrane, thus acquiring the lipid envelope containing the SU/TM trimers. Final maturation occurs outside the cell with the cleavage of the precursor protein Gag-Pol by PR and posterior structural rearrangement and repositioning of the viral proteins, giving rise to mature and infectious viral particles [65, 70].

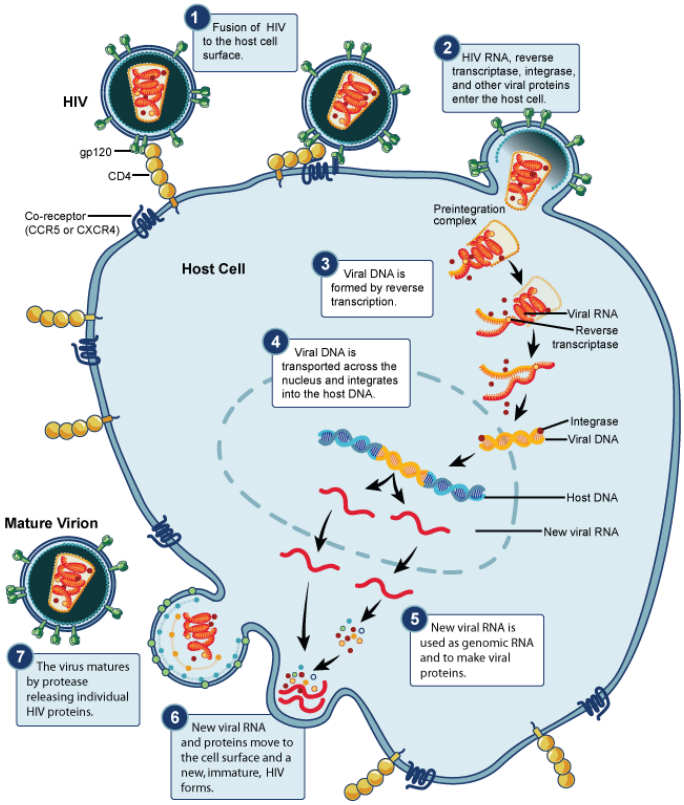

Figure 1.3 – The life cycle of HIV. (Adapted from www.niaid.nih.gov)

HIV-2 envelope

Molecular and structural organization

As mentioned above, the envelope glycoproteins SU and TM are encoded by the *env* gene, and are responsible for the fusion between viral and host cell membranes, allowing the release of the viral capsid into the host cell cytoplasm. The SU and TM glycoproteins are associated by non covalent bounds forming trimers or spikes [71, 72]. HIV-2 spikes have been reported to be more prominent and stable after budding [73-75], whereas in HIV-1 they drop immediately after budding and during maturation [71, 76].

Despite the high variability of SU, some structural and functional elements are conserved, which allowed the classification of five hypervariable regions (V1 to V5), separated by five more conserved regions (C1 to C5). This glycoprotein has a complex secondary structure, with variable regions V1 to V4 forming loops stabilized by disulphide bridges. In its native conformation, SU has two domains, one internal and one external [77]. The external domain is highly glycosylated, has most of the antigenic determinants, including neutralizing epitopes, and is involved in the interaction between the SU and the cellular receptor and coreceptors. The internal domain is hydrophobic and is essential for the SU and TM association. Connecting the external and internal domains there is a smaller domain designated bridging sheet [65, 72, 77, 78].

The TM glycoprotein is divided in one extracellular ectodomain, one transmembrane region (insertion in the host cell membrane) and one intracytoplasmatic domain. The ectodomain has several domains common to other fusion proteins: a hydrophobic region rich in glycines at the N-terminal end called fusion peptide, followed by two α-helices containing leucine-zipper motifs designated heptad repeat 1 (HR1) and 2 (HR2). The fusion peptide is essential for attachment to the host cell membrane. The HR motifs present repeated patterns of seven amino acids, being the first and fourth residues hydrophobic, mainly leucines. These motifs are arranged in a thermostable structure in sextuple helix, formed by trimers of HR1 and HR2. This structure is directly involved in the fusion to the host cell. The

intracytoplasmatic domain mediates the binding of the envelope to the matrix protein, necessary for the maturation of new viral particles [65, 72, 77, 79].

Interaction between the Env protein and the cell

The entry of HIV into the host cell generally involves three steps mediated by the envelope glycoproteins: binding of the SU to the CD4 receptor, binding of the SU to the coreceptor and fusion of the viral envelope with the host cell membrane (Figure 1.4).

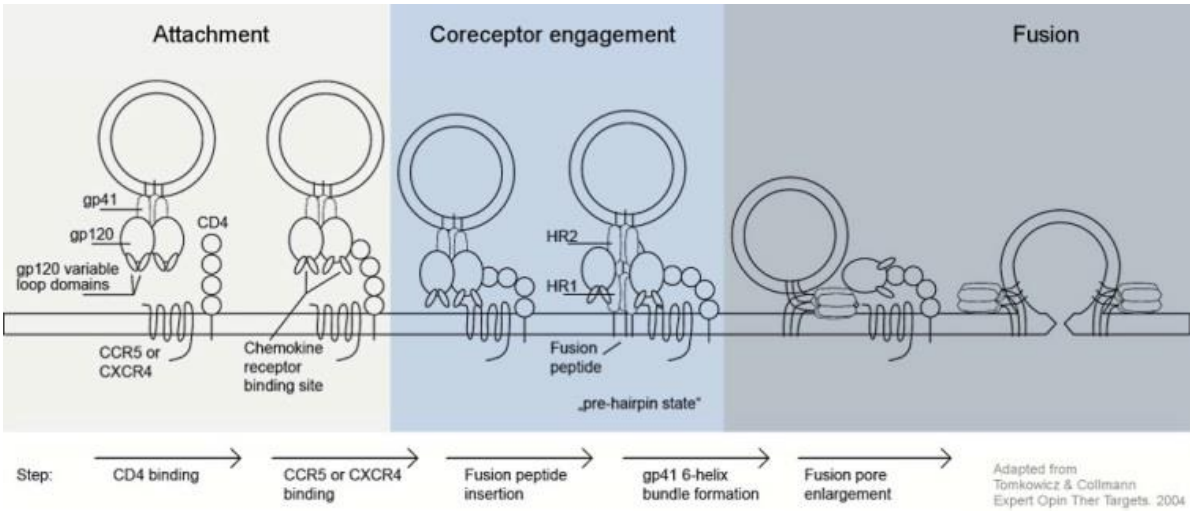

Figure 1.4 – Model of the multi-step process of HIV entry. (Adapted from http://www.virology.uzh.ch/)

The process begins with the interaction between the external domain of the SU and the CD4 receptor. After binding to the CD4, SU suffers major conformational changes, with the formation of a bridging sheet and increased exposure of V1, V2, V3 and C4 regions (Figure 1.5) [65, 72]. The folding of the bridging sheet is necessary to form the conserved part of the coreceptor binding site and the repositioning of the V1/V2 loop is thought to uncover this site [80]. All these rearrangements allow the stabilization of the SU-CD4 bond and leads to an approximation between the viral envelope and the host cell membrane and consequent interaction with the coreceptor [65, 72, 79, 81, 82]. Some HIV-2 primary isolates are known to infected cells independently of CD4, which implies that the coreceptor binding site in these isolates is already formed or exposed prior to the CD4 binding [82, 83]. Therefore it is

thought that the V3 region in some HIV-2 has a more open and exposed conformation, which allows it to induce conformational changes in the V1/V2 loop during fusion without the need to previously bind to the CD4 [82, 84, 85]. The SU-CD4 binding also induces changes in the TM glycoprotein: the fusion peptide becomes exposed and is inserted in the host cell membrane, and HR1 and HR2 fold in an antiparallel form, originating a six-helix bundle. The viral envelope and the host cell membrane are brought together during this process, leading to the formation of the fusion pore, allowing the entrance of the viral capsid into the cytoplasm of the target cell [65, 86, 87]. After CD4 binding, the exposure of the coreceptor binding site seems to be faster in HIV-2, dues leading to a more rapid fusion between the viral envelope and the host cell membrane, despite the lower affinity of gp125 (HIV-2) to the CD4 receptor compared to gp120 (HIV-1) [85, 88]. This faster exposure of the coreceptor binding site might be due to differences in orientation of the V1/V2 loop and folding of the bridging sheet, as mentioned above [80].

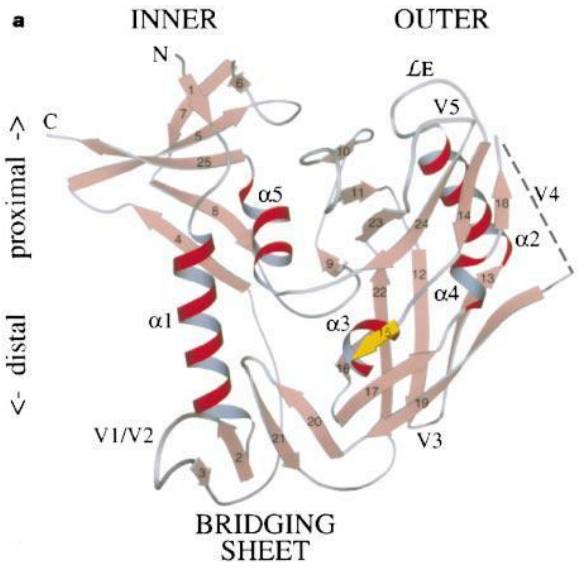

Figure 1.5 – Secondary Structure of gp120 after CD4 binding. (Adapted from Kwong *et al*, Nature 1998 [77])

HIV-2 coreceptor usage and pathogenesis

In vivo, the main coreceptors of HIV are the CCR5 and CXCR4. However, HIV has been shown to use other coreceptors [89, 90]. HIV's tropism to cells expressing one of the coreceptors is related with the replication capacity of the virus in different cell lines. Viruses that infect preferentially macrophage cell lines, without the capacity to induce syncytia and with low/slow replication rates typically use the CCR5 coreceptor and are called R5 viruses. On the other hand, viruses that infect mainly lymphocytic cell lines, with the ability to induce syncytia and with high/fast replication rates, are named X4 viruses and use the CXCR4 coreceptor. Finally, R5X4 viruses are variants with identical capacity of replicating in macrophage and lymphocytic cell lines and use indifferently the CCR5 and the CXCR4 coreceptors [91-94].

HIV-2, like HIV-1, uses mainly CCR5 and/or CXCR4 as coreceptors to enter $CD4^+$ T lymphocytes. However, many HIV-2 isolates can use a wider variety of alternative coreceptors (like CCR1, CCR2, CCR3 and CCR8), though less efficiently than CCR5 and CXCR4 [95]. The use of a broader range of coreceptors does not seem to be related to HIV-2's pathogenicity and the relevance of using alternative coreceptors *in vivo* is not clarified [83, 89, 91, 95, 96].

In HIV-2 infection, R5 viruses are isolated from asymptomatic patients or in early stages of disease, and X4 variants are found in advanced AIDS [97, 98]. The emergence of X4 variants in HIV-2 infection seems to be related with escape from neutralizing antibodies (Nabs) directed against the V3 region since X4 viruses are more resistant to neutralization [97]. In HIV-1 infection, R5 viruses are also found in acute and asymptomatic phases. In 50% of infected patients, the viruses evolve to an X4 phenotype with a rapid decline of $CD4^+$ T cells and progression to AIDS. However, R5 viruses can also be responsible for $CD4^+$ T cell depletion and persist in advanced disease. Contrarily to HIV-2, R5X4 and X4 HIV-1 variants seem to be more sensitive to Nabs, which might explain why the phenotype change does not always occur, and when it does it's in a stage of immune system failure [93, 99-101].

The major determinants for CCR5 or CXCR4 coreceptor usage by HIV-2 are located in the C-terminal region of the V3 loop. An increase in charge on this region, by the presence of

positively charged amino acids is correlated with CXCR4 phenotype [84, 102-104]. The most relevant residues are in positions 18, 19 and 24 [104]. In HIV-1 the glycosylation pattern of V3 and V1/V2 regions also seem to influence the coreceptor phenotype [105-108]. The degree of glycosylation is lower in HIV-2 but the impact on coreceptor usage is still elusive since no clear association has been made between glycosylation of V1/V2 or V3 and coreceptor phenotype of HIV-2 [84, 109].

Transmission

HIV spreads through sexual contacts, contaminated blood or blood products (medical injections, blood transfusions and injection drug usage) and from mother to child (pregnancy, delivery and breast feeding) [57]. The most common route of transmission of HIV-2 is through heterosexual contact, as is for HIV-1, but transmission rates are 3 to 6 folder lower [60]. Similarly, MTCT is rare in HIV-2 with a rate below 5% compared to almost 25% in HIV-1 in untreated pregnant women [110-113]. A Portuguese study found a MTCT rate of 1.5% for HIV-2 and 3.4% for HIV-1 between 1999 and 2005 and transmission was associated with absence of ART [114]. The reasons for the reduced transmission rates of HIV-2 are still not fully understood, but are probably linked to the lower plasma viremia [49, 110] and reduced viral shedding in the genital tract seen during HIV-2 chronic infection [115, 116]. A study from The Gambia found a 37-fold difference in plasma RNA levels between HIV-2 and HIV-1 infected untreated pregnant woman (410 *versus* 15,100 RNA copies/ml) [110]. On the other hand, a study in Ivory Coast observed that 24% of HIV-1 infected women had detectable RNA in vaginal secretions compared to only 5% of HIV-2 infected pears [117]. Detectable RNA in the semen is also lower in HIV-2 compared to HIV-1 infected men ($2.6\log_{10}$ *versus* $4.4\log_{10}$ RNA copies/ml) [115].

Despite being a rare event, HIV-2 MTCT has been document in both epidemiologic [111, 112, 118-122] and molecular studies [123-125]. There is evidence that the survival rate of HIV-2 infected is higher than their HIV-1 infected pears [126]. In fact, contrarily to HIV-1 infected children [127-129], disease progression and the first clinical manifestations might take many years to arise in HIV-2 infected infants [122, 130]. Furthermore, and also in opposition to

HIV-1 [127, 128], maternal plasma viral load does not seem to determine disease progression [126]. In HIV-1 infection, immaturity of the children's immune system is thought to account for the fast disease progression, though time of transmission (intrauterine, intrapartum or postpartum) also seem to have a major influence [127, 128]. There is no information about immune responses in MTCT of HIV-2.

Pathogenesis of HIV-2 infection

The course of HIV infection can be divided into three stages: the acute phase, the chronic phase and AIDS [131]. The acute phase, or primary HIV infection, lasts four to eight weeks and is characterized by intense HIV replication and massive loss of $CD4^+$ T cells mainly in gut associated lymphoid tissue (GALT). When transmission occurs through the blood (transfusion, IDUs or MTCT) the virus is probably removed from circulation by the reticuloendothelial system of the spleen, liver and lungs, with consequent infection of lymphoid tissue , HIV replication and dissemination within these organs [132]. Infection is also possible through rectal and genital mucosa (heterosexual and homosexual contacts). In this case DCs seem to have a central role in capturing and transporting viruses to draining lymph nodes and secondary lymphoid tissue where high levels of activated $CD4^+$ T cells are present [131, 132]. $CD4^+$ T cells depletion is a consequence of direct viral infection, activation induced cell death and host cytotoxic responses [133]. As a result of the immune responses directed against the infection, infected individuals experience in this phase flu like symptoms (acute HIV syndrome), including fever, body ache and lymphadenopathy [132]. Viral load, or plasma viremia, usually peaks at three to four weeks after infection but is eventually suppressed to a semi steady state level (viral set point) due to HIV-specific cytotoxic T lymphocytes (CTL) and Nabs. At this point $CD4^+$ T cells are partially restored. The viral setpoint is also dependent on the individual genetic background and is an important determinant of disease progression in HIV-1 infection [38, 132]. In HIV-2 the viral set point is usually much lower compared to HIV-1 (2500 *versus* 70000 RNA copies/ml) [134].

During this acute phase and early chronic phase, HIV-1 infected patients can be categorized into Fiebig stages I to VI based on a stepwise gain of positivity in clinical diagnosis assays

(Figure 1.6). The first HIV infection marker to be detected is viral RNA that can be assessed by polymerase chain reaction (PCR). The HIV-1 p24 (from *gag*) usually peaks 20 days after infection and can be detected by enzyme-linked immunosorbent assay (ELISA). Afterwards, it is possible to detect specific antibodies by ELISA and western blot and finally HIV-1 p31 is also detectable by ELISA (Figure 1.6) [135].

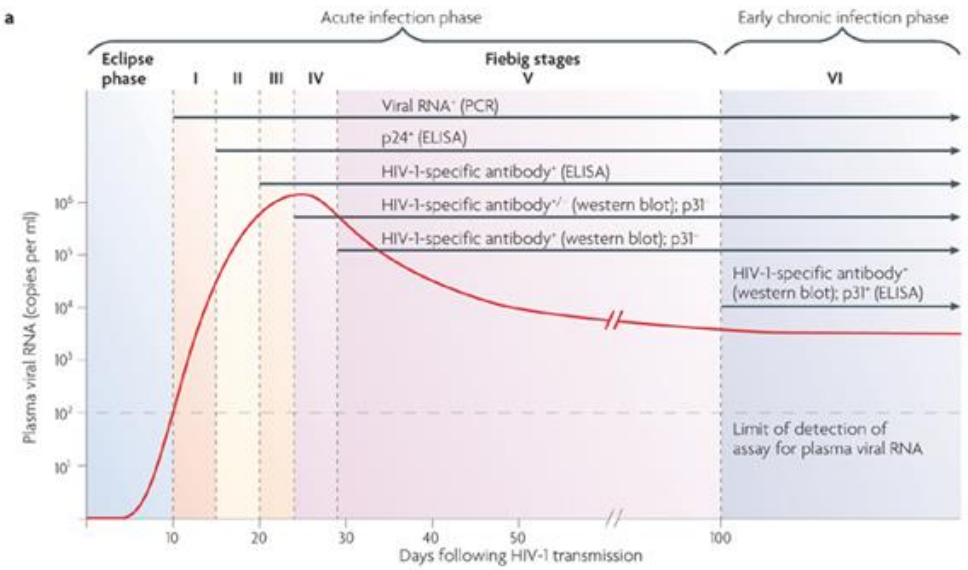

Figure 1.6 – Fiebig clinical stages of HIV-1 infection. (Adapted from McMichael *et al*, Nature Reviews Immunology 2010 [136])

The chronic phase is asymptomatic and in untreated patients lasts about ten to 25 years in HIV-2 and is much faster in HIV-1 (eight to ten years) [132, 137]. At this stage, infected patients usually present generalized lymphadenopathy caused by persistent follicular hyperplasia. This period corresponds to clinical latency with low but persistent HIV replication in the viral reservoirs (lymphoid tissue) and constant antigen stimulation that drives immune activation [132]. Chronic immune activation is manifested by increased cell turnover, abnormal activation and differentiation of lymphocytes, increased terminal differentiation of B cells and increased activation-induced apoptosis of $CD4^+$ T cells, $CD8^+$ T cells and B cells, thus leading to cellular exhaustion, senescence, and low renewal potential [131, 132]. HIV-2 infection is characterized by a lower state of immune activation, which

might account for the slower disease progression [138-141]. Immune activation, however, does not seem to be directly linked to viral load in HIV-2 infection, since several studies reported that, despite the absence of detectable viremia, levels of immune activation were the same in HIV-1 and HIV-2 infected individuals when patients were matched for the same degree of CD4 depletion [138, 141].

In the late phase of the infection, the immune system slowly gets exhausted by the chronic immune activation and depletion of $CD4^+$ T cells and eventually collapses. This leads to occurrence or reactivation of opportunistic infections (like tuberculosis, candidiasis and pneumonia) and virus induced tumours (Epstein-Barr virus related lymphomas, Kaposi's sarcoma and cervical cancer caused by Human Papillomavirus). These diseases mark the onset of AIDS. The Centers for Disease Control (CDC) in the USA periodically revises the list of clinical situations that define this stage. Another criterion that defines AIDS is the drop of $CD4^+$ T cell levels below 200 cells/µl of plasma [132].

As mentioned above, HIV-2 infection is characterized by a slower disease progression, longer survival and reduced mortality rates compared to HIV-1 [57, 137, 142-147]. In fact, most HIV-2 infected patients have normal $CD4^+$ T cell counts, undetectable viral loads and absence of clinical disease [57, 134, 137, 139, 148]. Several studies showed that the probability of being AIDS free was near 100% in HIV-2 up to five years post seroconversion compared to 67% in HIV-1 infected patients [142, 149]. Mortality rates were estimated to be 2.5 to 3.5-fold lower per year of follow-up in HIV-2 compared to HIV-1 infection [147, 150]. Another important difference between the two viruses is the lower replication capacity of HIV-2 [151, 152], despite similar proviral loads at the same disease stage [137, 139, 153, 154]. With disease progression, most immunological differences between HIV-1 and HIV-2 are lost, the level of immune activation is the same and $CD4^+$ T cell depletion is similar [57, 137, 138, 145-147]. Nonetheless, after the onset of AIDS, median time to death in HIV-2 infection was twice of that in HIV-1 (12.6 *versus* 6.3 months) and mortality rates were lower (52% *versus* 87% per year of follow-up in HIV-2 and HIV-1 infected individuals, respectively). When patients were adjusted for $CD4^+$ T cell counts, mortality rates were similar in HIV-2 and HIV-1 infected individuals (86% *versus* 130% in HIV-2 and HIV-1 infected patients with $CD4^+$ T counts bellow

200 cells/ml; 62% *versus* 73% in patients with $CD4^+$ T counts equal or above 200 cells/ml) [147].

HIV-2 molecular evolution

Mechanisms of viral evolution

HIV and other RNA viruses shares common characteristics that make them good models to study evolution: high mutation rates, small genomes, large population sizes, short generation times and high number of offspring [155, 156]. Due to these factors HIV is one of the fastest evolving organisms, with an exceptionally high mutation rate of $2.4x10^{-5}$ mutation/replication in HIV-1 [156]. It is worth mentioning that a recent study with Portuguese and Swedish patients found that the rate of nucleotide substitutions in HIV-2 gp125 and V3 is higher than in HIV-1 when patients were matched for disease stage and CD4 dynamics ($10.20x10^{-3}$ *versus* $6.40x10^{-3}$ substitutions/nucleotide/year in gp125 and $29.37x10^{-3}$ *versus* $12.36x10^{-3}$ substitutions/nucleotide/year in V3) [157]. These mutations include substitutions, insertions and deletions and can appear during several steps of the replication cycle. Most of the point mutations are generated during reverse transcription due to the lack of 3'-5' exonuclease activity of the RT enzyme [158, 159]. In addition, host enzymes may contribute to the mutation process, particularly the host APOBEC3 family cytidine deaminases that induce G-to-A hypermutations in retroviral RNA [160, 161]. When a mutation in a gene passes on to the offspring and coexists with the original form is called a polymorphism. In a population, two or more variants circulate simultaneously at a polymorphic site. In an HIV infected individual, the viral population consists of a pool of closely related variants known collectively as quasispecies [162].

The second major contribution to HIV genetic diversity is recombination between different viral variants. During one replication cycle, an estimate of $4.9x10^{-4}$ and $3.6\ x10^{-4}$ recombinations per site can happen in HIV-2 [163] and HIV-1 [164, 165], respectively. When a cell is infected by two different strains, the newly produced virions may comprise a heterodimeric DNA, meaning an RNA molecule from each strain. Once a new cell is infected

by these virions, a recombinant proviral DNA with a mosaic genome will be generated due to the template switch of the RT enzyme during reverse transcription. As a consequence, large evolutionary and antigenic leaps may occur in just one round of replication [159, 165, 166]. Despite the high recombination rate seen in HIV-2, contrarily to HIV-1, CRFs are rare (for a review on HIV-1 CRFs see references [167] and [168]). As mentioned above, only one CRF has been described so far, the CRF01_AB [43, 46, 47]. The geographic restriction of the infection is probably a major constrain to the development of CRFs in HIV-2. Due to the simultaneous prevalence of both HIV-1 and HIV-2 infections in some West African countries and Portugal, there were concerns that an HIV-1/HIV-2 recombinant could emerge and produce an even more aggressive virus. In fact, recombination between the two viruses has been demonstrated to be possible *in vitro* [169]. However, *in vivo*, there are important differences between HIV-1 and HIV-2 in the signals and mechanisms of genomic RNA packing that impede recombination between the two viruses [170-173].

The high replication and production rates combined with the ability to mutate and recombine are the bases for the high diversity of HIV; however there are other selective forces (natural selection) and chance events (genetic drift) that shape viral evolution [162, 174]. Natural selection is a highly deterministic evolutionary process that promotes the elimination of deleterious mutations by reducing their incidence in the population (negative selection) and favours the fixation of beneficial mutations by increasing their frequency (positive selection). A mutation is deleterious if it decreases the ability of the virus to survive and/or reproduce in the present environment (fitness), beneficial or advantageous if it increases viral fitness compared to the wild type and neutral if it has no significant effect. Neutral mutations are not affected by natural selection. So, whenever a new mutation is generated it becomes either fixed or is eliminated from the population accordingly to the way it affects viral fitness in that given environment [162, 174, 175]. In HIV-2 and HIV-1 infections, natural selection is a major driver of molecular evolution, due to the strong selective pressures imposed by the host's immune system, mainly Nabs over the *env* gene [176-180].

Mutation frequencies are affected not only by natural selection but also by random genetic drift [162, 174]. The latter is a stochastic process in which mutation frequencies fluctuate

randomly through time until the mutation becomes fixed or is eliminated. These random events are highly affected by the population size: the smaller the population, the larger the effect of chance events and thus the role of genetic drift in the frequency and fate of mutations is more important [162, 174]. In fact, a given mutation under negative selection might not be entirely deleterious and become fixed in the population due to genetic drift, only this requires a few more generations than in the case of a neutral mutation [158, 162, 174, 175].

Fixed mutations can either change the encoded amino acid, and are called *nonsynonymous* substitution, or be neutral, without change in the amino acid, and named *synonymous* substitution. Comparison between the rate of *nonsynonymous* substitution (dN) and the rate of *synonymous* substitutions (dS) is commonly used to characterize viral evolution and to investigate possible codons under positive selective pressure [162]. In this context, a dN/dS ratio below one implies a higher accumulation of *synonymous* mutations compared to *nonsynonymous*, amino acid sequences are more homogenous and there is a tendency to conserve the protein structure, corresponding to negative selection or purifying evolution. When the rate is similar or equal to one, the gene is under neutral selection. Values higher than one mean positive selection or adaptive evolution, and sequence variability is favoured and there a higher adaptation to the environment [181, 182]. In a protein coding sequence, conservative forces usually dominate because the functions of the proteins must be preserved in order for the organism to survive. However, in the context of negative selection, some codons might be under strong positive selection [178, 181].

Evolution of the *env* gene and disease progression

Few studies have evaluated the HIV-2 *env* gene evolution in association with disease progression. A few years back, Sankalé and co-workers observed that fast disease progression was associated with higher genetic diversity (genetic variability at one time point) in the V3 region [183]. A transversal comparative study found that the *env* gene is under negative selection, despite the detection of strong positively selected sites across the gene [177]. Another transversal study, described a direct association between the number of

infecting years and the genetic diversity of HIV-2, meaning that patients infected longer ago had higher genetic diversity of C2V3C3 region than virus from patients recently infected [184]. These authors detected a marked tendency for negative evolution in this region. In a longitudinal study, MacNeil and collaborators described that sequence diversity of C2V3C3 region in HIV-2 infected patients increased over time and diversification rate was associated with $CD4^+$ T cell depletion [185]. They also found evidence of negative selection in this region. However, they observed that diversity and divergence (genetic change from a point of reference) rates were significantly lower in HIV-2 infected individuals compared to HIV-1 infected patients with high viral setpoints. In agreement, Lemey *et al* found that the dS was similar between HIV-2 infected patients and HIV-1 slow progressors ($2.41x10^{-4}$ and $3.59x10^{-4}$ substitutions/nucleotide/month, respectively), but significantly lower compared to HIV-1 moderate progressors ($5.63x10^{-4}$ substitutions/nucleotide/month). They also observed that the dN was in the same range in HIV-2 and HIV-1 (both moderate and slow progressors) infections ($7.19x10^{-4}$, $7.07x10^{-4}$ and $8.68x10^{-4}$ substitutions/nucleotide/month, respectively) [186]. Borrego and co-workers, also in a longitudinal study, observed an increase in genetic diversity in C2V3C3 region through infection, but without significant association with viral load or clinical stage [179]. However, they showed an inverse correlation between the C2V3C3–specific binding antibodies [(immunoglobulin G (IgG)] and the diversity of this region. More recently, Skar *et al.* showed that the genetic evolution in gp125 and V3 (see above) and the rate of *synonymous* substitutions in gp125 are significantly higher in HIV-2 compared to HIV-1 ($13.38x10^{-3}$ *versus* $7.06x10^{-3}$ substitutions/nucleotide/year) [157]. The major difference between this study and those from MacNeil *et al* [185] and Lemey *et al* [186] is that Skar and collaborators [157] adjusted the samples for $CD4^+$ T cell counts and disease stage, and this might account for the opposing results. Summarizing all these data, one concludes that the HIV-2 *env* gene is under negative selection, it evolves at a higher rate compared to HIV-1 and genetic diversity increases through infection in association with $CD4^+$ T cell depletion and decrease in IgG binding antibodies [157, 177, 179, 183-186].

As for HIV-1 infection, several studies have compared the intrapatient evolution of the *env* gene with disease progression. During chronic infection, genetic diversity and divergence of the *env* gene have been associated with progression to AIDS in opposed ways [129, 187-

201]. Recently, some authors explained these different observations by proposing a model where HIV evolution is a dynamic rather than a constant process [186, 196, 202]. In fact, Lee and collaborators developed a sequence evolution model that reflects the dynamics of divergence and diversity throughout infection, suggesting a direct association between the evolutionary rate and the changes in $CD4^+$ T cell counts [202]. Overall, globally HIV-1 *env* gene was found to be under negative selection [203-206].

Studies that comprised HIV-1 transmission pairs (both sexual and MTCT) found that in acute infection (newly infected partner or newborn) the *env* gene is characterized by lower diversity, smaller V1-V2 regions, fewer glycosylation sites and sensibility to NAbs compared to chronic infection (chronically infected partner or mother) [206-210]. In HIV-2 infection, acute infection is difficult to find, since patients are diagnosed years after seroconvertion [122, 211]. Therefore, there is no information on evolution of the *env* gene in HIV-2 acute infection (either adults or infants).

Human responses against HIV-2

Innate responses

The human immune system is capable of detecting invasion by a pathogenic intruder and to activate defence mechanisms in order to eliminate the infection. The innate immune response is the first line of defence against invading pathogens, before the development of an adaptive immune response [212, 213]. When encountering the immune system for the first time, HIV triggers innate immune receptors such as the toll-like receptor (TLR) 7, TLR8 and TLR9, leading to the potent activation of DCs and the release of high amounts of type 1 interferons (IFNs) and tumor necrosis factor α (TNF-α) [214-216]. IFNs and TNF- α are involved in shutting down viral replication in infected cells and in promoting the immune response by recruiting other immune cells to the sites of infection and enhancing the functions of macrophages, Natural Killer (NK) cells, T cells, B cells and macrophages [213, 217]. In addition, the activation of DCs and other cells expressing TLRs induces the release of proinflammatory cytokines (IL-2, IL-12, IFNγ, IL-4, IL-10 and IL-15) and chemokines that use

the receptor CCR5 [213, 217, 218]. In HIV-2 infection, as in HIV-1 infection, responsiveness to TLR9 is defective [219] and, despite absence of viremia, plasmacytoid DCs are decreased in association with CD4 depletion and immune activation [220]. Plasmacytoid DCs are major producers of IFN-α upon TLR9 stimulation, and therefore production of IFN-α is diminished in HIV-2 infection [220]. However, plasmacytoid DCs are less susceptible to infection by HIV-2 compared to HIV-1, suggesting that other mechanisms besides direct viral infection determine the depletion of plasmacytoid DC during chronic HIV-2 infection [220, 221]. On the other hand, myeloid DCs are also less susceptible to HIV-2 and these cells' function remains intact through infection [221, 222]. Likewise, and because myeloid DCs produce IL12 upon TLR7/8 stimulation, responsiveness to this receptors as well as IL-12 production are preserved in HIV-2 infection [219]. However, a loss in circulating levels of myeloid DCs was detected in advanced disease stages in association with increase in viral load, CD4 depletion and immune activation [223]. Another set of cells that are better preserved during HIV-2 infection are NK cells [224]. The cytolytic activity of these cells as well as their ability to release TNF-α and IFNγ during chronic HIV-2 infection is similar to that of uninfected donors. However, with $CD4^+$ T cell decrease, cytolytic and chemokine-suppressive activity of NK cells drop to the levels seen in HIV-1 infected patients [224].

A robust innate immune response is mounted against HIV, but the intensity and magnitude of it may contribute to an early state of immune activation that promotes viral replication, particularly in HIV-1 infection [213, 225, 226]. Despite the better preservation of most innate immune responses during chronic HIV-2 infection, as mentioned above, with $CD4^+$ T cell loss and increase in immune activation, most immunological differences between HIV-1 and HIV-2 are lost and disease progression is equivalent [138, 147, 223, 224].

Another important component of the innate immune system that is activated upon HIV encounter is the complement cascade (Figure 1.7). This system is thought to participate in the early events of infection by recruiting DCs and macrophages to the site of infection. Also, it makes the bridge between the innate and adaptive immune systems by opsonisation and enhancement of humoral responses, and is therefore also important in the chronic phase of infection [227-232].

HIV replication cycle can also be blocked by several host restriction factors like Trim5-α, APOBEC3G and tetherin proteins [233]. Trim5-α belongs to the tripartite motif protein family and blocks HIV uncoating through interactions with the viral capsid [131, 137]. APOBEC3G is a member of the cytidine deaminases family. These proteins are packed within viral proteins and induce G-to-A hypermutation and degradation of the nascent proviral DNA. However, Vif impairs APOBEC3G's activity [65, 131, 137]. HIV-2 seems to be more sensitive to Trim5-α [234] and more resistant to APOBEC3G [235] compared to HIV-1. Tetherin is a recently identified host restriction factor that inhibits the release of new viral particles, but HIV-2 Env proteins can block its activity by interacting with the tetherin cytoplasmic ectodomain instead of with the transmembrane domain as HIV-1 does [236-241]. Furthermore, HIV-2 Env sequesters tetherin from the cell surface but, unlike HIV-1, does not seem to destroy it [238]. Other host genetic characteristics, such as HLA types, have been associated with viral replication and disease progression [242-244]. Of note, the HLA types considered to be protective in HIV-1 infection (HLA-B*27 and HLA-B*57) [242-246] showed no influence in HIV-2, whereas HLA-B*35, HLA-B*1503 and HLA-B*0801 were found to increase the risk of disease progression since individuals with these alleles had higher viral loads and lower $CD4^+$ T cell counts [245, 246].

Cellular responses

Most cellular responses against HIV are mediated by $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTL) [132, 247]. These cells recognise viral determinants at the surface of infected cells through antigen presentation by HLA class I and induce direct apoptosis of these cells [132, 247]. CTL responses against HIV-2 are thought to be more polyfunctional and hence have a higher avidity and a more preserved capacity of producing TNF-α, IFNγ and other pro-inflammatory cytokines compared to HIV-1 infection [248, 249]. The preservation of $CD8^+$ T cell functions was found to be associated with CD4 counts and decreased immune activation, despite the absence of detectable viral load, and is therefore thought to account for the slow disease progression of HIV-2 infected individuals [248]. In HIV-1 infection, CTL responses are thought to play a major role in the initial decline in plasma viral load and in

establishing and maintaining the viral set point [244, 250-252]. Usually, the virus rapidly escapes these early responses, forcing the T cells to become broader and target more conserved epitopes [250, 253, 254]. However, as disease progresses, the role of $CD8^+$ T cells responses in controlling viral replication remains controversial since they have been positively and negatively correlated to plasma viral load [255-259].

$CD4^+$ T cells recognise viral determinants in the context of antigen presentation by HLA class II and respond by proliferating and releasing cytokines (particularly IL-2). They are essential in maturation of B cells (and antibody class switching) and in the activation and growth of CTL and macrophages. $CD4^+$ T are the main target of HIV infection and hence are progressively depleted and functionally impaired through infection [132, 260]. In HIV-2 infected individuals, the HIV-specific $CD4^+$ T cell are thought to be more frequent, maintain the proliferative capacity and be more polyfunctional by sustaining the production of high levels of IL-2 and IFNγ compared to HIV-1 infection [249, 261-263]. HIV-1 infection is characterized by an early loss of $CD4^+$ T cell responses and a decrease in proliferative capacity and in the ability to secrete IL-2 [264]. In chronic infection, these responses are severely impaired or even absent [265, 266].

Humoral responses

Humoral immune responses are mediated by antibodies (or immunoglobulin – Ig) produced by B cells [267]. The Ig is composed by two heavy chains and two light chains. The amino terminal regions of both light and heavy chains are hypervariable between different antibodies and together they form the antigen binding domain. The variable regions of both chains along with the constant regions of the light chain and the amino terminal part of the constant heavy chain form the fragment antigen-binding (Fab) region. The carboxyl terminal regions of the heavy and light chains are constant between antibody isotypes (subclasses). The constant regions of the heavy chains are brought together to form the fragment crystallisable (Fc) region that is responsible for the effector functions of the antibody, namely binding to Fc receptors (FcγRs). According to their Fc portion, antibodies can be classified as IgM, IgD, IgG, IgA and IgE. Naïve B cells (previously to antigen exposure) express

IgM and IgD on their surface. Upon antigen encounter, $CD4^+$ T cells induce B cells maturation and isotype switch to IgG, IgA or IgE. The antibody isotype determine the effector function, whereas antigen specificity is determined by the antigen binding domain. The specificity and variability of antibodies is achieved by affinity maturation through somatic hypermutation and clonal selection. At the time of isotype switch, random mutations (somatic hypermutation) are inserted in the antigen-binding coding sequences of the Ig. The higher affinity mutants are selected (clonal selection) in detriment of the ones with decreased affinity to the antigen because they provide the cell with a proliferative advantage in response to that antigen [267].

Studies on the impact of HIV-2 infection over the B cell compartment are lacking. Since humoral responses are common in most HIV-2 infected patients [97, 109, 180, 268, 269], one could think that the B cell compartment is not affected by HIV-2. However, recently, Tendeiro *et al* described a depletion in memory B cells (unswitched and switched) in association with $CD4^+$ T cell decrease, despite the absence of detectable viremia [270]. This imbalance, like in HIV-1 infection [271-273], was not recovered by ART [270]. No other studies have addressed the B cell compartment during HIV-2 infection or the relationship with humoral responses. Defects in global B cell function have been described in HIV-1 infection, with a major reflexion on opportunistic infections and vaccination since humoral responses to other pathogens are compromised by the impairment of B cell populations caused by HIV-1 [274, 275]. One of the hallmarks of HIV-1 infection is hyperactivation of B cells in association with immune activation, with increased polyclonal B-cell activation [272], increased cell turnover [276, 277], increased expression of activation markers [278, 279], increased differentiation of B cells to plasmablasts [272, 276, 280] and decrease in memory B cells [271-273, 281]. Ultimately, ongoing viral replication leads to the expansion of several aberrant B-cell populations and B cell exhaustion [282, 283]. The consequences of B cell defects during HIV-1 infection in HIV-specific B cells have been the subject of recent studies [284, 285]. In HIV-1 infection the frequency of HIV-specific B cells was found to be quite low [284, 286, 287], despite the marked hypergamablobulinemia seen in these patients [272, 279, 280, 288]. In fact, Buckner and collaborators recently reported that the cells responsible for the hypergamablobulinemia are plasmablasts but the majority of the IgGs produced are

not HIV-specific [285]. Furthermore, these unspecific plasmablasts arise early in infection, which might help to explain the inadequacy of antibody response in HIV-1 infected individuals [285]. Nonetheless, some HIV-1 infected patients are able to produced cross-neutralizing antibodies that do not seem to impair in quality or quantity by the B cell imbalances [284, 289, 290]. B cell activation and hypergamablobulinemia are also present during HIV-2 infection, though to a lesser extent, in association with decrease in $CD4^+$ T cell counts [291-293]. Furthermore, contrarily to HIV-1 infection [294, 295], this hypergamablobulinemia does not seem to be polyclonal since it affects only IgGs, with no changes in the levels of IgA production compared to uninfected individuals [291, 293]. However, nothing is known about which subset of B cells is responsible for the production of these IgGs and whether they are HIV-2-specific.

Humoral responses against Env

Humoral immune responses against HIV-1 Env develop as early as eight days post-infection, with the first antibodies being IgM against gp41 region, followed by IgG and IgA (Figure 1.7) [296]. These antibodies mainly form immune complexes and generally do not control viral replication and are also not responsible for the initial decline in plasma viral load [296]. However, escape mutants have been reported to arise during this acute phase of infection [297, 298], implying that very low titers of Nabs are developed very early post infection [299]. The first autologous Nabs arise weeks after infection (13 weeks in HIV-1 clade B and 3 to 8 weeks in HIV-1 clade C infections), target mainly the variable loops of gp120 and are strain specific [296, 300], but the virus rapidly evolves to escape neutralization [296, 300]. This leads to the development of new Nabs, and the cycle repeats itself with continuous Nab production and viral escape [298, 301-303]. Because of the ability of HIV-1 to escape neutralization, Nabs are thought to have a limited effect on the control of virus replication and disease course. In fact, in HIV-1 infection, only about 20% of infected individuals develop antibodies able to neutralize some variants from different subtypes but they take three to four years to develop, suggesting that antibody affinity maturation and chronic antigen exposure are key factors [284, 304-308]. Consistent with this, in most studies there is a

positive correlation between the breadth of Nab response and viral diversity [176, 268, 298, 301-303, 306, 309, 310]. The development of highly potent antibodies that neutralize the majority of clades is a rare event that occurs in 1% of infected patients, these individuals being called “elite neutralizers” [311].

In contrast to HIV-1, most HIV-2 infected patients in the chronic phase of infection have a potent autologous and heterologous Nab response [97, 109, 180, 268, 269]. Weiss and co-workers were the first to describe the presence of Nabs in HIV-2 infected patients [312]. The studies that followed presented some limitations regarding the number of patients, the use of laboratory adapted strains and inconsistency in the neutralization assays used [84, 313]. Nonetheless, the work by Björling *et al* was the first to demonstrate that production of autologous Nabs was common in HIV-2 infection and to suggest an association between the slow disease progression of HIV-2 infected individuals and the presence of these antibodies [313]. More recently, Rodriguez *et al* found a broader Nab response in HIV-2 individuals but of a lower potency compared to HIV-1 (after adjusting for age and $CD4^+$ T cell counts) and, in both infections, Nab titers were directly associated with viral load [268]. Kong *et al* found that Nab responses were both broad and potent, but not correlated with $CD4^+$ T cells, viral load or disease outcome, and neutralization escape was rare [180]. On the other hand, Özkaya Sahin and co-workers reported that Nabs were both broader and more potent in HIV-2 infected patients and the association with viral load was only present in HIV-1 infection [269]. de Silva *et al* showed that autologous and heterologous Nabs were present in HIV-2 infected individuals but found no association with viral load [109]. This study also found that higher sequence diversity and variation in putative glycosylation sites conferred some level of resistance to NAbs [109]. More recently, Marcelino *et al* in a longitudinal study, demonstrated that disease progression is associated with loss of breath and potency of NAbs in patients infected with R5 viruses and that neutralization resistance occurs in late stages of the disease and is associated with X4 tropism [97]. X4 tropism was also associated with plasma viremia. Table 1.1 summarizes the most important findings of these studies, as well as the populations and methods used.

Table 1.1 - Recent studies on Nab response in HIV-2 infected patients

Patients	ART	Median $CD4^+$ T cells/µl	Median viral load (RNA copies/ml)	Viruses	Plasma samples	Neutralization assay	Main findings	Cross-neutralization with HIV-1	Ref
20 patients from Guinea-Bissau 9 patients from Portugal	not mentioned	not mentioned	not mentioned	- 1 lab adapted strain	IgGs and IgAs	- analysis of PBMC supernatants by capture ELISA - infectivity after 7 days	- first demonstration of IgA neutralization in HIV-2 infection - no association between IgA or IgG concentration and neutralization titers - higher potency of IgGs compared to IgAs	not assessed	[314]
35 female sex workers from Senegal	drug naïve	760	2.6	- 8 pseudoviruses (1 lab adapted)	whole plasma	- luciferase reporter assay using U87 cells (CD4+/CCR5+/CXCR4+) - infectivity after 72H	- highly potent (median titer 2.07log_{10} IC50) and broad Nabs - **positive** association between Nab titers and viral load - **no association** between Nab titers and $CD4^+$ T cell counts	very low breadth and potency (against 7 HIV-1 pseudoviruses)	[268]
64 patients, the majority from Senegal	drug naïve	480.5	2.1	- 3 pseudoviruses	whole plasma and IgGs	- β-galactosidase and luciferase reporter assay using TZM-bl cells - infectivity after 48H	- highly potent (median titer 4.89log_{10} IC50) and broad Nabs - **no association** between Nab titers and viral load - **no association** between Nab titers and CD4+ T cell counts - neutralization mediated by IgGs - no Nab escape	no neutralization (against 1 HIV-1 pseudovirus)	[180]

Table 1.1 (Continued)

Patients	ART	Median $CD4^+$ T cells/µl	Median viral load (RNA copies/ml)	Viruses	Plasma samples	Neutralization assay	Main findings	Cross-neutralization with HIV-1	Ref
20 HIV-2+ 11 HIV-1/HIV-2+ from Guinea-Bissau	not mentioned	422	72% <3 22% 3-4 6% >4	- 5 primary isolates CCR5-tropic	whole plasma	- plaque reduction assay in U87 cells (CD4+/CCR5+) with hematoxylin staining - infectivity after 72H	- highly potent (median titer 3.18log_{10} IC50) and broad Nabs - **no association** between Nab titers and viral load	very low breadth and potency (against 5 HIV-1 primary isolates)	[269]
40 patients from Guinea-Bissau	drug naïve	508	4.1	- 54 pseudoviruses (autologous) - 8 pseudoviruses (heterologous)	whole plasma	- luciferase reporter assay using TZM-bl cells - infectivity after 48H	- highly potent (median autologous titer > 4log_{10} IC50; median heterologous titer > 3.85log_{10} IC50) and broad Nabs - positive association between heterologous Nab titers and viral load - **no association** between autologous Nab titers and viral load	not assessed	[109]
28 patients from Portugal (followed for 4 years – total of 41 samples)	treated and naïve	363	undetectable in 24 patients 4 patients with median 3.8	- 8 primary isolates CCR5-tropic + 4 primary isolates CXCR4-tropic	IgGs	- luciferase reporter assay using TZM-bl cells - infectivity after 48H	- highly potent (median autologous titer 3.91log_{10} IC50) and broad Nabs - **positive association** between Nab titers and CD4+ T cell counts - **positive association** between plasma viremia and X4 tropism - first association between susceptibility to neutralization and HIV-2 tropism	not assessed	[97]

Table 1.1 (Continued)

Patients	ART	Median $CD4^+$ T cells/µl	Median viral load (RNA copies/ml)	Viruses	Plasma samples	Neutralization assay	Main findings	Cross-neutralization with HIV-1	Ref
10 HIV-2+ 8 HIV-1/HIV-2+ from Guinea-Bissau	drug naïve	493	80% <2.5 (data missing for 2 patients)	- 1 primary isolate CCR5-tropic	IgGs and IgAs	- plaque reduction assay in Ghost cells (CD4+/CCR5+) - analysis of Green Flourescent Protein expression - infectivity after 72H	- highly potent IgG (median titer = 5.2log_{10} IC50) and IgA (median titer = 2.1log_{10} IC50) neutralization - **positive association** between IgG and IgA titers - **no association** between IgG titers and $CD4^+$ T cell counts	no IgG or IgA neutralization (against 1 HIV-1 primary isolate)	[293]

Direct comparison between all the studies remains difficult due to the use of pseudoviruses or clinical isolates and different neutralization assays. Most importantly, the differences in the populations studied may justified the discordant associations, or lack of, between Nabs and viral load and/or $CD4^+$ T cell counts found in these studies. Some authors found no association between Nab titers and $CD4^+$ T cell counts but the patients included in their studies did not represent the full spectrum of disease being in general asymptomatic and with high median $CD4^+$ T cell count [180, 268]. The inclusion of patients in more advanced disease stages, with lower $CD4^+$ T cell counts, was important to show for the first time, an association between decrease in Nab titers and $CD4^+$ T cell depletion [97]. The same conclusions can be drawn regarding plasma viremia: the studies with patients presenting the lower median viral load (i.e. in which most patients had undetectable viral load) found no associations between this marker and Nab titers [180, 268], whereas those that included infected patients with higher plasma viremia found a positive association between viral load and neutralizing activity [97, 109]. Furthermore, despite being observed in two studies [97, 109] Nab escape was shown to be really significant in the one study that enrolled patients in advanced disease stages [97]. This clearly points the importance of including in these type of studies patients with the full spectrum of disease and $CD4^+$ T cell counts.

In HIV-2 infection anti-Env antibodies are predominantly of IgG1 subclasses, like in HIV-1, although IgG3 might also be found in significant concentrations [291]. IgA antibodies are also thought to have an important protective role not only at the mucosal level, as in HIV-1, but also in the plasma [291, 293, 314, 315]. In fact, recent studies have demonstrated that plasma IgA in HIV-2 infection, contrarily to HIV-1, have potent neutralizing activity (Table 1.1) [293, 314]. During acute and chronic HIV-1 infection, antibodies against Env, as mentioned above, are mainly IgG1 type and higher titers of this immunoglobulin have been associated with better control of viral replication [316, 317]. Anti-Env IgG3 is the second most predominant immunoglobulin found in HIV-1 infected patients, and has in fact a greater *in vitro* neutralization capacity compared to IgG1 [316]. IgA is the predominant antibody in mucosal surfaces and several studies have suggested that IgA present in vaginal fluids and saliva can protect against HIV-1 infection [318-322].

The majority of the neutralizing epitopes in HIV-2 are located in the surface of gp125 glycoprotein. Nabs are directed against the CD4 and coreceptor binding sites (conformational epitopes that include selected amino acids in the C4 and V4 regions), V3 (the linear epitope LMSGLVF and the conformational epitope comprising amino acids FHSQ in positions 315-318 and WCR in positions 329-331) and V4 (conformational epitope involving the C-terminal portion of V4) [323, 324]. Other epitopes have also been identified in regions V1, V2 and gp36 [314, 325-327]. Table 1.2 summarizes, in a chronological order, the neutralizing epitopes defined so far in HIV-2 Env.

Can HIV-2-specific neutralizing antibodies neutralize HIV-1 and prevent HIV-1 acquisition? The early reports of modest cross-neutralization of HIV-1 by HIV-2 sera [312, 328] lead several authors, particularly West African groups, to investigate if HIV-2 infection provides cross protection against HIV-1 infection. Contrarily to initial findings [329-331], HIV-2 infection does not seem to protect against HIV-1 acquisition and may in fact increase the risk of dual infection [332-336]. However, it is not clear if this higher risk is due to increased biological susceptibility or just risky sexual behaviour [337]. More recent studies on cross-neutralization concluded that HIV-1 is poorly neutralized by plasmas from HIV-2 infected patients (Table 1.1) [268, 269]. Rodriguez *et al* found that neither HIV-1 nor HIV-2 were cross-neutralized by plasmas from HIV-2 and HIV-1 infected patients, respectively [268]. Özkaya-Sahin *et al* also found that HIV-1 was not neutralized by HIV-2 infected plamas even when using sera from dually infected donnors [269]. Furthermore, they found that plasmas from HIV-1 infected individuals neutralized HIV-1 and HIV-2 with the similar potency, and they atributed this phenomenon to the more neutralization-sensitive phenotype of HIV-2. Interestingly, however, a recent study found that disease progression of HIV-1 infected individuals is delayed by contemporaneous HIV-2 infection [338]. The authors found a significantly lower HIV-1 diversity, higher estimated time to the development of AIDS and higher $CD4^+$ T cell percentage in dually infected patients than in HIV-1 infected individuals, and these differences were even higher in patients with HIV-2 seroconversion prior to HIV-1 infection. However, no significant differences were found in HIV-1 divergence, *nonsynonymous* substitutions or *synonymous* substitutions rates between HIV-1 infected

patients and dually infected individuals [338]. Nab responses were not evaluated in these patients and the mechanism of protection against HIV-1 disease progression provided by HIV-2 was not defined.

Nabs are thought to be of major importance in preventing the mother-to-child transmission (MTCT) of HIV-1 because mothers who transmit the virus to their infants tend to have lower titers of NAbs against autologous viruses than those how do not transmit [339-342]. Consistent with this, the homogenous population of viruses that are usually transmitted perinatally are thought to be neutralization escape mutants and are more resistant to neutralization by maternal plasma [210, 339, 340, 342-347]. However, the passive transfer of maternal Nabs or the development of *de novo* antibodies by the HIV-1 infected infant does not seem to influence disease outcome [129]. Regarding HIV-2, MTCT is an exceedingly rare event [110-113] which justifies the absence of studies on the role of maternal neutralizing antibodies in transmission or on the *de novo* development of Nabs in the infected infant.

In addition to Nabs, humoral responses against HIV comprise non-neutralizing antibodies that bind to HIV antigens present at the surface of cells through their Fab region and recruit cells from the innate immune system that have an Fc receptor (FcγRs). Cells with an FcγRs include B cells, NK cells, DCs, neutrophils, and monocyte macrophages. The effector functions of these antibodies can result in one or more of the following events: antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated viral inhibition (ADCVI), antibody-dependent complement-mediated inactivation (ADCMI) and phagocytosis (Figure 1.7) [348-358]. In HIV-2 infected patients both ADCC and ADCMI responses seem to be stronger compared to HIV-1, but no association has been made with $CD4^+$ T cell counts or viral load [354, 359]. ADCC and ADCVI responses have been positively associated with $CD4^+$ T cell counts and negatively with viral load and disease progression in HIV-1 infection [360-364]. A recent study evaluated the effect of complement on humoral responses in chronic HIV-1, HIV-2 and dual infections and found that the antiviral effect of ADCMI is intratype specific and more potent in HIV-2 infection than in HIV-1 infection, suggesting that the efficient use of complement might contribute to the strong antiviral

activity seen in HIV-2 infection [354]. The effector functions of antibodies might have an important role in controlling HIV-2 and therefore more studies addressing ADCC, ADCVI and ADCMI during HIV-2 infection are needed.

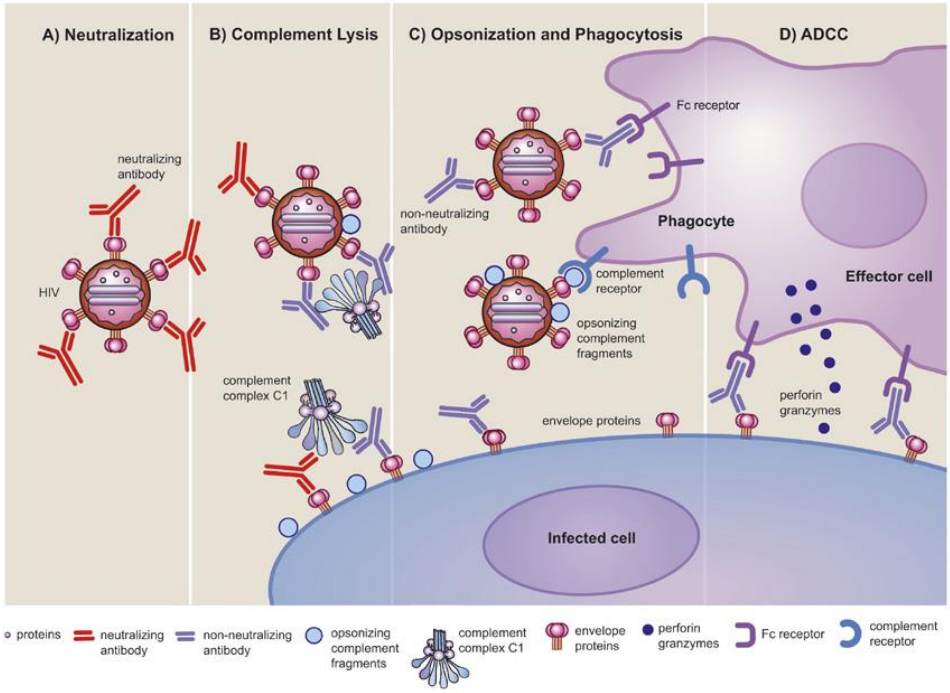

Figure 1.7 – Antibody effector functions. (a) Neutralization of free virus by antibodies, (b) ADCMI, (c) opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, (d) ADCC. (Adapted from Huber and Trkola, Journal of Internal Medicine 2007 [365])

Table 1.2 - Neutralizing epitopes on HIV-2 Env

Epitope region (Env)	Neutralizing epitope	Epitope characteristics	Epitope mapping system	Ref
V3	CRRPENKTVVPITLMSGRRFHSQKIINKKPRQAWC	Linear epitope	ELISA with synthetic peptides	[328]
V1	AVATTSPSGPDMINDTDPC	Linear epitope	Guinea-pigs Immunized with synthetic peptides	[325]
V3	SGRRFHSQKIINKKPRQAWC	Linear epitope		
V3	QKIINKKPRQAWCRFKGEWR	Linear epitope		
gp36	CHTTVPWVNDTLTPEWNNMT	Linear epitope		
gp36	HIHKDWEQPDREETEE(C)	Linear epitope		
C-terminal end of gp125	ELGDYKLVEVTPIGFAPTAEKR(C)	Linear epitope		
C-terminal end of gp125	TAEKRYSSAPGRHKRGVLVLG(C)	Linear epitope		
V3	Undefined	Potential conformational epitope	ELISA with synthetic peptides	[366]
V3	FHSQ and WCR	Conformational epitope	Guinea-pigs immunized with synthetic peptides	[324]
V3	HYQ	Potential conformational epitope	Mice immunized with synthetic peptides	[367]
C3	APGK	Not determined		
V2	FNMTGL	Linear epitope	Competition radioimmunoassay with overlapping synthetic peptides	[326]
V3	LMSGHVFHSHYQ	Linear epitope		
Not V1/V2 or V3	Undefined	Conformational epitope		
V2	CQFNMTGLERDKKKQYNETWYSKDWC	Linear epitope	Guinea-pigs immunized with synthetic peptides	[327]
V4	CNMTWFLNWIENKTHRNYAPC	Linear epitope		
gp36	MYELQKLNSWDVFGN(C)	Linear epitope		
gp36	MYELQKLNSWDVFGN(C)	Linear epitope	Guinea-pigs Immunized with synthetic peptides	[314]
C2V3C3	Undefined	Not determined	Mice immunized with synthetic peptides	[368]
V3	(T)LMSGLVF	Linear epitope	Pepscan peptide-binding ELISA with linear and cyclic synthetic peptides	[323]
V3	CLMSGLVFHSQPINKRC	Linear epitope dependent on conformation		
C-terminal end of V4	Undefined	Conformational epitope		
CD4 binding site	Undefined	Conformational epitope		
coreceptor binding site	Undefined	Conformational epitope		

CHAPTER 2

Aim of the Studies and Work Plan

HIV-2 was identified as one of the causative agents of AIDS is 1986, although its first transmission to humans is thought to have occurred around 1938. Presently, this virus affects 1 to 2 million people, the majority living in West Africa. Besides being endemic in several countries in West Africa, like Guinea-Bissau, Ivory Coast and Cape Verde, HIV-2 is only found in countries with historical and socio-culture relations with these West African regions, contrarily to HIV-1 responsible for the global pandemic. Apart from the confined geography, HIV-2 is seen as an attenuated virus compared to HIV-1, due to lower *in vitro* replication capacity and the characteristic slow disease progression and low transmission rate. Furthermore, human immune responses are thought to be more preserved in HIV-2 infected individuals since most patients have strong cytotoxic responses to Env and Gag proteins and raise autologous and heterologous Nabs. Studying HIV-2 infection and understanding what is behind the better immune responses can be crucial to know how to control HIV-1.

Several recent studies evaluated Nab responses in HIV-2 chronically infected patients and found them to be essential in controlling viral replication and disease progression. However, there is very little information on how these responses shape viral evolution in the early stages of the infection. Studying HIV-2 early infection might provide crucial information on how this virus is better controlled by the immune system compared to HIV-1. Hence, the first aim of these studies was to characterize the Nab response and molecular and phenotypic viral evolution in early infection. Because most HIV-2 infected patients are diagnosed many years after seroconversion, cases of MTCT represent a unique opportunity to evaluate Nab responses and the consequences to the virus soon after transmission. In Chapter 3, two children infected with HIV-2 through MTCT were followed longitudinally since birth for nine and twelve years. The entire *env* gene was amplified, cloned and sequenced in order to compare genetic distances, selective pressure, nucleotide evolutionary rates and putative N-linked glycosylation sites throughout infection. Viral tropism was assessed in viruses from initial and subsequent sampling years in both children. Nabs responses present in the infants were determined against autologous and heterologous viruses using a luciferase reporter gene assay in TZM-bl cells. Structural models of the Env glycoprotein were generated by

homology modelling using the children's *env* sequences from birth and subsequent years. The evolution of the *env* gene and Nab responses were compared between the two infants through infection to investigate the factors behind the differences seen in the clinical progression of the children. Nab responses were also evaluated in parallel with viral tropism and structural models of C2V3C3 region to assess the importance of Nabs in tropism switch.

Memory B cells are responsible for a rapid and specific antibody response on a second encounter with an antigen. These cells are known to be severely impaired in both HIV-1 and HIV-2 infections, in association with CD4+ T cell depletion. However, in contrast to HIV-1 infected individuals, Nabs are produced by most HIV-2 chronically infected patients. What is the relation between Nab production and the memory B cell depletion and which B cell subsets are responsible for maintaining Nab production in HIV-2 infected patients? The answer to these questions lead to the second aim of these studies: to characterize Nab responses in HIV-2 chronically infected patients with several degrees of memory B cell disturbances and assess the correlation between these two parameters. In Chapter 4, Nab responses were evaluated in 37 HIV-2 chronically infected patients, which had previously been reported to have memory B cell imbalances. This cohort of patients included treated and untreated individuals, with diverse CD4+ T cell counts and varied degrees of memory B cell depletion. Patients' serum was used to neutralize a panel of four heterologous R5 tropic HIV-2 clinical isolates in a TZM-bl reporter assay. Nab breadth and potency was then compared with C2V3C3 and gp36 specific binding antibodies to determine the main targets for neutralization in diverse stages of infection. Nab titers were also compared with the frequency of unswitched and switched memory B cells in the different groups of patients to establish the relation between Nab production and memory B cell depletion.

HIV-2 resistance to antibody neutralization is a rare event that has mainly been detected in two X4 variants isolated early in infection from infants infected perinatally (Chapter 3) and four X4 strains from patients in late stage disease. Are all X4 viruses intrinsically resistant to Nabs? The third aim of this thesis was to characterize the neutralization phenotype of X4 strains from HIV-2 infected individuals in diverse disease stages. In Chapter 5, HIV-2

neutralization susceptibility was assessed in a new set of X4 viruses and compared with R5 variants used as controls. X4 viruses were obtain from two vertically infected children (early infection) and five chronically infected adults with advanced disease (late infection). Viruses were neutralized with a panel of 16 plasmas from unrelated HIV-2 chronically infected patients in a luciferase reporter gene assay in TZM-bl cells. Amino acid sequences and structural models of the V3 loop (generated by homology modelling) of all strains were also compared to assess possible differences between R5 and X4 viruses and between X4 strains from early and late infections. A model of the evolution of HIV-2 V3 loop conformation through infection in close association with Nab escape and disease progression was proposed.

CHAPTER 3

Neutralizing antibodies drive the molecular and phenotypic evolution of the human immunodeficiency virus type 2 envelope

Neutralizing antibodies drive the molecular and phenotypic evolution of the human immunodeficiency virus type 2 envelope

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Abstract

Background: Differently from HIV-1, HIV-2 disease progression usually takes decades without antiretroviral therapy and the majority of HIV-2 infected individuals survive as elite controllers with normal $CD4^+$ T cell counts and low or undetectable plasma viral load. Neutralizing antibodies (Nabs) are thought to play a central role in HIV-2 evolution and pathogenesis. However, the dynamic of the Nab response and resulting HIV-2 escape during acute infection and their impact in HIV-2 evolution and disease progression remain largely unknown. Our objective was to characterize the Nab response and the molecular and phenotypic evolution of HIV-2 associated with Nab escape in the first years of infection in two children infected at birth.

Results: $CD4^+$ T cells decreased from about 50% to below 30% in both children in the first five years of infection and the infecting R5 viruses were replaced by X4 viruses within the same period. With antiretroviral therapy, viral load in child 1 decreased to undetectable levels and $CD4^+$ T cells recovered to normal levels, which have been sustained at least until the age of 12. In contrast, viral load increased in child 2 and she progressed to AIDS and death at age 9. Beginning in the first year of life, child 1 raised high titers of neutralizing antibodies that neutralized primary R5 isolates more effectively than X4 isolates, both autologous and heterologous. Child 2 raised a weak X4-specific Nab response that decreased sharply as disease progressed. Rate of evolution, nucleotide and amino acid diversity, and positive selection, were significantly higher in the envelope of child 1 compared to child 2. Rates of R5-to-X4 tropism switch, of V1 and V3 sequence diversification, and of convergence of V3 to a β-hairpin structure were closely related with rate of escape from the neutralizing antibodies.

Conclusion: Our data strongly suggests that neutralizing antibodies drive the molecular and phenotypic evolution of the HIV-2 envelope providing further support for a model in which the neutralizing antibodies play a central role in HIV-2 pathogenesis.

Keywords: Vertical HIV-2 infection; evolution of the neutralizing antibody response; escape from neutralization; molecular evolution, tropism.

Background

Infection with human immunodeficiency virus type 2 (HIV-2) affects 1-2 million individuals mostly living in West Africa, India and Europe [57, 369]. Eight different HIV-2 groups named A through H have been reported but only viruses from groups A and B are known to cause human epidemics [42, 45]. Among those, viruses from group A are responsible for the vast majority of HIV-2 infections worldwide.

Even though HIV-1 and HIV-2 are closely related viruses and share a high degree of similarity, infections by these viruses lead to very different immunological and clinical outcomes. HIV-2 infection eventually leads to CD4 depletion, AIDS and death [145, 149, 370]. However, differently from HIV-1, HIV-2 disease progression usually takes decades without antiretroviral therapy and the majority of HIV-2 infected individuals survive as elite controllers with normal $CD4^+$ T cell counts and low or undetectable plasma viral load [134, 142, 148, 153, 291, 371-374]. Understanding of the factors involved in the effective control of viral replication and disease progression in HIV-2 infected individuals might prove crucial to devise the best strategy to prevent and treat HIV-1.

Enhanced immune control could explain the mild outcome of most HIV-2 infections. Unlike HIV-1 infected patients, most HIV-2 patients in chronic stage produce potent and broad neutralizing antibodies [109, 180, 268, 269, 313]. Recent evidence has shown that the viruses isolated from HIV-2 infected patients with advanced disease are characterized by increased resistance to entry inhibitors, including the CCR5-antagonist maraviroc [375] and neutralizing antibodies [97], and by a remarkably high evolutionary rate [157, 179]. These results suggest that neutralizing antibodies play a central role in HIV-2 evolution and pathogenesis. However, in contrast to HIV-1, still nothing is known about the neutralizing antibody response and the molecular and phenotypic features of HIV-2 in acute/early infection because HIV-2 patients are usually diagnosed many years after seroconversion.

Most neutralizing epitopes in the HIV-2 envelope glycoprotein complex are located in the surface gp125 glycoprotein. Neutralizing epitopes in gp125 have been identified in V1, V2, V3, V4 and C5 regions, and in the CD4-binding site [97, 109, 180, 323, 324, 326, 327]. These epitopes are well exposed in the envelope complex of CCR5-using isolates that are usually highly sensitive to antibody neutralization [180, 323]. However, X4 isolates that emerge in

late stage infection in some HIV-2 patients when C2V3C3-specific neutralizing antibodies wane are highly resistant to antibody neutralization [97]. The V3 loop sequence, size and conformation of the X4 isolates are markedly different from those of R5-neutralization sensitive isolates supporting a direct role of this region in escape from neutralization and a direct role of the neutralizing antibodies in shaping the evolution of V3 in progressive HIV-2 infection. The neutralizing domains expressed in the envelope glycoproteins in acute/early infection and the role of the neutralizing antibodies and neutralization escape in shaping the evolution of the HIV-2 envelope in this period remains to be determined.

Perinatal transmission of HIV-2 is a rare event that in Europe has only been documented in Portugal [114, 122-125] and France [376]. Vertical transmission cases constitute a unique opportunity to study the phenotypic and molecular evolution of HIV-2 Env in acute and early infection as well as the role of Nabs in this process. Our objective was to characterize the evolution of the Nab response in two children infected with HIV-2 at birth in association with the molecular and phenotypic evolution of the virus. We show that broad and potent Nabs can be elicited very early after infection and that HIV-2 Env evolves at a very high rate in the first years of infection, this rate being directly associated to the potency of the Nab response. R5-to-X4 tropism change, increased diversity in V1 and V3, and selected changes in V3 conformation were associated with escape from antibody neutralization. The data fully supports the hypothesis that the main driver of the rapid molecular and phenotypic evolution of the HIV-2 envelope in the first years of infection is the selective pressure imposed by the neutralizing antibodies.

Materials and methods

Study subjects and ethics

Two children infected by vertical transmission were studied. Blood samples were collected from child 1 (patient PTHDECT), 39 days after birth in 1998, in 1999, 2000, 2001, 2003, 2006 and 2010, and from child 2 (patient PTHDESC), 27 days after birth in 1992, in 1997 and 2001. Clinical and immunological characteristics of the patients are shown in Table 3.1. Child 1 started ART (stavudine+lamivudine+lopinavir/ritonavir) in November 2003. Presently, the

child is taking lamivudine+abacavir+lopinavir/ritonavir; his viral load is undetectable and he is clinically and immunologically stable. Child 2 started ART with zidovudine immediately after birth and lamivudine was added in 2001. In 2001, viral load increased slightly and $CD4^+$ T cells decreased sharply leading to the child's death. Ethical approval was obtained from the Ethics Committee of Hospital Curry Cabral and written informed consent was obtained from the children's parents before entry into the study.

HIV-2 env gene PCR amplification, cloning and sequencing

Chromossomal DNA was extracted from infected PBMC's using Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer recommendations. A 2600 bp fragment encompassing the entire *env* gene was amplified by nested Polymerase Chain Reaction (PCR) using the Expand Long Template PCR Systemkit (Roche) and newly designed primers (Table S3.1). The PCR protocol consisted of denaturation at 95^{o}C for 5 min, 35 amplification cycles of 15 sec at 94^{o}C, 30 sec at 59^{o}C and 3 min at 68^{o}C with 5 sec increments and a final elongation step at 68^{o}C for 30 min. 5 µl of PCR product was used as the template for nested PCR. The amplification profile of the nested PCR was identical to the first PCR, except for annealing temperature and extension time (61^{o}C and 2 min respectively). PCR amplicons were purified with a JETQUICK Gel Extraction Spin Kit (Genomed). For each sample, PCR products were cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen), using the TOPO TA Expression Kit (Invitrogen) according to the manufacturer's instructions. At least eight clones from each patient/year were sequenced using the BigDye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems); sequencing primers are displayed in Table S3.1. Sequencing was performed on an ABI 3100 - Avant Genetic Analyzer (Applied Biosystems).

Table 3.1 - Virological and immunological characterization of the patients

Patient	Sampling year	Age (Years)	Viral load (RNA copies/ml plasma)	Viral tropism[a]	Number of $CD4^+$ T cells/µl (%)	Drug regimen	Disease stage[b]
Child 1	1998	0.11[c]	<200	R5	5342 (47)	-	N
	1999	1	<200	nd	2992 (50)	-	N
	2000	2	1355	R5	2919 (43)	-	A1
	2001	3	nd	nd	3253 (51)	-	A1
	2003	5	20968	X4	595 (27)	d4T+3TC+LPV/RTV	B2
	2006	8	<200	nd	1895 (55)	d4T+3TC+LPV/RTV	A1
	2010	12	<40	nd	1878 (54)	3TC+ABC+ LPV/RTV	A1
Child 2	1992	0.07[d]	<200	R5[e]	1670 (52)	AZT	C1
	1997	5	<200	X4	1050 (25)	AZT	C1
	1999	7	13883	nd	127 (15)	-	C1
	2001	9	1250	X4	44 (5)	AZT+3TC	C3 (death)

[a] As determined phenotypically in TZMbl cells [375] or genotypically based on V3 loop sequence patterns [104]; [b] According to the CDC revised classification system for HIV infection in children; [c] First blood collection was at day 39 after birth; [d] First blood collection was at day 27 after birth; [e] Isolate obtained at birth was syncytium inducing as determined in PBMCs and several cell lines [124]; d4T – stavudine, 3TC – lamivudine, LPV – lopinavir, RTV – ritonavir, ABC – abacavir, AZT – zidovudine; nd – not done.

Sequence analysis

Clustal X 2.1 [377] software was used to construct alignments of HIV-2 *env* sequences. Reference HIV-2 sequences were obtained from the Los Alamos National Laboratory HIV sequence database [378]. Maximum likelihood phylogenetic analyses were performed using the best-fit model of molecular evolution estimated in PAUP by Modeltest using likelihood ratio tests [379]. The chosen model was GTR+I+G. Tree searches were conducted in PAUP using nearest-neighbour interchange (NNI) and tree-bisection plus reconnection (TBR) heuristic search strategies [380], and bootstrap resampling with 1000 replicates [381]. The genetic distances between sequences were calculated by averaging pairwise tree distances using all sequences obtained for each patient at each time point, as previously described [202]. Putative recombinants were identified using the Phi-statistic [382] available in SplitsTree version 4.10 [383] by performing 10 randomized reductions of putative recombinants. Putative recombinant sequences were removed before doing the evolutionary rate analyses. These were: 00PTHDECT_9, 00PTHDECT_16, 00PTHDECT_22, 00PTHDECT_6, 00PTHDECT_24, 00PTHDECT_19, 00PTHDECT_8, 00PTHDECT_12, 03PTHDECT_17, 03PTHDECT_33, 03PTHDECT_21, 01PTHDESC_13, 01PTHDESC_6 and 01PTHDESC_14.

Selective pressure on the HIV-2 Env was examined with the DATAMONKEY web-server [384], after removing all positions containing gaps and missing data from the dataset. All estimations were performed using the MG94 codon substitution model [385] crossed with the nucleotide substitution model GTR, previously selected with Modeltest (see above). The single-likelihood ancestor counting (SLAC) method was used to infer the ratio of nonsynonymous to synonymous nucleotide substitutions (dN/dS) averaged over all codon positions of the alignment. To identify individual codons under selective pressure, site-specific dN/dS rates were estimated by the relaxed-effects likelihood (REL) method, with a cut-off value for the Bayes factor of 50 [386].

The Bayesian program BEAST was used to estimate the nucleotide evolutionary rates [387]. The SRD06 model [388] of substitution was used and two different clock models were used, relaxed lognormal and strict clock. A constant parametric demographic model as well as the non-parametric Skyline plot with 3 groups was tested. The MCMC chains were chosen so

that the effective sample size for all parameters exceeded 300 and convergence was assessed by inspecting the traces in the program Tracer [389]. Appropriate demographic and molecular clock models were chosen by examining the marginal posterior distributions of relevant parameters.

Potential N-linked glycosylation sites were identified using the N-Glycosite software [390] and the entropy at each position in protein alignment was measured with Shannon's entropy [391], both available at the Los Alamos National Laboratory HIV sequence database [378].

Virus isolation and tropism characterization

Primary virus isolates were obtained from both patients using the co-cultivation method as described previously [124]. Viral tropism (CCR5 and/or CXCR4 usage) was determined in TZM-bl cells in the presence of CCR5 or CXCR4 antagonists as described previously [375]. Tropism was also determined genetically using the V3 loop clonal sequences and the algorithm described by Visseux*et al*[104] which is based in the sequence, size and charge of the V3 loop.

Neutralization assay

The neutralizing activity present in patients serum was analyzed against autologous and heterologous primary virus isolates using a luciferase reporter gene assay in TZM-bl cells, as described previously [303, 368, 369]. Briefly, the cells [10,000 cells in 100 µl of complete growth medium (GM) that consists of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS)], were added to each well of 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight. One hundred µl of each virus (corresponding to 200 TCID50) were incubated for 1 h at 37°C with 2-fold serial dilutions of heat-inactivated patients sera in a total volume of 200 µl of GM containing DEAE-Dextran (20 µg/ml). The lowest serum dilution used in the assays was 1:80. Forty-eight hours later, plates were analyzed for luciferase activity on a luminometer (TECAN) using the One-Glow Luciferase Assay System (Promega, Madison, WI). Medium only control wells were measured as background, and virus-only control wells were included as 100% infection. Neutralization titers were expressed as the reciprocal of the plasma dilution that inhibited virus infection by

50% (IC50). IC50 was estimated by the sigmoidal dose–response (variable slope) equation in Prism version 5.0 [392]. Nonspecific inhibition was assessed by testing all HIV-2 isolates against HIV-negative plasma and all plasma samples against HIV-1 strain SG3.1 and HIV-1 SG3.1 pseudotyped with VSV envelope (using pSG3Δenv as backbone).

Structural models

Structural models of the C2-V3-C3 domain in gp125 were produced with SWISS-MODEL homology modelling server in automated mode, using PDB file 2BF1 (SIV) as template [393, 394]. Accelrys Discovery Studio 2.1 (Accelrys Inc., San Diego, USA, 2008) was used to produce three dimensional images of the obtained models and perform the secondary structure analysis of the V3 loop.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 [392] with a level of significance of 5%. F test was used to compare best fit values of IC50 slopes obtained with CT00 and CT03 isolates from child 1. Non parametric Mann Whitney test was used to compare autologous Nab responses (mean IC50s) between child 1 and child 2. To compare evolutionary rates we computed the posterior probability (PP) that one rate exceeded the other and the probability was determined numerically by randomly sampling from the empirical posterior distributions [157]. Kruskal-Wallis test was used to compare mean Shannon's entropies between variable Env regions of both patients.

GenBank accession numbers

Full-length envelope sequences generated in this study are available from GenBank under the following accession numbers: GU983917-GU983940 and JX219591-JX219614.

Results

Clinical and virological progression is very fast in the first years of infection

Child 1 infection was diagnosed with HIV-2 infection by PCR and virus isolation in the first month of life in 1998. To confirm the vertical transmission event and characterize the transmitted/founder virus population, 8 clonal full-length *env* gene sequences were obtained from samples collected in 1998, 2000 and 2003 (in total 24 *env* sequences) and from his mother (mother 1 - PTHCC20) in 2000 and 2003 (16 *env* gene sequences). We were unable to obtain 1998 samples from the mother.

Child 2 infection was diagnosed in 1992 at day 39 after birth by PCR and virus isolation, and vertical transmission was confirmed by phylogenetic analysis of partial *env* sequences from the mother and the child [123, 124]. Eight new clonal full-length *env* sequences were obtained from samples collected in 1992, 1997 and 2001.

Phylogenetic analysis showed that all sequences belonged to HIV-2 group A and that mother and child sequences shared a common ancestor, being more closely related to each other than to any other sequences, which confirms the two vertical transmission events (Figure 3.1). The sequences showed patient-specific clustering, forming sub-clusters corresponding to each year of infection. The sequences from the first sample from both children segregated into one (child 2) or two (child 1) sub-clusters supported by high bootstrap values indicating that one or two virus variants were transmitted from the mothers to the children (Figure 3.1).

Child 1 was born with normal CD4 percentage (47%) which was sustained until age 3 without ART. The transmitted/founder virus was CCR5-tropic according to V3 loop sequence analysis of *env* gene clones obtained in 1998 and to phenotypic analysis of virus isolated in 2000 (Table 3.1 and Figure 3.1). At age 5, in 2003, CD4 levels decreased to 27%, plasma viral load increased significantly and the virus changed to CXCR4-tropic as determined by phenotypic analysis. Antiretroviral therapy (ART) was initiated at that time leading to a decrease in viral load to undetectable levels and to an increase in $CD4^+$ T cells to normal levels. Presently, this child is clinically and immunologically stable and remains asymptomatic.

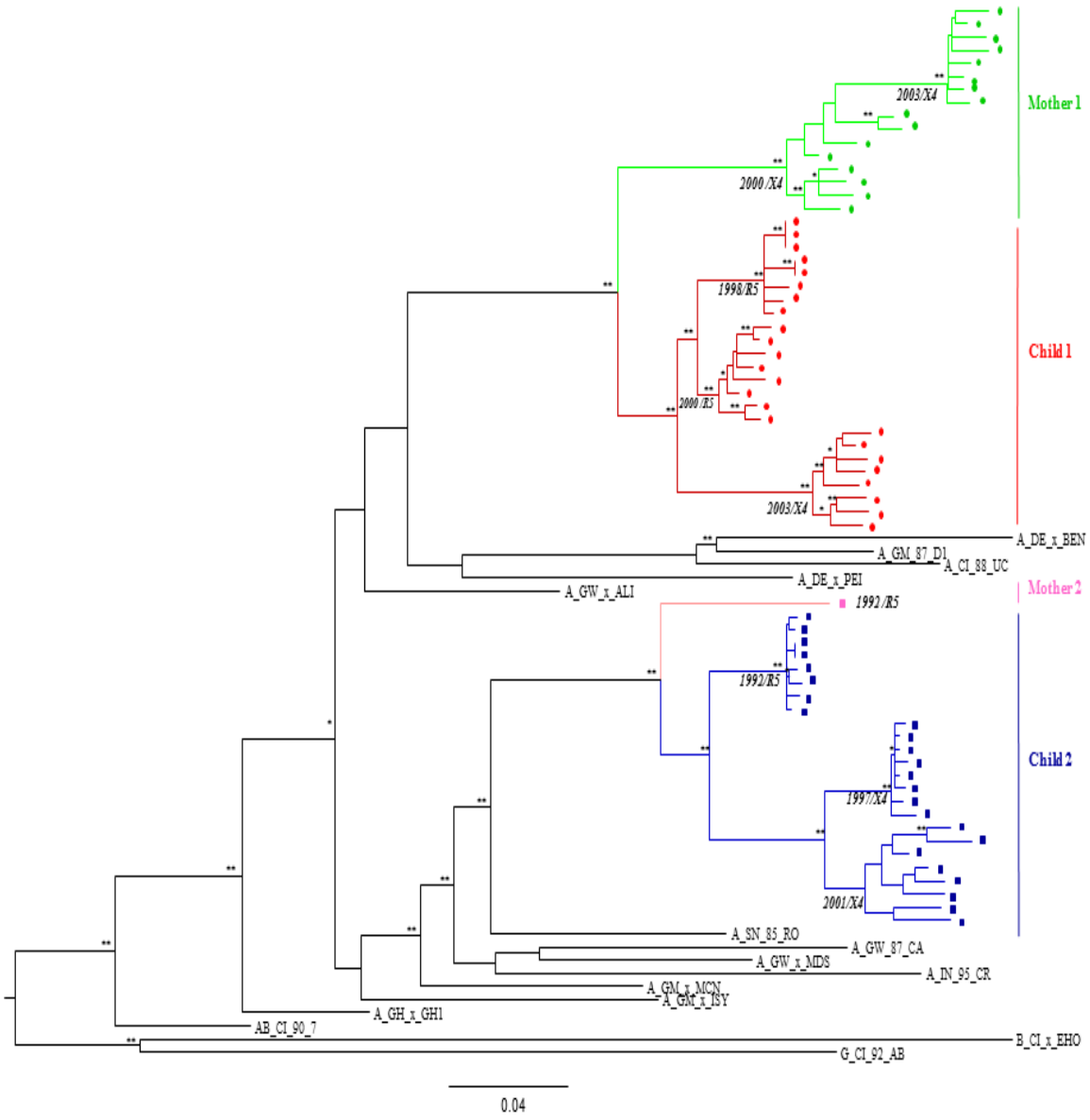

Figure 3.1 - - Evolutionary relationships between mother and child env sequences. A maximum likelihood phylogenetic tree was constructed using alignments of clonal env sequences obtained from the children in successive years and from their mothers with reference sequences from HIV-2 groups A, B and G. The bootstrap values supporting the internal branches are shown as follows: * bootstrap >70, ** bootstrap >90. The scale bar represents evolutionary distance in number of substitutions per site. Sequences from child 1 (PTHDECT) are represented by red circles, with each year of sample collection in orange (1998), red (2000) and dark red (2003); sequences from mother 1 (PTHCC20) are represented by green circles, with each of sample collection in light green (2000) and dark green (2003); sequences from child 2 (PTHDESC) are represented by blue squares with each year of sample collection in light blue (1992), blue (1997) and dark blue (2001); sequence from mother 2 has been published before [32] and is represented by a pink square. The tropism of the viruses is indicated to the right of each cluster. Evolutionary relationships between mother and child env gene sequences.

Child 2 was born with encephalopathy (CDC clinical stage C1) but with normal $CD4^+$ T cell percentage (52%) and undetectable viral load [123, 124] (Table 3.1). The transmitted/founder virus was CCR5-tropic, as determined by our V3 loop sequence analysis, but induced syncytia formation in peripheral blood mononuclear cells [123, 124]. At age 5, CD4 percentage decreased to 25% and the virus changed to CXCR4-tropic, as determined by V3 loop sequence analysis. AZT therapy (1992 up to 1997) and AZT + 3TC therapy (in 2001) did not prevent increase in viral load and further $CD4^+$ T cell decline and the child died of AIDS at age 9.

Potent neutralizing antibodies are produced since the first year of infection and selects for Nab-resistant X4 isolates

Nabs were detected in child 1 against both autologous virus isolates (CT00 and CT03) since day 27 of birth. Nab titers against the R5 isolate CT00 rose continually until age 8 (Figure 3.2). In contrast, after an initial increase, Nab titers against the X4 isolate (CT03) dropped at age 2 (year 2000) and decreased continuously from age 5 onwards, the titers being significantly lower compared to those raised against the R5 isolate (CT00). Considering all time points together, Nab titers were significantly higher against the R5 isolate (CT00) compared to the X4 isolate (CT03) [median (range) of reciprocal $\log_{10}$ IC50 neutralization titers against CT00 and CT03 were 4.6 (3.7-5.4) and 4.1 (3.2-4.4), respectively, P = 0.0472, Mann-Whitney test]. The close correlation between the rates of Nab escape and R5-to-X4 phenotypic switching suggests that phenotype transition in this infant was driven by the Nabs.

Notably, child 1 also produced neutralizing antibodies that potently neutralized several heterologous primary HIV-2 isolates. Again, the heterologous Nabs were significantly more effective against R5 strains than against X4 strains [median (range) of reciprocal $\log_{10}$ IC50 neutralization titers against R5 and X4 isolates were 3.5 (1.6 - 4.0) and 2.5 (1.6 - 4.0), respectively, P = 0.0041] (Figure 3.3).

In child 2 we could only analyse the evolution of Nab response against the autologous X4-isolate (SC01) from age 5 onwards. Comparing Nab response at age 5 in both patients (the only age-matched data point), we found that it was significantly weaker in child 2 than in

child 1 [median (range) of reciprocal $\log_{10}$ IC50 neutralization titer of child 2 against SC01 was 3.5 (3.4 - 3.6), and those of child 1 against autologous CT00 and CT03 isolates were .3 (5.0 - 5.5) and 4.2 (4.0 - 4.4), respectively, P<0.001] (Figure 3.2). Moreover, in contrast to child 1, Nab titer decreased steadily with infection time as viral load increased and disease progressed to AIDS and death at age 9 (Figure 3.2 and Table 3.1). Considering all time points together, average Nab titers were lower than those of child 1 against the age-matched X4 isolate CT03 [median (range) of reciprocal $\log_{10}$ IC50 neutralization titer against isolates SC01 and CT03 were 3.3 (3.0-3.5) and 4.1 (3.2-4.4), respectively, P = 0.057] and against the R5 isolate CT00 [median (range) of reciprocal $\log_{10}$ IC50 neutralization titer against isolates SC01 and CT00 were 3.3 (3.0-3.5) and 4.6 (3.7-5.4), respectively, P = 0.0106].

HIV-negative plasmas failed to neutralize HIV-2 strains and HIV-2 plasmas failed to neutralize HIV-1SG3.1 or viruses pseudotyped with VSV envelope indicating the absence of nonspecific inhibitory activities in these samples.

Overall, the results obtained with child 1 demonstrate that potent neutralizing antibodies (autologous and heterologous) can be elicited very rapidly after HIV-2 vertical infection. Neutralizing antibodies are highly effective against the transmitted R5 isolates but rapidly select for X4 isolates that escape neutralization. In the absence of effective antiretroviral therapy, as was the case of child 2, increased replication of the Nab-resistant X4 isolates likely contributed to rapid $CD4^+$ T cell depletion and progression to AIDS.

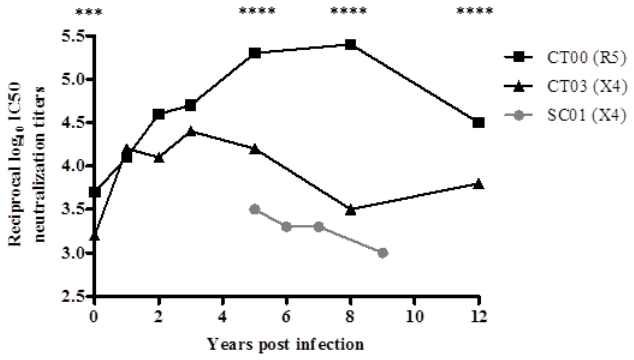

Figure 3.2 – Evolution of the autologous neutralizing antibody response in the children over the course of infection. The neutralizing activity present in patients serum was analyzed against their primary virus isolates using a luciferase reporter gene assay in TZM-bl cells; sera from child 1 from years 1998, 1999, 2000, 2001, 2003, 2006 and 2010 were tested against autologous viruses from 2000 [CT00 (R5) - black squares] and 2003 [CT03 (X4) – black triangles] and sera from child 2 from years 1997, 1998, 1999 and 2001 were tested against autologous virus from 2001 [SC01 (X4) – grey triangles]. The F test was used to compare IC50 values obtained for CT00 (R5) and CT03 (X4) isolates. ***P=0.0008, ****P<0.0001.

(a)

Years of infection	Reciprocal log_{10} IC50 neutralizing titers against heterologous primary HIV-2 isolates (tropism)				
	03PTHCC6 (R5)	03PTHCC12 (R5)	03PTHCC19 (R5)	03PTHCC20 (X4)	03PTHSM9 (X4)
0	>3.7	1.9	>3.7	2.5	2.5
1	3.4	2.4	n.d.	2.6	n.d.
2	>3.7	2.9	n.d.	3.1	2.0
3	>3.7	3.2	n.d.	2.8	<1.9
5	>3.7	3.3	n.d.	2.7	<1.9
8	>3.7	3.5	n.d.	1.9	<1.9
12	>3.7	3.5	2.7	2.3	<1.9

n.d. – not done due to lack of plasma.

(b)

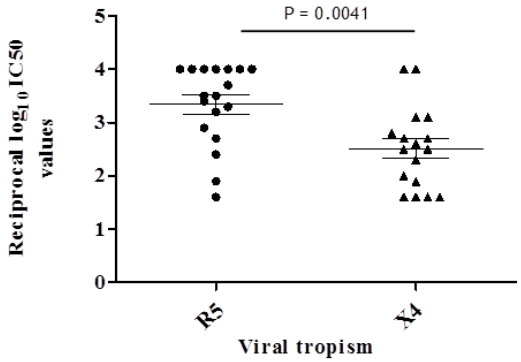

Figure 3.3 –Neutralizing antibody response against heterologous primary isolates in child 1 over the course of infection. A) A heat map of the reciprocal log-transformed IC50 value of each plasma sample from child 1 (left) against a panel of five heterologous primary virus isolates with respective tropism (top) is shown. The reciprocal log_{10} IC50 value is colour-coded. The darkest colour indicates that neutralization above 50% was still detected with the highest plasma dilution tested (1/5120). The lightest colour indicates that there was no detectable neutralization above 50% with the lowest plasma dilution tested (1/40). n.d. – not done (due to lack of plasma); B) Dot-plot graphic showing the mean and standard deviation of the reciprocal log_{10} IC50 values obtained against R5 and X4 isolates indicated in A. Mann-Whitney U test was used to compare the median log_{10} reciprocal IC50 values.

HIV-2 Env evolution in acute/early and late infection

At birth, nucleotide diversity in child 1 was twice that of child 2 in *env* and five times in C2V3C3 region (Table 3.2). At age 5, nucleotide diversity increased 2-fold in *env* and C2V3C3 in child 1 while in child 2 it only increased in C2V3C3 (2-fold) leading to an even higher difference in *env* and C3V3C3 diversity (3- and 6-fold, respectively). Interestingly, in the last 4 years of infection in child 2, diversity increased significantly both in *env* (4-fold when compared to diversity at age 5) and C2V3C3 (11-fold) exceeding that of child 1 at age 5. However, in contrast to the first years of infection, most substitutions occurring in this period were of a synonymous nature as indicated by the sharp decrease in the ω value both in *env* and C2V3C3.

The evolutionary rate of *env* was significantly higher in child 1 than in child 2 (0.0141 *vs* 0.0073 substitutions/site/year, posterior probability (PP) value <5%) (Table 3.2). When focusing on the C2V3C3 region, the evolutionary rates were not significantly different (0.0142 *vs* 0.0105 substitutions/site/year, PP =20%). There was a trend towards positive selection in child 1 (non-synonymous rate: *env*, 0.0143 and V3, 0.0152; synonymous rate: *env*, 0.0137 and V3, 0.0124 substitutions/site/year) and purifying selection in child 2 (non-synonymous rate: *env*, 0.0069 and V3, 0.0092; synonymous rate: *env*, 0.0082 and V3, 0.0132 substitutions/site/year) in both *env* and V3.

Table 3.2 - Nucleotide diversity and divergence rates in the *env* gene and C2V3C3 region

Patient	Sampling year	ω[a]		Nucleotide diversity[b] (SD)		Evolutionary rate[c] (95% HPD)	
		env	C2V3C3	env	C2-V3-C3	env	C2V3C3
Child 1	1998	0.96	1.52	0.013 (0.0061)	0.014 (0.0063)	0.0141 (0.0075, 0.0211)	0.0142 (0.0082, 0.0208)
	2000	0.88	5.78	0.027 (0.0047)	0.015 (0.0074)		
	2003	0.65	0.50	0.027 (0.0057)	0.031 (0.0118)		
Child 2	1992	1.15	0.38	0.007 (0.0022)	0.003 (0.0026)	0.0073 (0.0036, 0.0115)	0.0105 (0.0053, 0.0174)
	1997	0.99	2.60	0.008 (0.0025)	0.005 (0.0026)		
	2001	0.66	0.62	0.035 (0.0122)	0.055 (0.0157)		

[a] Ratio of nonsynonymous to synonymous substitution rates; [b] Within-patient genetic distances and standard deviation (SD) as determined by averaging pairwise tree distances over all the sequences obtained for each patient at each time point; [c] Nucleotide substitutions per site per year (HPD, highest posterior density).

We also analysed the evolution of amino acid diversity, as determined by the sum of Shannon's entropy, in variable regions of gp125 which contains most of the neutralizing domains [109, 180, 323, 324]. At birth, amino acid diversity was higher in child 1 than in child 2 (Table 3.3). At age 5, amino acid diversity increased significantly only in V1 and V3 in both patients, this being much more pronounced in child 1. In child 2, from age 5 to age 9 (death), amino acid diversity increased in V1 (9.3-fold), V3 (2.1-fold) and V4 (1.6-fold), though never reaching the level of diversity observed in child 1 at age 5. Amino acid changes observed after the first year in V1 and V3 are shown in Figure 3.4. In V1 there was no clear pattern of change except for the 2-4 amino acids deletion detected at year 5 in both patients. This

deletion was maintained along the full course of infection in child 2. In child 1, three mutations occurring at age 3 were fixed (were kept in year 5) and 4 mutations reversed back to the original residue suggesting that these changes affected viral fitness; in child 2, ten mutations were fixed over the course of infection and there were no reversions suggesting that the mutations did not reduce the fitness of the virus or that compensatory mutations occurred in other regions. Three of the fixed mutations in child 2 were located in the putative neutralizing epitope. In child 1 two mutations of a potentially disruptive nature emerged in the neutralizing epitope (N to K and T to E/G).

Table 3.3 – Evolution of amino acid diversity in variable Env regions in the first five years of infection

Patient	Variable regions	Sum of entropy		Fold increase
		year 1	year 5	
child 1	V1	1.324	8.657	6.5
	V2	0	1.885	na
	V3	0.754	3.614	4.8
	V4	0.939	0.662	na
	V5	0	3.402	na
	V1-V5	3.017	18.22	6.0
child 2	V1	0.377	0.754	2.0
	V2	1.131	0.377	na
	V3	0.377	1.131	3.0
	V4	0	1.316	na
	V5	0.377	0	na
	V1-V5	2.262	3.578	1.6

na- not applicable

In V3, mutations occurred almost exclusively within the neutralizing epitopes, and at residues 18, 19 and 27 that have been associated with R5 and X4 tropism [84, 102, 104]. One amino acid insertion occurred in the same position in both children and involved a hydrophobic residue (V in child 1; I in child 2). This type of insertion has also been associated to R5-to-X4 tropism switch [84, 104].

Env adaptation to Nab pressure is usually associated with positive selection of specific amino acids that might be located in neutralizing domains [301]. At year 5 of infection there were 10 positively selected sites in Env of child 1 (seven in gp125) (Table 3.4). Most sites (6 out of

10) were located in confirmed neutralizing domains (V2, V3 and C5 in gp125 and MPER in gp36). In contrast, positively selected sites were absent in child 2 at year 5 of infection and there were only 2 selected sites in the final year of infection. These results reveal a much better adaptation to Nab pressure in child 1 compared to child 2.

In all, these results show that HIV-2 *env* can evolve and diversify very rapidly in the first years of infection. The positive correlation between the rate of Env evolution, in terms of nucleotide divergence from the founder virus, nucleotide diversity, amino acid diversity, and positive selection, and the rate of Nab response and escape indicates that the neutralizing antibodies drive HIV-2 Env evolution in the first years of infection.

Table 3.4 – Positive selective pressure on the Env glycoproteins in both children over the course of infection

Env glycoprotein	**Codons under selective pressure (location)**[1]					
	Child 1			**Child 2**		
	1998	**2000**	**2003**	**1992**	**1997**	**2001**
gp125	none	5, 7 (SP)	178 (V2), 255, 259 (C2), 320 (V3), 459 (V5), 467, 471 (C5)	none	none	395 (C3)
gp36	none	none	552 (HR1), 672, 673 (MPER)	none	none	562 (HR1)

[1]Codons identified as being significantly (P <0.05) under selective pressure are indicated; SP, signal peptide; V2, variable region 2; C2, conserved region 2; C3, conserved region 3; V3, variable region 3; V5, variable region 5; C5, conserved region 5; HR1, helical region 1; MPER, membrane proximal external region.

Tropism and susceptibility to antibody neutralization are closely associated with V3 structure

In long-term HIV-2 infected individuals the envelope V3 region adopts a significantly different structure in Nab-resistant isolates as compared to Nab-sensitive isolates, supporting a direct role of V3 conformation in the different susceptibility of these viruses to antibody neutralization [97]. To gain some insight into the structural evolution of the V3 region in the first years of HIV-2 infection and try to relate it to tropism and susceptibility to antibody neutralization, model structures of C2-V3-C3 regions from both children were generated by homology modelling using the three-dimensional structure of an unliganded

SIV gp120 envelope glycoprotein as template. Remarkably, the V3 loop, which was characterized by a high content of irregular secondary structure in the first year of infection, converged to an similar β-hairpin structure at year five of infection in both infants and remained in this conformation until the last year of infection in child 2 (Figure 3.5 and Table S3.2). The rate of acquisition of the β-hairpin conformation fully correlated with the rate of R5-to-X4 tropism transition and with the rate of escape from antibody neutralization.

Discussion

There is limited knowledge on the natural history of HIV-2 infection and on the molecular and phenotypic evolutionary dynamics of HIV-2 because no study has investigated the full course of infection from the time of seroconversion. The current study is the first characterization of the Nab response and molecular and phenotypic evolution of HIV-2 followed from acute infection to late stage infection. Our studies were based on two children infected by vertical transmission and spanned the first 12 years of infection in one case and the complete infection period in the other (9 years). We show that a potent Nab response is raised very early after infection and that the rate and pattern of molecular and phenotypic evolution of the HIV-2 Env are closely associated to the rate of Nab escape.

Child 2 was born severely ill and with a low number of CD4 cells [123, 124] whereas child 1 was born asymptomatic and with normal CD4 levels. Despite the contrasting clinical conditions at birth, major CD4 depletion and disease progression occurred in both children in the first 5 years of infection. This fast disease course is typical of HIV-1 infected children in the absence of antiretroviral treatment [395] but is highly unusual in HIV-2 infected individuals [122, 373, 396, 397]. Both patients were infected with R5 strains but transitions to X4 tropism occurred rapidly, being detected after only 5 years of infection. This is the first time that a full R5-to-X4 tropism switch is observed in HIV-2 infected patients and it was unexpected to find it in paediatric patients. Like in some adult HIV-2 patients with advanced disease [84, 97, 152], the emergence of X4 viruses in our patients was associated with high viral load, marked CD4 depletion and disease progression. Hence, the rapid disease course in the two infants may have been determined by the early emergence of X4 isolates.

(a)

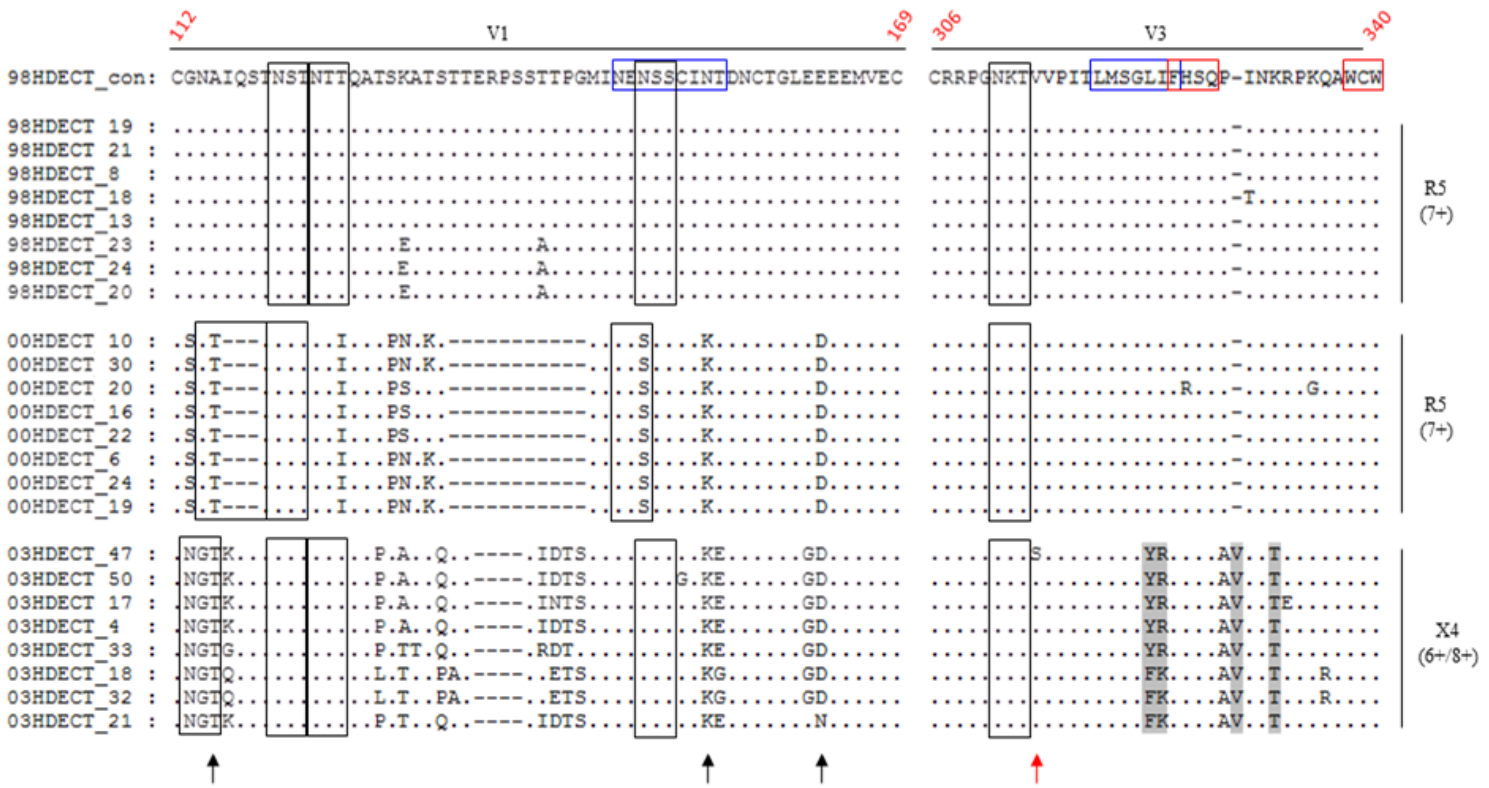

Figure 3.4 – Evolution of V1 and V3 regions. Clonal V1 and V3 amino acid sequences obtained over the course of infection from child 1 (panel A) and child 2 (panel B) were aligned against consensus sequences from the transmitted/founder isolate. Dots in the alignments indicate sequence identity to the consensus; dashes indicate deletions. Potential N-linked glycans (occurring at NXT/S) are represented in open boxes. Amino acids highlighted in light grey are involved in tropism change [104]. Blue boxes in the consensus sequence represent linear neutralizing epitopes in V1 [326] and V3 [324]; red boxes in the consensus sequence represent a conformational epitope in V3 [324]. Viral tropism is indicated at the right of the alignment as determined phenotypically or genotypically based on V3 loop sequence patterns. Black arrows signal fixed mutations.

(b)

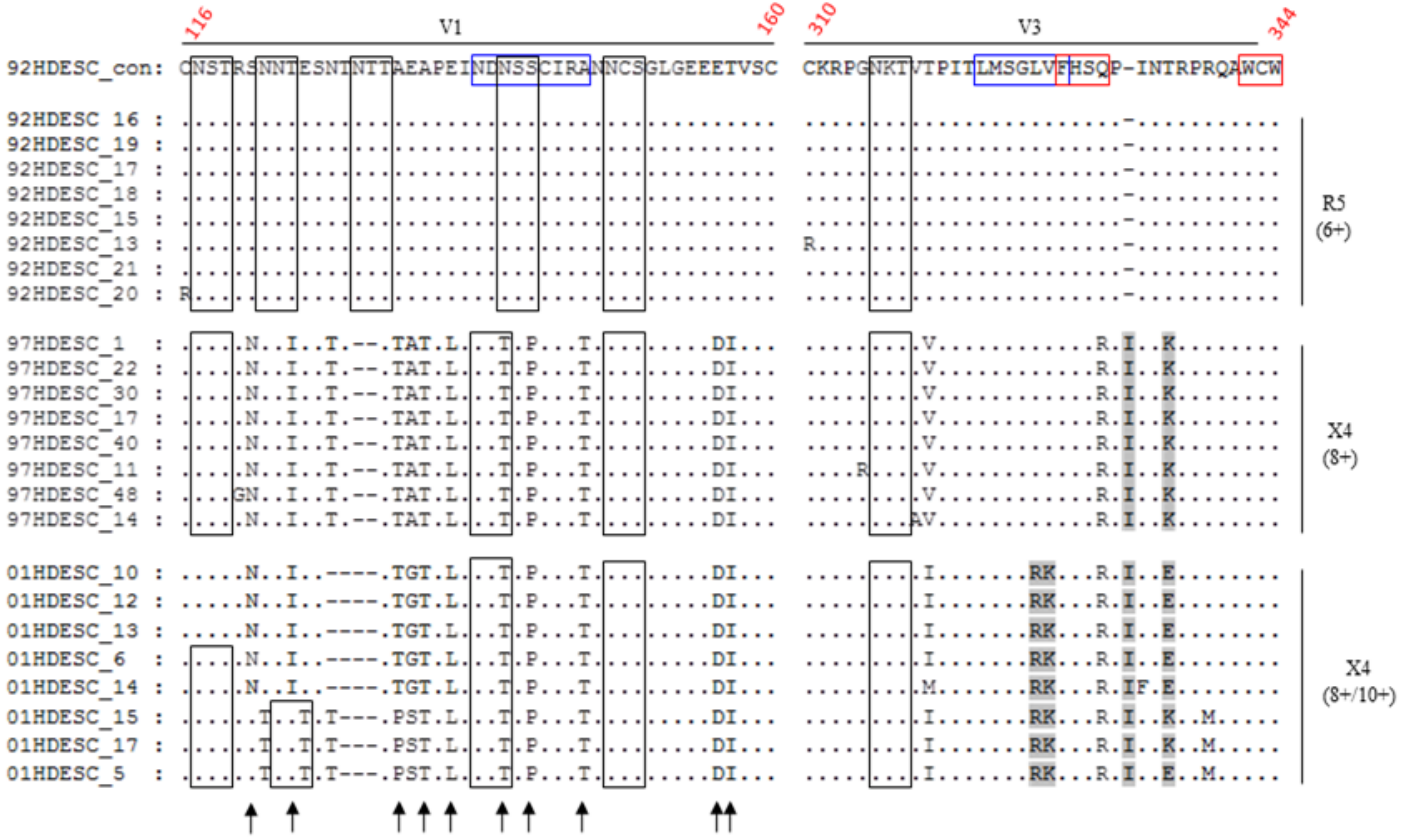

Figure 3.4 (*Continued*)

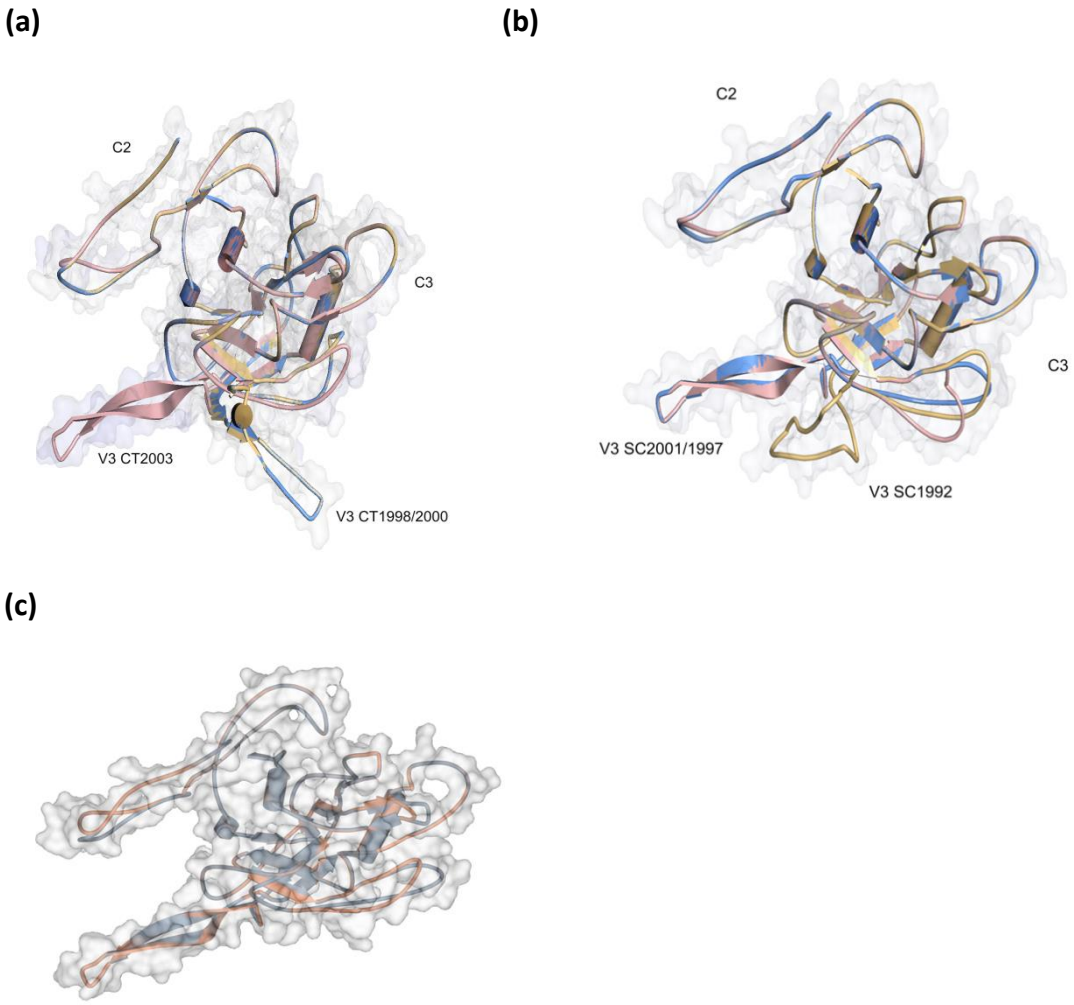

Figure 3.5 – Evolution of the structure of C2-V3-C3 envelope region. Three-dimensional structures of C2-V3-C3 amino acid sequences from child 1 and 2 were generated by homology modelling using the three-dimensional structure of an unliganded SIV gp120 envelope glycoprotein as template. A) Superimposition of the structures of C2-V3-C3 of child 1 in 1998 (yellow), 2000 (blue) and 2003 (pale red); B) Superimposition of the structures of C2-V3-C3 of child 2 in 1992 (yellow), 1997 (blue) and 2001 (pale red). V3 loop and C2 and C3 stretches are indicated in the figures. C) Three-dimensional structures of C2-V3-C3 from child 1 (CT) in 2003 (blue) and child 2 (SC) in1997 (red). At this time both viruses were X4 and showed resistance to Nabs.

At birth, HIV-2 nucleotide diversity in child 1 was 2-fold higher than in child 2 both in *env* and C2V3C3. Nucleotide diversity in child 1 was also 2-fold higher than in HIV-1 infected children in the first weeks after birth [206, 210, 398] and in HIV-1 adult patients during seroconversion [399]. Envelope diversity also increased more significantly, both at the nucleotide and amino acid levels, in child 1 than in child 2 in the first 5 years of infection. Consistently, the evolutionary rate of the *env* gene in child 1 was almost two times higher than child 2 and similar to that found in chronic HIV-2 infected patients under ART (0.0102

substitutions/site/year) [157]. These results reveal a remarkably high rate of molecular evolution of the HIV-2 envelope in child 1 during the first 5 years of infection and a moderate rate of evolution in child 2. The evolutionary rate in child 2 was similar to that in HIV-1 patients who, when untreated, have a disease progression generally similar to that of child 2. Thus, the higher rate in child 1 is consistent with the better immune control.

Previously, we have shown that production of gp36-specific and gp125-specific antibodies occurred during the first year of age in child 1 and that, at age 2, levels of binding antibodies to these glycoproteins were already similar to those found in HIV-2 infected patients in the chronic stage of disease [291]. In child 2, although gp36-specific antibodies were produced to near normal levels, there was a remarkably weak production of gp125-specific binding antibodies. Consistently, in this work we found that child 1 produced a much stronger Nab response than child 2. In child 1 autologous neutralizing antibodies appeared within the first year of infection, increased over time to levels that were similar to chronically infected patients [109, 180, 269], and were sustained until at least the age of 12. Moreover, child 1 also produced a potent Nab response against several heterologous virus isolates. As for child 2, the autologous Nab titer was significantly lower compared to child 1 at age 5 and decreased continuously to very low titers following the rapid decline of CD4 cells and progression to AIDS and death at age 9. These differences in Nab response correlate well with the magnitude and rate of envelope evolution in the infants which suggest a close association between the neutralizing antibody response and the evolution of the HIV-2 envelope in these infants.

Several lines of evidence further suggest that escape from Nab response is a major determinant of the evolution of the HIV-2 envelope in these infants, especially in child 1. First, complete replacement of virus quasispecies was noted in phylogenetic analysis of *env* sequences produced at the different time points which is compatible with a situation of ongoing viral escape from antibody neutralization [400]. Second, amino acid diversity increased significantly with infection time, especially in V1 and V3 which are major neutralizing domains in HIV-2. This is a major HIV escape mechanism as a single polymorphism can alter epitope sequence and/or conformation and reduce recognition and/or binding affinity by neutralizing antibodies [129, 176, 301, 400-402]. Third, increase in

dN/dS ratio and positive selection in the envelope were closely related to rate of Nab escape in child 1 [176, 301]. Finally, the similar gain of secondary structure in V3 in both patients fully correlated with the rate of escape from antibody neutralization. This has been recently associated to HIV-2 resistance to antibody neutralization in chronic HIV-2 infected patients [97].

Nabs were significantly more potent against R5 isolates than against X4 isolates (autologous and heterologous) confirming the inherent resistance of X4 viruses to antibody neutralization [97, 368]. More importantly, increase in Nab resistance in child 1 preceded the emergence of X4 variant suggesting that tropism switch was driven by the neutralizing antibodies. Given the immunodominance of the V3 region in HIV-2 infected patients [139], the location of two of the three amino acid residues that are associated to R5 and X4 tropism (positions 18 and 19) [84, 102, 104] within the first neutralizing epitope in V3 [324] and the major difference in V3 conformation of R5 and X4 strains [97], the close association between HIV-2 susceptibility to antibody neutralization and tropism is not surprising.

The main limitations of this study are the small number of patients and the inexistence of viral isolates from all time points in both children. However, worldwide it has been impossible to find individuals acutely infected with HIV-2. Moreover, due to the low or absent viral load it is often impossible to isolate virus from HIV-2 infected patients. Therefore, we believe that even with these limitations our results are a major contribution to our understanding of the natural history of HIV-2 infection and of the role of the immune system in controlling and shaping HIV-2 evolution.

Conclusions

In conclusion, we show that a potent Nab response is elicited very early after HIV-2 infection and that the HIV-2 envelope evolves at a high rate in the first years of infection, this rate being directly correlated to the potency of the Nab response. R5-to-X4 tropism switch, increased nucleotide and amino acid diversity in V1 and V3, and convergence of V3 to a β-hairpin structure were closely associated with escape from the Nab response. The data strongly suggests that neutralizing antibodies are the main driver of the rapid molecular and phenotypic evolution of the HIV-2 envelope in HIV-2 infection. Our studies provide further

support to a model of HIV-2 pathogenesis in which the neutralizing antibodies play a central role and have clear implications for the vaccine field.

Competing interests

The authors have no commercial or other type of association that might pose a conflict of interest.

Authors' contributions

NT and TL designed the study; CR, HS, TL, AQ and NT analysed the data and wrote the paper; CR performed the experiments; CR, HS and TL, performed the evolutionary analysis;, CR, RC, PB, JMM, IB and HB provided analytical reagents and nucleotide sequences; CR, PB and NT performed the statistical analysis; CF and AQ performed the structural analysis; PG performed the viral load assays; LR and PCS contributed clinical data from the patients. The final text was read and approved for submission by all authors.

Acknowledgements

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Additional material

Table S3.1 – PCR and sequencing primers for the HIV-2 env gene.

Primer	Position	Sequence 5' – 3'
CR1[a]	5927 – 5946	aggaaacag(c,t)gg(a,c)gaagaga
CR2[a]	9391 – 9369	tctacatcatccatattttg(c,t)tg
CR3[b]	6673 – 6692	ctcat(c,t)cgtcttctgcatca
CR4[b]	9286 – 9268	tcacaggagggcgatttct
CRSEQ2[c]	7363 – 7345	atcccaatagtgctt(a,g)tca
CRSEQ3[c]	7313 – 7334	cattgcaacacatcagtcatca
CRSEQ4[c]	7918 - 7898	ccaattgaggaaccaagtcat
CRSEQ5[c]	7859 – 7879	atgtggactaactgcagagga
CRSEQ6[c]	8360 – 8344	gctgttgctgttgctgc
CRSEQ7[c]	8344 – 8360	gcagcaacagcaacagc
CRSEQ8[c]	8835 – 8817	gagaaaacaggcctatagc
CRSEQ9[c]	8817 – 8835	gctataggcctgttttctc
CRSEQ10[c]	7159 – 7173	agacaattgcacagg
CRSEQ11[c]	7424 – 7410	tggtatcattgcatc

[a]Outer PCR primer; [b]Inner PCR primer; [c]Sequencing primer

Table S3.2 - Percentage of major secondary structure motifs present in the V3 loop of HIV-2 isolates obtained from child 1 and 2

Secondary structure	Child 1 (CT)			Child 2 (SC)		
	1998 (R5 virus)	2000 (R5 virus)	2003 (X4 virus)	1992 (R5 virus)	1997 (X4 virus)	2001 (X4 virus)
α-helix	11.8 %	11.8 %	0.0 %	0.0 %	0.0 %	0.0 %
β-sheet	41.2 %	41.2 %	71.4 %	5.9 %	71.4 %	71.4 %
Turn	11.8 %	11.8 %	11.4 %	20.6 %	11.4 %	11.4 %
Random coil	35.2 %	35.2 %	17.2 %	73.5 %	17.2%	17.2 %

CHAPTER 4

Potent and broadly neutralizing antibodies despite marked memory B cell depletion in chronic HIV-2 infection

Potent and broadly neutralizing antibodies despite marked memory B cell depletion in chronic HIV-2 infection

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Research article

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Abstract

Objective: HIV-2 infection has been shown to course with significant levels of neutralizing antibodies (Nabs). Nevertheless, HIV-2 disease progression leads to marked memory B cell loss. Here, we investigated Nab responses in HIV-2 infected patients and their relationship with memory B cell imbalances.

Design and methods: Thirty seven chronically infected HIV-2+ individuals were studied, 73% of which were antiretroviral (ART) drug naive, 76% had undetectable viral load and 59% had ≥350 $CD4^+$ T cells/µl. Memory B cell populations were analysed by flow cytometry. Neutralizing antibody titers were assessed on TZM-bl cells against a panel of four primary R5 isolates and Env-specific binding antibodies were determined using the ELISA-HIV2 assay.

Results: Nabs were present in all patients (median reciprocal log_{10} IC50 titer: 2.86; interquartile range: 2.64-3.10). Apart from two exceptions, all individuals neutralized at least two viruses (55% neutralized three or four viruses). Potency of the Nab response was unrelated to viral load and $CD4^+$ T cell counts. Nab titers were positively associated with binding activity of C2V3C3-specific antibodies in patients with ≥350 $CD4^+$ T cells/µl, and of gp36-specific antibodies in patients with more advanced disease. Remarkably, Nab titers correlated inversely with the frequency of unswitched and switched memory B cells in the untreated group of patients.

Conclusion: The potent neutralizing antibody response elicited throughout HIV-2 infection occurred despite the loss of memory B cells observed with disease progression. The data suggests a role for long lived plasma cells in the production and perpetuation of neutralizing antibodies and may have important implications for the vaccine field.

Keywords: HIV-2 infection, neutralizing antibodies, memory B cells

Introduction

HIV-2 disease is characterized by low-to-undetectable viral load in the plasma and genital tract of HIV-2 infected individuals throughout infection [115, 117, 134, 403-405], a feature that possibly accounts for HIV-2's lower transmissibility and pathogenic potential [110, 115]. Nevertheless, similarly to HIV-1, HIV-2 infection causes progressive $CD4^+$ T cell depletion and AIDS, albeit at a much slower rate [138, 142, 143, 146, 406].

Suppression of viral replication in HIV-2 disease has been suggested to be related to well-preserved innate and specific immune responses [57, 220-222, 224, 249, 261, 263, 285]. Indeed, during the chronic (asymptomatic and aviremic) phase of infection, high $CD4^+$ T cell counts have been associated with the production of highly polyfunctional HIV-specific $CD4^+$ and $CD8^+$ T cells [249, 261], efficient cytolytic activity of natural killer cells [224], and preserved dendritic cell function and interferon-α production [220, 222, 285]. Of note, most HIV-2 positive individuals are able to produce and maintain high levels of broad and potent neutralizing antibodies, in striking contrast with their HIV-1 counterparts [109, 180, 269, 291, 313]. This preserved Nab response seems to be related to particular features in the envelope glycoproteins of HIV-2 that favour antibody recognition [109, 180, 269, 291, 313, 407, 408].

HIV-2, like HIV-1 infection, induces polyclonal B-cell activation and hypergammaglobulinemia [291, 292], and disease progression occurs in direct association with increased T cell activation [138, 406]. Importantly, we have shown that the progression of HIV-2 disease was associated with a marked depletion of both switched and unswitched memory B cells not recovered by ART [270]. Additionally, HIV-2 disease progression leads to a significant decrease in the breadth and potency of Nab responses [97]. The relationship between B cell dysfunctions and Nab response in HIV-2 infection has yet to be determined.

Here we evaluated Nab responses in HIV-2 infection and assessed their association with the previously described memory B cell disturbances [270]. All patients were found to produce potent and broadly neutralizing antibodies even in the presence of marked memory B cell depletion. Interestingly, whilst C2V3C3-specific Nabs were mainly produced before significant $CD4^+$ T cell depletion occurred, gp36-specific antibodies were more characteristic of later disease stages. Our results suggest an important role for B cell subsets, other than the memory pool, in maintaining envelope-specific Nab responses in HIV-2 infection.

Materials and Methods

Study subjects and ethics

The study involved 37 previously studied HIV-2 infected individuals [270]. Clinical and immunological data of the patients are detailed in Supplemental Digital Content 4.1. Twenty seven patients (73%) were untreated; 28 (76%) had undetectable viral load and 22 (59%) had $CD4^+$ T cell counts above 350 cells/µl. Informed consent for blood collection and participation in the study was obtained from all participants. The study was approved by the Ethical Board of the Faculty of Medicine, University of Lisbon.

Plasma viral load assessment

HIV-2 levels of circulating virus (viremia) were quantified by RT-PCR (detection threshold: 200 RNA copies/ml), as described [154].

Neutralization assay

The neutralizing activity present in patients' plasma was analysed against a panel of four heterologous primary R5 tropic isolates (03PTHCC6, 03PTHCC12, 03PTHCC19, 03PTHSM2) showing different sensitivity to IgG neutralizing antibodies [97] using a luciferase reporter gene assay in TZM-bl cells, as described previously [303, 368, 409]. Briefly, 10,000 cells in 100 µl of complete growth medium (GM: DMEM supplemented with 10% heat-inactivated fetal bovine serum, FBS) were added to 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight. 100 µl of each virus (corresponding to 200 TCID50) were incubated for 1 h at 37°C with 2-fold serial dilutions of heat-inactivated patients' sera in a total volume of 200 µl of GM containing DEAE-Dextran (20 µg/ml). The lowest plasma dilution used in the assays was 1:40. Forty-eight hours later, plates were analysed for luciferase activity on a luminometer (TECAN) using the One-Glow Luciferase Assay System (Promega, Madison, WI). Medium-only control wells were measured as background, and virus-only control wells were included as 100% infection. Nonspecific inhibition was assessed by testing all viruses against HIV-negative plasmas and all plasma samples from HIV-2

patients against HIV-1 pseudotyped with the vesicular stomatitis virus (VSV) envelope (using pSGΔenv plasmid as a backbone). HIV-negative plasmas failed to neutralize HIV-2 strains and HIV-2 plasmas failed to neutralize VSV envelope pseudotyped HIV-1, indicating the absence of nonspecific inhibitory activities in these samples. Neutralization titers were expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC50). IC50 was estimated by the sigmoidal dose–response (variable slope) equation in GraphPad Prism version 5.0 [392].

Env-specific binding antibodies

Quantification of specific antibodies against HIV-2 envelope glycoproteins gp36 and gp125 (C2V3C3 region) was performed in plasma samples from 22 patients using the ELISA-HIV2 assay, which is a dual-antigen ELISA assay, as previously described [270, 407]. Briefly, microwells were independently coated (100µl/well) with the polypeptides rpC2-C3 (containing the C2, V3 and C3 regions) and rgp36 (containing the gp36 ectodomain) and incubated overnight at 4°C. Microwells were blocked with 1% gelatin for 1 h and 100 µl of a 1/100 dilution of each plasma sample was added to the wells and incubated for one hour at room temperature. After washing, a 1:2,000 dilution of goat anti-human immunoglobulin G (Fc specific) conjugated to alkaline phosphatase (Sigma-Aldrich) was added and incubated for one hour at room temperature. The colour was developed using p-nitrophenylphosphate tablets (Sigma-Aldrich) as a chromogenic substrate, and the optical density(OD) was measured with an automated LP 400 microplate reader (Bio-Rad) at 405 nm against a reference wavelength of 620 nm. The results of the assay are expressed quantitatively as ODclinical sample/ODcut-off (S/CO) ratios. For ratio values >1 the sample was considered seroreactive.

Flow cytometry

Memory B cell populations were assessed based on the expression of CD27 and surface IgD by flow cytometry, as previously described [270], in freshly collected peripheral blood.

Statistical analyses

Medians were estimated with interquartile range (IQR). The one-sample Kolmogorov-Smirnov test for normality was used for continuous variables. SPSS (version 19; SPSS Inc., Chicago, IL, www.spss.com) was used. P values were 2-tailed and significance was defined as $P < 0.05$. Non parametric Mann Whitney U test was used to compare medians of clinical and immunological data between treated and untreated patients, Nab titers (reciprocal $\log_{10}$ IC50) against the different viruses and Nab titers between the different groups of patients. To study how two variables varied together, linear regression was performed and Spearman correlation coefficients were computed. For linear regression analysis, influential points were identified by Cook's Distance and Centered Leverage Values and excluded from the analyses.

Results

Nabs are present in all HIV-2 infected patients, independently of viral load, $CD4^+$ T cell counts or antiretroviral treatment

We investigated the Nab responses in a previously described cohort of HIV-2 infected patients [270]. Untreated patients (n=27) had significantly higher $CD4^+$ T cell counts compared to the 10 treated individuals (P=0.0043) (Supplemental Digital Content 4.1), in agreement with the poor response to ART that has been described in HIV-2 infected patients [410, 411]. All patients produced Nabs against a panel of four primary R5 isolates (Supplemental Digital Content 4.2). Considering all viruses, the median reciprocal $\log_{10}$IC50 neutralization titer was 2.86 (interquartile ranged 2.64-3.10) (Table 4.1). There were, however, significant differences between the susceptibility of the four isolates to antibody neutralization ($P<0.05$) (Supplemental Digital Content 4.2). With two exceptions, all plasmas neutralized two or more viruses, and the majority (20/37, 55%) neutralized at least three of the viruses (Table 4.1). Nab titers did not differ significantly in untreated and treated individuals (P=0.0971), viremic and aviremic patients (P=0.5471), and patients with more or less than 350 $CD4^+$ T cells/µL (P=0.6096) (Supplemental Digital Content 4.3). Considering all patients, there was a positive association between Nab potency and breadth (N=37,

Spearman rank, r=0.4055, P=0.0128) (Figure 4.1). Notably, however, this association was only maintained in the untreated group of patients (untreated: N=27, Spearman rank, r=0.4916, P=0.0092; treated: N=10, Spearman rank, r=0.3290, P=0.3487).

Table 4.1 – Potency and breadth of antibody neutralization in HIV-2 infected patients

	Neutralizing titers [Median reciprocal log_{10}IC50 (interquartile range)]	Breadth of neutralization [Number (%) of plasmas that neutralize the viruses]
All plasmas/all viruses	2.86 (2.64-3.10)	37 (100)
Neutralize 100% (4/4) viruses	3.20 (2.77-3.49)	5 (14)
Neutralize 75% (3/4) viruses	2.82 (2.76-3.05)	15 (40.5)
Neutralize 50% (2/4) viruses	2.86 (2.61-2.97)	15 (40.5)
Neutralize 25% (1/4) viruses	1.72 (1.65-1.79)	2 (5)

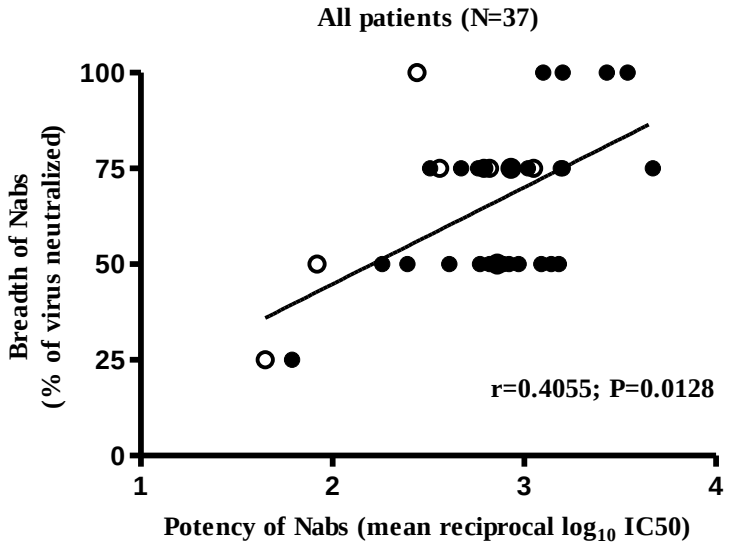

Figure 4.1 - Association between potency and breadth of antibody neutralization. Spearman's rank correlation coefficient was used to assess associations between Nab titers and breadth in all patients (N=37). Untreated patients are represented by closed circles and treated patients by open circles.

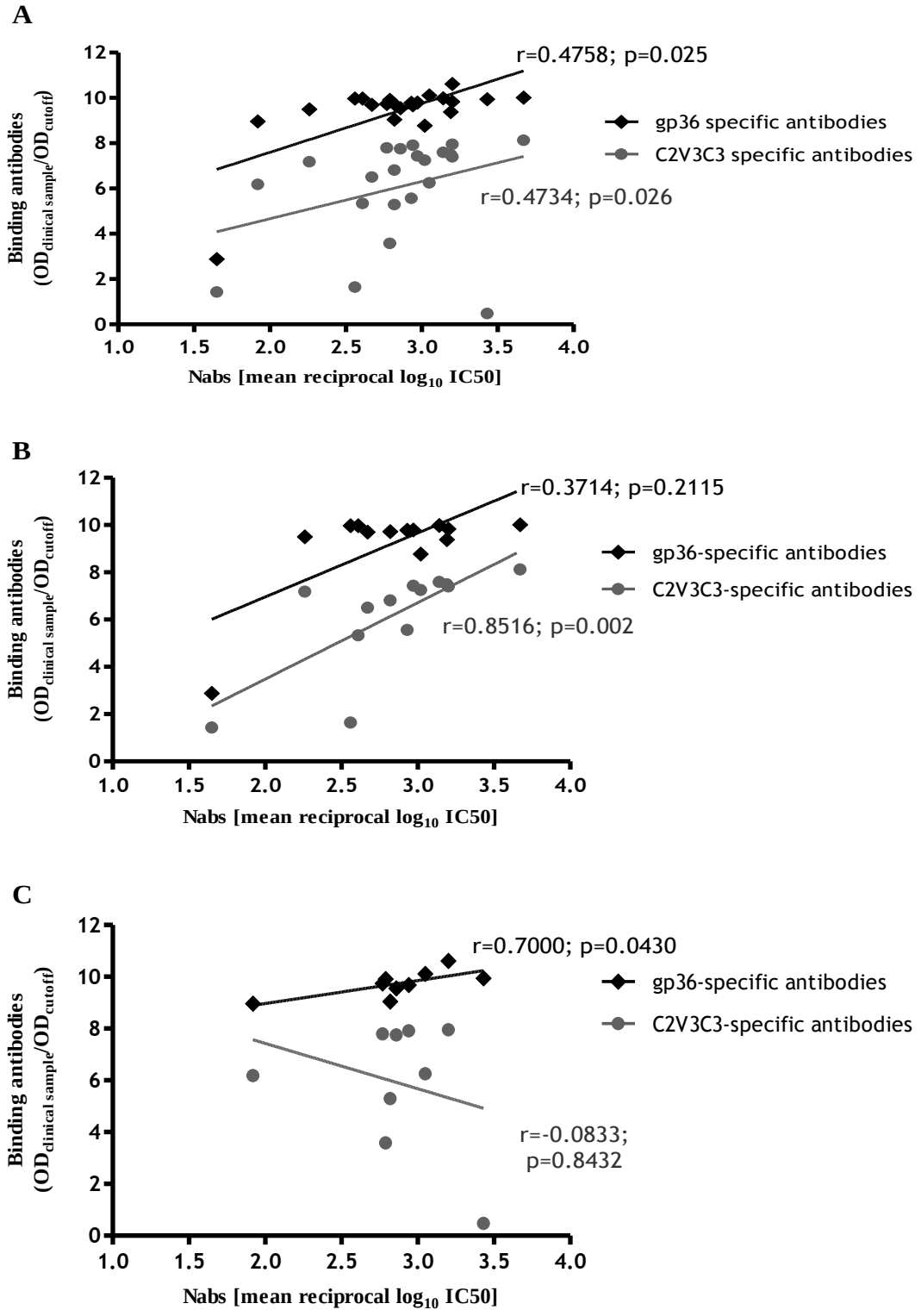

Figure 4.2 – Association between neutralizing and binding antibodies. Spearman's rank correlation coefficient was used to assess associations between Nab titers and levels of C2V3C3- (grey) and gp36-specific binding antibodies (black) in all patients (panel A), patients with $CD4^+$ T cells ≥ 350 cell/µl (panel B) and patients with $CD4^+$ T cell < 350 cells/µl (panel C).

Nabs target the C2V3C3 region of gp125 in chronic infection and the gp36 ectodomain in late infection

Nab titers were positively associated with the binding activity of C2V3C3-specific antibodies (N=22, Spearman rank, r=0.4734, P=0.026) and gp36-specific antibodies (N=22, Spearman rank, r=0.4758, P=0.025), indicating that they target both these regions in the HIV-2 Env (Figure 4.2). Notably, for the C2V3C3-specific antibodies, this association was only observed in patients with > 350 $CD4^+$ T cells/µl (N=13, Spearman rank, r=0.8516, P=0.0002), whereas for gp36-specific antibodies it was observed only in patients with < 350 $CD4^+$ T cells/µl (N=9, Spearman rank, r=0.700, P=0.043) (Figure 4.2 and Supplemental Digital Content 4.4). The erosion of C2V3C3-specific Nab response in later infection may be due to neutralization escape following sequence and/or conformational changes in V3, as previously suggested [291, 408]. Conversely, the presence of gp36-specific Nabs in advanced disease is consistent with the high conservation of this envelope region and with its overall immune dominance in HIV-2 infection [407].

Nab titers are inversely associated with memory B cells in untreated HIV-2 infection

HIV-2 infected individuals showed significant memory B cell disturbances as previously described [270]. Both unswitched and switched memory B cells were significantly depleted in these patients, particularly in those with pronounced $CD4^+$ T-cell loss, detectable viral load or on antiretroviral treatment [270]. Untreated patients had significantly higher levels of memory B cells [both unswitched (P=0.0210) and switched (P=0.0004)] compared to treated individuals [270] (Supplemental Digital Content 4.1).

In the untreated group of patients (N=27), Nab titers were inversely related with both unswitched (Spearman rank, r=-0.4162, P=0.0308) and switched memory B cell frequencies (Spearman rank, r=-0.0436, P=0.0003) (Figure 4.3 and Supplemental Digital Content 4.5). Notably, however, these associations were maintained only in the groups of untreated patients with > 350 $CD4^+$ T cells/µl and/or undetectable viral load (Supplemental Digital Content 4.5). Consistent with this, the strength of the correlation between Nab titers and frequency of memory B cell populations was higher in the untreated group of patients, where about 21% and 25% of the variability in Nab titers could be explained by the

variability in unswitched and switched memory B-cell frequencies, respectively (Figure 4.4). In the treated individuals, no association was found between Nab titers and memory B cell frequency (Supplemental Digital Content 4.5). Hence, our data suggest that, in untreated HIV-2 infected individuals, potent Nab responses are associated with a reduced memory B cell pool.

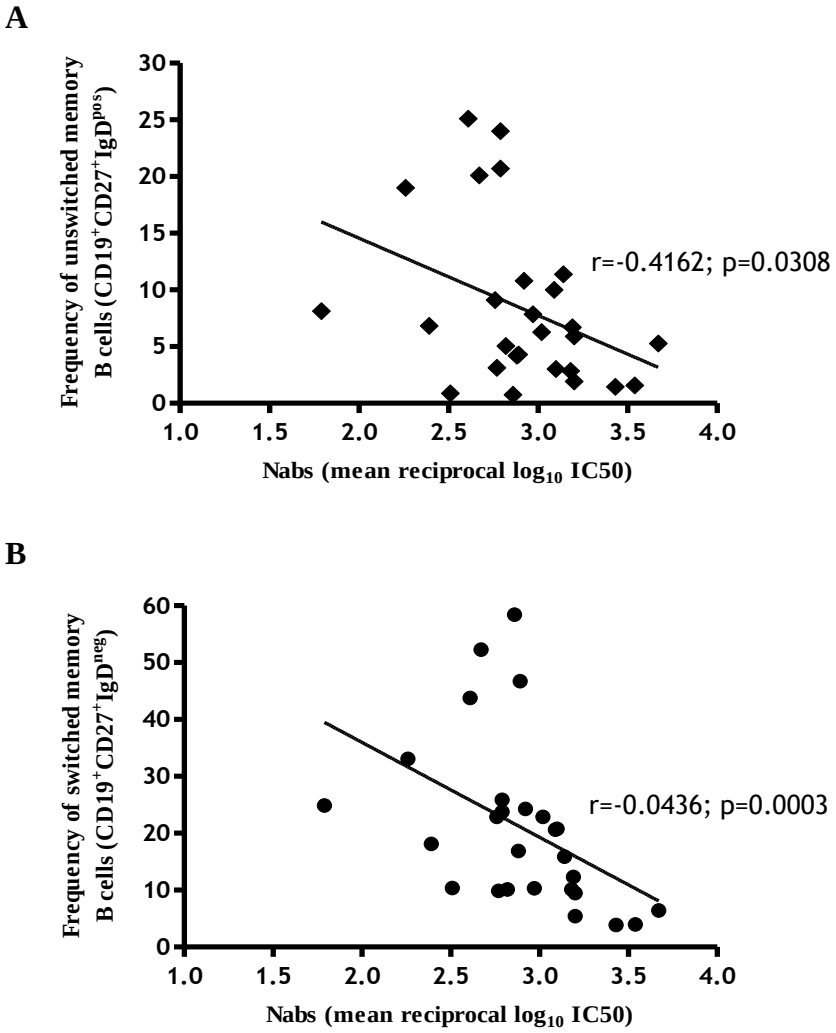

Figure 4.3 - Association between neutralizing antibodies and memory B cells. Spearman's rank correlation coefficient was used to assess associations between Nab titers and frequency of unswitched ($CD19^+CD27^+IgD^+$ cells shown in grey) (panel A) and switched ($CD19^+CD27^+IgD^-$ cells shown in black) (panel B) memory B cells.

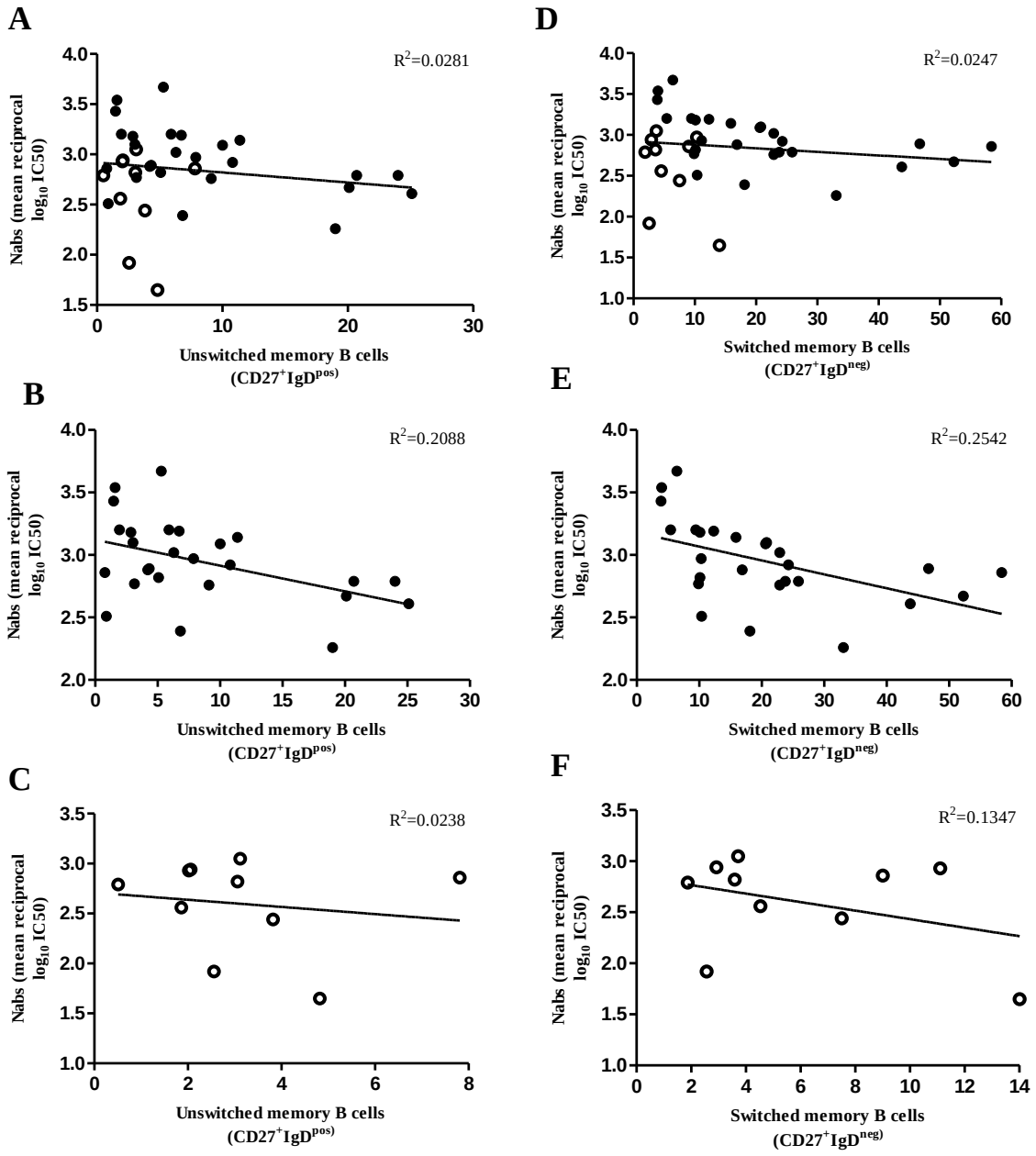

Figure 4.4 – Strength of the relationship between neutralizing antibodies and memory B cells. Linear regression was used to assess the strength of the relationship between Nab titers and frequency of unswitched memory B cells in all patients (panel A), in untreated patients (panel B) and in treated patients (panel C) and between Nab titers and switched memory B cells in all patients (panel D), untreated patients (panel E) and treated patients (panel F). The results of linear regression analysis are shown with the associated correlation coefficients for each comparison. In panels A and D untreated patients are represented by closed circles and treated patients by open circles.

Discussion

To our knowledge, this is the first study addressing the relationship of Nab responses in HIV-2 infected patients with memory B cell disturbances. We show that potent and broadly Nabs are produced in chronic HIV-2 patients, despite evidence of marked memory B cell loss.

The thirty seven chronically infected HIV-2 individuals enrolled in this study showed a significant depletion of both unswitched and switched memory B cells, which was particularly marked in patients in more advanced disease stages (CD4<350 cells/µl), with detectable viral load or on antiretroviral treatment [270]. Interestingly, despite the marked memory B cell loss, all patients produced Nabs that were both broad and potent. The median Nab titer obtained in this study was lower than in other studies (reciprocal $\log_{10}$ IC50, 2.9 vs 3.0-4.7) [109, 180, 269]. This was likely due to the inclusion in our study of a considerable number of patients in a more advanced disease stage, since Nab potency and breadth decrease as HIV-2 disease progresses [97]. Moreover, in this study we have used a panel of diverse primary isolates instead of the pseudoviruses and cloned viruses used in other studies, which are generally easier to neutralize [412].

A positive association was found between Nab titers and breadth, meaning that patients with the most potent Nabs were also those with higher neutralizing breadth, which is in agreement with previous reports, both in HIV-1 [289, 413] and HIV-2 [97, 269] infections. Nab titers were also found to positively correlate with levels of C2V3C3-specific binding antibodies, confirming that this region contains a dominant neutralizing epitope, likely V3, that is expressed in most HIV-2 infected individuals [97]. Additionally, there was a direct association of the Nab titers with the levels of gp36-specific binding antibodies, indicating that the patients also produced heterologous Nabs targeting the gp36-ectodomain. Remarkably, Nab titers correlated with C2V3C3-specific binding antibodies only in patients with higher number of CD4 cells (>350 cells/µl), whereas a similar correlation with gp36-specific binding antibodies was present only in patients in later stage disease with lower number of CD4 cells (<350 CD4 T cells/µl). This is consistent with the observation that C2V3C3-specific binding antibodies wane as disease progresses much more drastically than gp36-specific antibodies and is likely related to major sequence changes that occur in the V3 neutralizing epitope during the disease course and eventually lead to R5-to-X4 tropism

switch and resistance to antibody neutralization [97, 291]. In contrast, the lower diversification of the gp36-ectodomain, likely due to a lower Nab pressure, might explain the more pronounced gp36-specific Nab response in late stage disease.

Unlike HIV-1 infection, where no significant association seems to exist between Nab production and frequency of memory B cells [284, 290, 414], we found Nabs to correlate inversely with the frequency of both unswitched and switched memory B cells in the untreated groups of patients showing that, in HIV-2 infection, Nabs are produced regardless of memory B cell loss. The mechanisms underlying the correlation found between efficient production of neutralizing antibodies and reduced memory B cells in HIV-2 infected individuals require further investigation. Previous reports on several viral infections have shown an absence of correlation between antibody levels and circulating memory B cells, assuming that antibody production could have been maintained by bone marrow long-lived plasma cells that do not require continuous antigen exposure [415, 416]. Hence, in chronically infected untreated HIV-2 patients, production of neutralizing antibodies might also be predominantly maintained by long lived plasma cells [415, 417]. Furthermore, recent findings showed that plasmablasts are responsible for the elevated levels of IgGs seen in HIV-1 infected individuals [285]. These same cells might also be responsible for maintaining antibody production in HIV-2 infection, with the difference that they most likely are HIV-specific, and produce high levels of Nabs, whereas in HIV-1 most IgGs are not HIV-specific [285].

In conclusion, this study provides new insights into how Nabs are produced and maintained during chronic HIV-2 infection. We show that Nabs are produced throughout the course of disease, either against the C2V3C3 envelope region or, in advanced stage, against the gp36 ectodomain, and that potent Nab responses occurred in the context of marked memory B cell loss. These data suggest a role for B cell subsets other than memory B cells, such as long-lived plasma cells, in the production and perpetuation of neutralizing antibodies and may have important implications for the vaccine field.

Acknowledgements

NT and AES designed research. CR, JMM, PB, RC, RT and SF performed experiments. CR and JD analyzed and interpreted the data. CR, PB and CP performed the statistical analysis. EV and FA contributed with patients' data. NT, CR, JD and AES wrote the article. All authors revised and approved the final manuscript.

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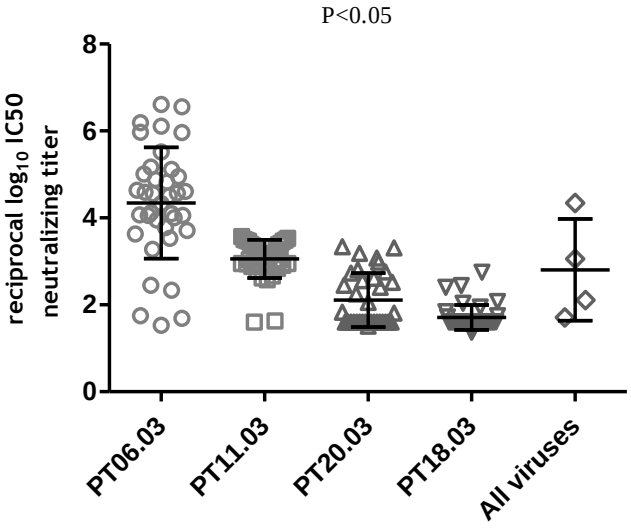
Supplemental digital contents

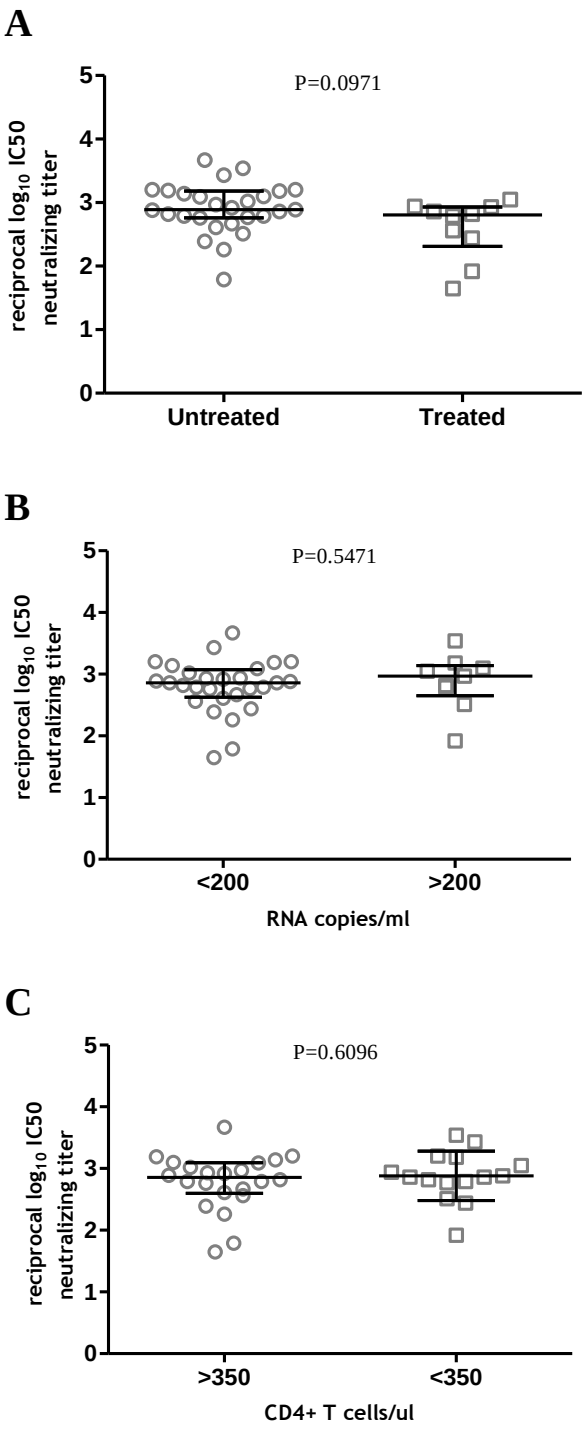
Supplemental digital content 4.1 – Virological and immunological characterization of the patients.

	Untreated (N=27)	Treated (N=10)	Pvalue
Age (years)	56 (19-78)	57.5 (34-62)	P=0.2802
$CD4^+$ T cells/µl	568 (52-1511)	288.5 (84-554)	**P=0.0043**
RNA copies/ml	200 (200-13627)	200 (200-34314)	P=0.5857
Frequency of unswitched memory B cells ($CD19^+CD27^+IgD^+$)	6.29 (0.77-25.1)	2.8 (0.51-7.8)	**P=0.0210**
Frequency of switched memory B cells ($CD19^+CD27^+IgD^-$)	18.1 (3.87-58.4)	4.12 (1.87-14)	**P=0.0004**
Specific antibodies against rgp36 (22 patients)[a]	9.799 (8.775-10.62)	9.682 (2.889-10.12)	P=0.3328
Specific antibodies against rpC2-C3 (22 patients)[a]	7.403 (0.479-8.129)	5.566 (1.434-7.911)	P=0.0714

Data are expressed as median with limits in brackets; Mann Whitney U test was used to compare medians between the treated and untreated groups; P values below 0.05 were considered statistically significant and are highlighted in bold. [a]ODclinical sample/ODcut-off (S/CO) ratios.



Supplemental Digital Content 4.2 – Neutralizing antibody response against a panel of heterologous primary isolates. The median Nab titer (reciprocal log-transformed IC50) of each plasma sample against a panel of four R5 heterologous primary virus isolate is shown. The overall median values and interquartile ranges are also shown. Non parametric Mann Whitney U test was used to compare median Nab titers. All medians were statistically significant (P<0.05).



Supplemental Digital Content 4.3 – Neutralizing antibody response in patients stratified according to treatment, viral load and CD4$^+$ T cell counts. The median Nab titer (reciprocal log-transformed IC50) of each plasma sample against a panel of four R5 heterologous primary virus isolate is shown. The overall median values and interquartile ranges are also shown. Patients were stratified according to treatment (panel A), viral load (panel B) and CD4$^+$ T cells category (below and above 350 cells/µl) (panel C). Non-parametric Mann Whitney U test was used to compare median Nab titers.

Supplemental Digital Content 4.4 – Association between Nab response and Env-specific binding antibodies.

		Env-specific binding antibodies			
		C2V3C3 (in gp125)		gp36 (ectodomain)	
Groups	**Number of samples**	**Spearman R**	**P value**	**Spearman R**	**P value**
Untreated patients	13	0.4209	0.1521	0.4759	0.1002
Treated patients	9	**0.7330**	**0.0311**	0.5667	0.1206
CD4 >350	13	**0.8516**	**0.0002**	0.3714	0.2115
CD4 <350	9	0.0833	0.8432	**0.7000**	**0.0430**
Viral load <200	17	0.4733	0.0550	0.4307	0.0844
Viral load >200	5	0.6000	0.3500	0.7000	0.2333
Untreated, CD4 >350	10	**0.8182**	**0.0058**	0.3212	0.3679
Untreated, viral load <200	11	0.4191	0.2030	0.4784	0.1375
Untreated, CD4 >350, viral load <200	8	**0.8333**	**0.0154**	0.3571	0.3894

Spearman's rank correlation coefficient was used to analyse associations between Nab titers and envelope specific binding antibodies in the different groups of patients; P values below 0.05 were considered statistically significant and are highlighted in bold.

Supplemental Digital Content 4.5 – Association between Nab response and memory B cell populations.

		Memory B cells ($CD27^+$)			
		Unswitched (IgD^+)		Switched (IgD^-)	
Groups	**Number of samples**	**Spearman R**	**P value**	**Spearman R**	**P value**
All samples	37	-0.1924	0.2541	-0.2573	0.1242
Untreated patients	27	**-0.4162**	**0.0308**	**-0.0436**	**0.0003**
Treated patients	10	-0.1273	0.7330	-0.1152	0.7589
CD4 >350	22	-0.2045	0.3614	-0.4181	0.0528
CD4 <350	15	-0.1519	0.5889	-0.0268	0.9244
Viral load <200	28	-0.2612	0.1794	-0.3656	0.0558
Viral load >200	9	0.2333	0.5517	0.2343	0.5517
Untreated, CD4 >350	19	**-0.4976**	**0.0301**	**-0.6795**	**0.0014**
Untreated, viral load <200	21	**-0.4756**	**0.0293**	**-0.6701**	**0.0009**
Untreated, CD4 >350, viral load <200	16	-0.4753	0.0628	**-0.6951**	**0.0028**

Spearman's rank correlation coefficient was used to analyse associations between Nab titers and memory B cell populations in the different groups of patients; P values below 0.05 were considered statistically significant and are higlighted in bold.

CHAPTER 5

Primary isolates of HIV-2 that use the CXCR4 co-receptor are intrinsically resistant to antibody neutralization

Primary isolates of HIV-2 that use the CXCR4 co-receptor are intrinsically resistant to antibody neutralization

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Research article

Short communication in preparation

Abstract

Objective: CXCR4-tropic HIV-2 viruses isolated from patients with advanced disease were found to be highly resistant to IgG antibody neutralization but whether this is an intrinsic characteristic of these viruses remains to be determined. In this study we aimed to characterize the neutralization phenotype of an extended set of primary X4 isolates obtained from HIV-2 patients in diverse disease stages.

Design and methods: Seven X4 variants were obtained from five chronically infected patients in late disease stages and two vertically infected children (early infection). Neutralization susceptibility of X4 isolates was determined using a luciferase reporter gene assay in TZM-bl cells with 16 plasmas from unrelated HIV-2 infected patients and compared to that of primary R5 isolates.

Results: Early and late infection X4 viruses were significantly more resistant to Nabs than R5 viruses (median reciprocal $\log_{10}$ IC50 neutralization titers: 1.60 vs 3.95, $P<0.0001$). Late infection X4 isolates were significantly more resistant to Nabs than early infection X4 isolates. Compared to R5 isolates, X4 viruses had all of the following characteristics in V3: a mutation at position 18, the mutation V19K/R, an insertion at position 24 and a higher global net charge. Nab resistance was also associated to a significant gain in secondary structure in V3.

Conclusions: Nab resistance is an intrinsic characteristic of X4 HIV-2 viruses that is likely determined by major sequence and/or conformational changes in the V3 region in the envelope glycoprotein.

Keywords: HIV-2 infection, neutralizing antibodies, R5 and X4 tropism

Introduction

In contrast to HIV-1, most HIV-2 infected patients have normal and stable $CD4^+$ T cell counts, low or undetectable viral loads and absence of clinical disease [134, 139, 148]. These features might be related to a more effective cellular and humoral immune response generated against HIV-2 [138, 140, 141, 151, 152, 268, 313, 314, 368]. With disease progression, $CD4^+$ T cell depletion occurs at a similar level relative to HIV-1 and the mortality rate is also equivalent to HIV-1 [145, 146].

HIV-2 cell entry usually requires the interaction of the envelope glycoproteins with the CD4 receptor and with co-receptors that belong to the family of chemokine receptors. Among those, CCR5 and CXCR4 are the most important HIV-2 co-receptors [83, 84, 95, 96, 98, 418] but some primary isolates from asymptomatic patients may infect peripheral blood mononuclear cells (PBMCs) independently of these co-receptors [419]. Other isolates may even infect CD4-negative cells using CCR5 or CXCR4 as main receptors [83]. Most chronically infected HIV-2 patients (usually asymptomatic and aviremic) harbour CCR5-using (R5) strains [89, 420]. CXCR4-tropic (X4) HIV-2 isolates have only been found in patients with advanced disease and low $CD4^+$ T cell counts and are strongly associated with faster disease progression compared to R5 isolates [84, 89, 97, 420]. The V3 loop in the gp125 envelope glycoprotein is the main determinant of HIV-2 co-receptor usage and tropism and its charge, size and structural conformation seem to directly influence interaction with CCR5 or CXCR4 [84, 97, 102, 368, 421].

In contrast to HIV-1, most HIV-2 patients in chronic stage of infection have a potent and broad Nab response against R5 isolates [84, 97, 109, 180, 268, 269, 313]. Nonetheless, the potency of the Nab response against R5 isolates decreases with $CD4^+$ T cell depletion and a minority of R5 isolates and all X4 viruses isolated from patients with advanced disease are highly resistant to plasma autologous and heterologous IgG neutralization [97]. Interestingly, mice immunized with envelope antigens from the reference R5 isolate (HIV-2ALI) produced antibodies that potently neutralized heterologous R5 primary isolates but not X4 isolates [368]. These results suggest that the susceptibility of HIV-2 to antibody neutralization is associated with co-receptor usage and that resistance to antibody neutralization is an intrinsic property of HIV-2 isolates that use the CXCR4 co-receptor.

In the present study we aimed to characterize the neutralization phenotype of an extended set of primary X4 isolates obtained from HIV-2 patients in diverse disease stages. We provide definitive evidence that Nab resistance is an intrinsic characteristic of X4 HIV-2 viruses that is likely determined by major sequence and/or conformational changes in the V3 region in the envelope glycoprotein.

Methods

Viruses, study subjects and ethics

Five X4 variants were obtained from five adult chronically infected patients in late disease stage (median $CD4^+$ T cells/µl=78; interquartile range=31.5-221) and two additional X4 variants were obtained from two vertically infected children at age 5 (early infection) (median $CD4^+$ T cells/µl=319.5; interquartile range=44-595). Three primary R5 isolates were obtained from chronically infected patients with median $CD4^+$ T cells/µl=275; interquartile range=66-615). Sixteen plasmas from unrelated HIV-2 infected patients (median $CD4^+$ T cells/µl=333; interquartile range=194.5-480) were used to neutralize the X4 and R5 viruses. Informed consent for blood collection and participation in the study was obtained from all participants or their mothers (in the case of the two vertically infected children). The study was approved by the Ethical Board of the Hospital de Curry Cabral, Lisbon.

Neutralization assay

Neutralization assays were performed using a luciferase reporter gene assay in TZM-bl cells as described previously [290, 368, 409]. Briefly, the cells [10,000 cells in 100 µl of complete growth medium (GM) that consists of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS)], were added to each well of 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight. 100 µl of each virus (corresponding to 200 TCID50) were incubated for 1 h at 37°C with 2-fold serial dilutions of heat-inactivated patients sera in a total volume of 200 µl of GM containing DEAE-Dextran (20 µg/ml). The lowest serum dilution used in the assays was 1:40. Forty-eight hours later, plates were analysed for luciferase activity on a luminometer (TECAN) using the One-Glow Luciferase Assay System (Promega,

Madison, WI). Medium-only control wells were measured as background, and virus-only control wells were included as 100% infection. Nonspecific inhibition was assessed by testing all viruses against HIV-negative plasmas and all plasma samples from HIV-2 patients against HIV-1 pseudotyped with the vesicular stomatitis virus (VSV) envelope (using pSGΔenv plasmid as a backbone). HIV-negative plasmas failed to neutralize HIV-2 strains and HIV-2 plasmas failed to neutralize VSV envelope pseudotyped HIV-1, indicating the absence of nonspecific inhibitory activities in these samples. Neutralization titers were expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC50). IC50 was estimated by the sigmoidal dose–response (variable slope) equation in Prism version 5.0 [392].

Statistical analyses

Statistical analysis was performed with GraphPad Prism 5.0 [392] with a level of significance of 5%. Non parametric Mann Whitney U test was used to compare medians of reciprocal $\log_{10}$ IC50 neutralization titers between X4 viruses, X4 viruses from late infection, X4 viruses from early infection and R5 viruses.

Results

X4 viruses from late and early infection are more resistant to antibody neutralization than R5 viruses

Nab sensitivity of a panel of seven X4 viruses, including five isolates from patients in late disease stage and two variants from early infant infection, was analysed in parallel with three R5 variants. $CD4^+$ T cell counts were not significantly different between patients providing the X4 and R5 strains (median, 78 vs 275 $CD4^+$ T cells/µl, P=0.2667). All patients infected with R5 variants and four out of five infected with X4 strains had undetectable viral loads (< 200 RNA copies/ml). One individual chronically infected with an X4 virus and the two children also infected with X4 variants had viral loads of 4792, 20968 and 1250 RNA copies/ml, respectively.

(a)

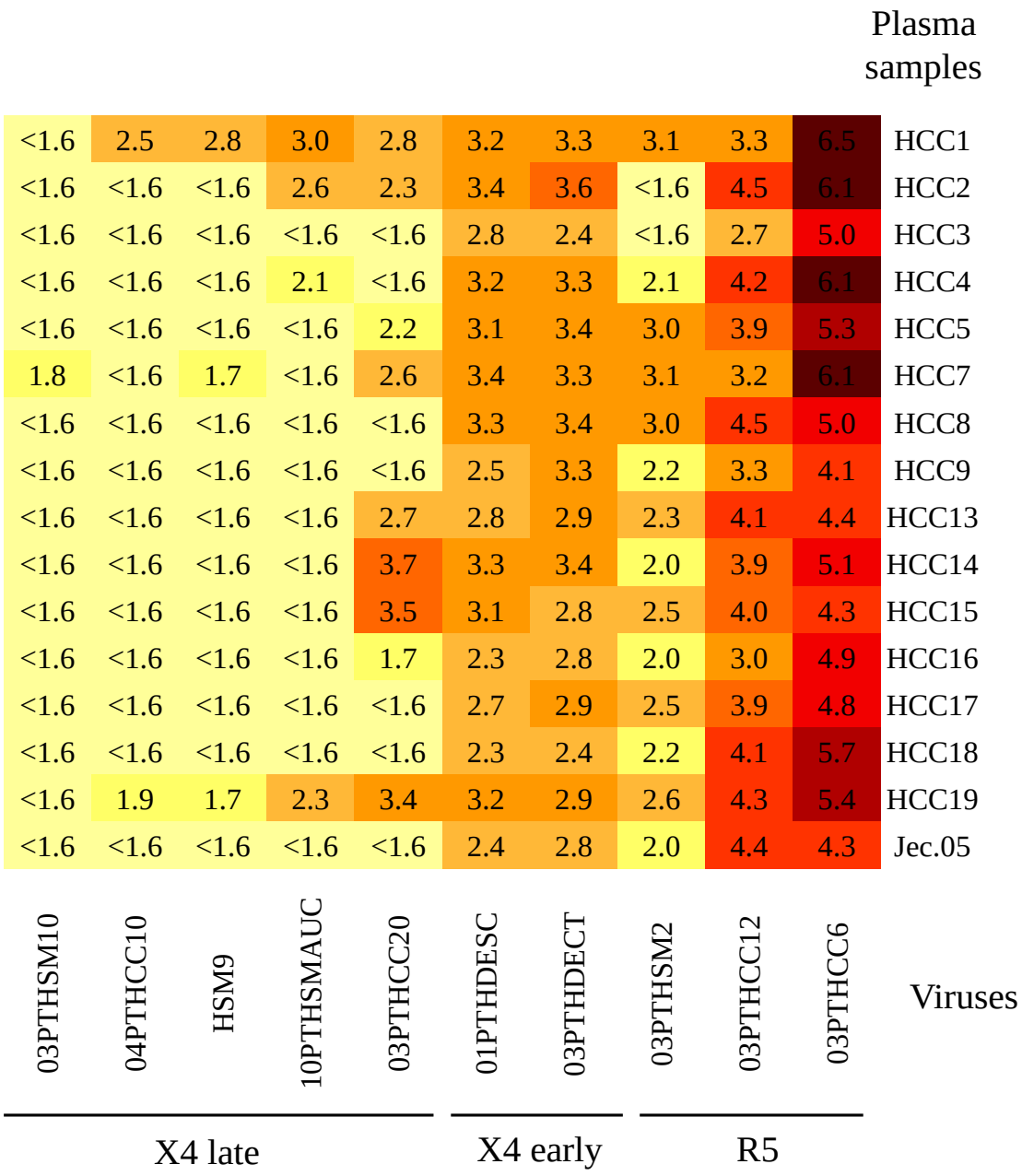

Figure 5.1 – Antibody neutralization of the X4 isolates. Panel A – A heat map of the median reciprocal log10 IC50 neutralizing titer of each heterologous plasma sample (right) against the seven X4 isolates and the three R5 isolates (bottom) is shown. The reciprocal $\log_{10}$ IC50 value is colour-coded. The darkest colour represents the highest neutralizing titer; the lightest colour indicates that there was no detectable neutralization above 50% with the lowest plasma dilution tested (1/40). **Panel B** – Dot-plot graphic showing the median reciprocal $\log_{10}$ IC50 neutralizing titers and interquartile range of 16 heterologous plasma samples against X4 isolates from early and late stage disease and R5 isolates as controls. Mann-Whitney U test was used to compare the median $\log_{10}$ reciprocal IC50 values.

(b)

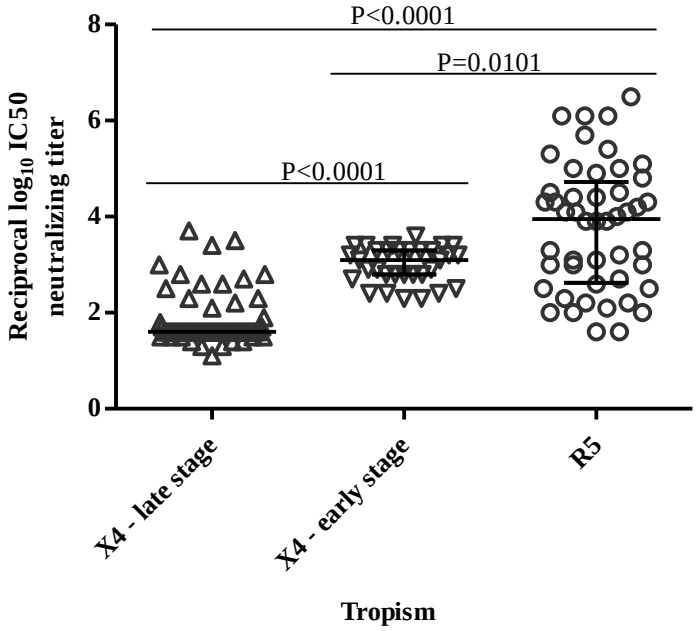

Figure 5.1 (continued)

All viruses were neutralized by at least one plasma sample (Figure 5.1A). R5 isolates and early infection X4 variants were neutralized by all plasma samples whereas X4 strains from late disease stages were only neutralized by 50% (8/16) of the plasma samples. Neutralizing antibody titers were significantly higher for R5 isolates compared to X4 viruses (median reciprocal $\log_{10}$ IC50: 3.95 versus 1.60, P<0.0001). Within the X4 isolates, median Nab titers were significantly higher for early infection isolates compared to late infection isolates (median reciprocal $\log_{10}$ IC50: 3.10 versus 1.60, P<0.0001). Nonetheless, early infection X4 viruses were still significantly more difficult to neutralize than R5 viruses (median reciprocal $\log_{10}$ IC50: 3.10 versus. 3.95, P=0.0101) (Figure 5.1B). These results show that X4 HIV-2 isolates are intrinsically more resistant to antibody neutralization than R5 isolates and suggest that this phenotypic feature in X4 isolates is acquired with infection time.

Features of the V3 neutralizing domain in R5 and X4 isolates

Tropism switch and susceptibility to antibody neutralization in HIV-2 has been associated with particular changes in the envelope V3 region [84, 104, 291]. We therefore compared sequences from the V3 regions of all X4 and R5 isolates. In contrast to R5 isolates, X4 viruses

had all of the following characteristics in V3: a mutation at position 18, the mutation V19K/R, an insertion in position 24 and a global net charge ≥ 7 (Figure 5.2). Moreover, the V3 region of early infection X4 viruses had a lower mean global net charge compared to late infection X4 isolates (+8 and +9, respectively).

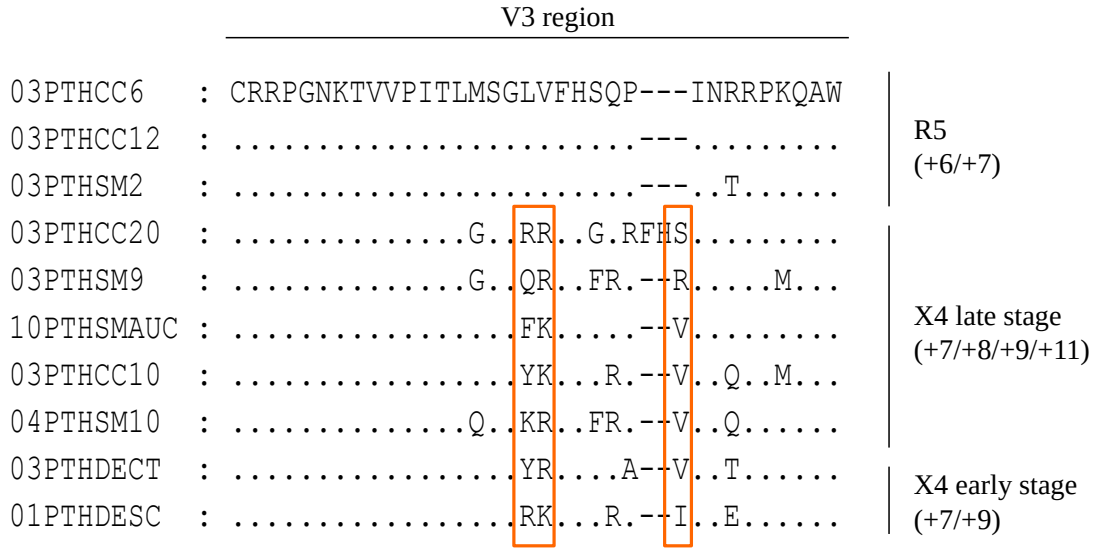

Figure 5.2 – Alignment of the amino acid sequences of the V3 loop of the X4 and R5 isolates. Dots in the alignments indicate sequence identity to the first R5 isolate sequence (03PTHCC6); dashes indicate deletions; amino acids in grey boxes are involved in tropism change [104]; viral tropism, as determined phenotypically [422] and genotypically based on V3 loop sequence patterns, and disease stage are indicated at the right of the alignment; global net charge of the V3 region is indicated at the right of the alignment.

Discussion

To investigate if resistance to antibody neutralization is an intrinsic characteristic of primary isolates of HIV-2 that use the CXCR4 co-receptor, we characterized the neutralization phenotype of a panel of X4 isolates obtained from recently infected patients and from patients with long-term chronic infection against 16 heterologous plasmas from unrelated HIV-2 infected patients. We found that X4 isolates from early and late infection are significantly more resistant to antibody neutralization compared to the R5 viruses. These results provide strong and definitive evidence that Nab resistance is an intrinsic characteristic of primary isolates of HIV-2 that use the CXCR4 co-receptor.

The V3 region in the HIV-2 gp125 envelope glycoprotein is a major neutralizing domain [180, 323, 324] and also contains the most important molecular determinants of CXCR4 and CCR5 usage [84, 97, 102, 368, 421]. To look for sequence correlates of Nab resistance we compared the V3 sequences of Nab-resistant X4 isolates with those of Nab-sensitive R5 isolates. Compared to the R5 isolates, the V3 region of all X4 strains had a mutation at position 18, the mutation V19K/R, an insertion in position 24 and a global net charge ≥7. These V3 features have been associated to CXCR4 usage [84, 97, 102, 368, 421] thus establishing a close link between CXCR4 usage and resistance to antibody neutralization and, more generally, between HIV-2 tropism and susceptibility to antibody neutralization.

Resistance to neutralizing antibodies can be developed soon after HIV-2 infection has shown in the X4 isolates obtained at year 5 of infection from the two infants. However, Nab resistance was significantly more pronounced in isolates from chronically infected patients suggesting that acquisition of Nab resistance it is a stepwise process that occurs over the course of infection and might be driven by the constant selective pressure exerted by the Nabs on the envelope glycoproteins. A similar stepwise process has been recently proposed for acquisition of maraviroc resistance in HIV-2 infected patients undergoing maraviroc therapy [422].

The V3 loop in the gp125 envelope glycoprotein of HIV-2 seems to acquire a higher level of secondary structure with disease progression. Indeed, the V3 loop of most R5 strains from chronically infected adult patients (asymptomatic and aviremic despite long-term infection) is characterized by the absence of a regular secondary structure whereas the V3 loop of X4 viruses found in late infection in adult patients either acquires a β-α-β conformation or a helix-loop-helix conformation (Figure 5.3) [97]. Likewise, the V3 loop of R5 strains from recently infected children also shows absence of regular secondary structure, and on the other hand, X4 viruses in early infection present a β-hairpin conformation (Figure 5.3) (Rocha *et al*, submitted). These different conformations of the V3 region are a consequence of the evolving changes at the amino acid level and might be responsible for the marked differences in Nab sensitivity of HIV-2 isolates over the course of infection. We propose a model were, throughout infection, the pressure exerted by Nabs over the V3 loop forces the virus to escape and change this region in order to conceal it from neutralization. These

changes over time also favour tropism switch and are closely associated with $CD4^+$ T cell decline (Figure 5.3).

In conclusion, our results show that Nab resistance is an intrinsic feature of CXCR4- tropic HIV-2 isolates that is acquired over the course of infection in close association with sequence and conformational changes in the envelope V3 loop that also favour R5-to-X4 switch.

Acknowledgements

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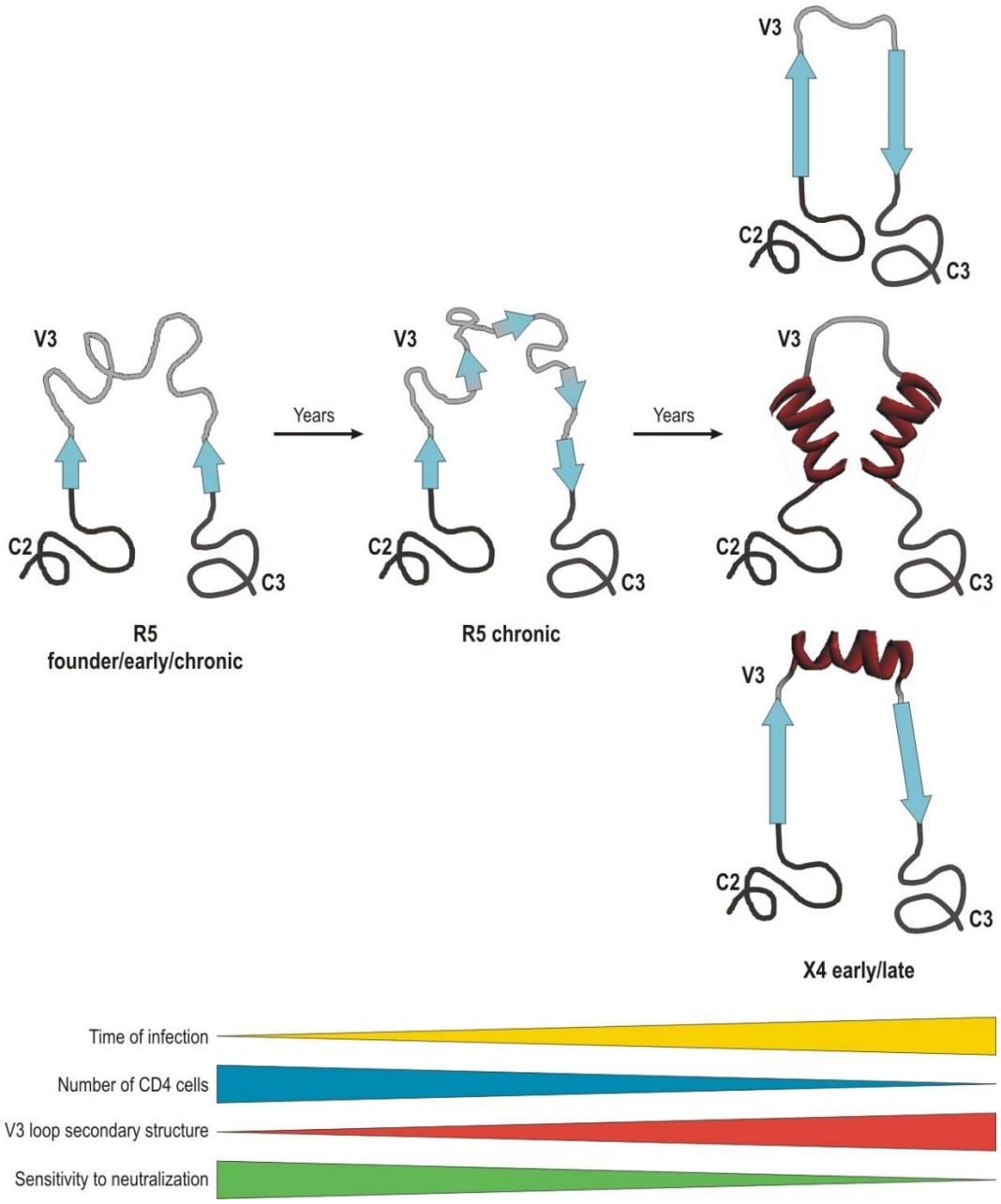

Figure 5.3 –Model of the evolution of HIV-2 V3 loop conformation with infection time and disease progression. The diagram shows a model of the evolution of the secondary structure of the V3 loop of R5 and X4 isolates with disease progression. The drawings were based on homology modelling data that has been published previously [97] or is submitted for publication (Rocha et al., submitted). The different secondary structures of V3 region are represented in grey, blue and red and the neighbouring C2 and C3 regions are represented in black. The V3 region evolves from not having a regular secondary structure R5 founder/early/chronic), to extended β-strands (R5 chronic), to a β-hairpin conformation (X4 early) or a β-α-β or helix-loop-helix conformation (X4 late). The yellow triangle represents increase in infection time; the blue triangle represents decrease in $CD4^+$ T cell counts through infection; the red triangle represents increase in secondary structure of V3 region; the green triangle represents decrease in Nab sensitivity through infection.

CHAPTER 6

General Discussion and conclusions

Many aspects of HIV-2 infection remain largely unknown, which poses as an obstacle for vaccine design, treatment and ultimately patient care. When trying to make a parallel between HIV-1 and HIV-2, one faces the problem of two completely different disease courses, incomparable natural history and unmatchable immune responses, despite both viruses belonging to the same genus. Furthermore, HIV-2 can be seen as an attenuated form of HIV infection and the differences in virus-host interactions are most likely responsible for the distinct outcomes. Studying and understanding these interactions can ultimately be used to infer the type of immune responses that a vaccine against HIV-1 needs to elicit in order to be efficient. One of the major contrasts between these two infections is the production of broad and potent Nabs by HIV-2 infected patients. These antibodies, in parallel with other immune responses, are thought to have a crucial role in controlling viral replication and disease progression in the chronic phase of infection. Yet, until very recently, information was lacking on the origin of the NAbs in HIV-2 patients, on the characteristics of the Nab response in acute and early HIV-2 infection and on the evolution of the virus to deal with the neutralizing antibodies. Hence, one objective of this work was to characterize the Nab response and viral evolution in early HIV-2 infection. It was found (Chapter 3) that Nabs can be detected very early in infection and have a major impact in viral evolution. In the setting of a broad and potent Nab response (child1), viral diversity and evolutionary rate were extremely high (similar to HIV-2 chronically infected patients undergoing treatment) [157], whereas in the presence of a weaker Nab response (child2) these evolution markers were similar to HIV-1 in newly infected children and adults [206, 210, 398, 399]. Escape from neutralization was detected at year five of infection, has shown by the increasing resistance to Nabs seen in both infants. Furthermore, and consistent with the escape, there was a complete replacement of virus quasispecies and an increase in diversity, dN/dS rate and positively selected sites throughout infection. The increased resistance to neutralization was associated with the R5-to-X4 tropism switch, providing further evidence for the previously described association between Nab escape and X4 tropism [97]. In this study, however, the emergence of X4 tropic viruses was abnormally fast, since these strains are usually detected in chronically infected patients in more advanced disease stages [97]. This difference towards infection in adults is probably related to the fact that these patients are children

and acute infection occurred when they were newborn. Hence, transposition of these results to adult early HIV-2 infection is limited and must be made with care. Another interesting finding in this study was that R5-to-X4 tropism switch was associated with amino acid mutations in V3 region that had a major impact in the secondary structure of this region. The V3 loop of R5 viruses from both children were characterized by the absence of regular secondary structure, whereas the X4 strains showed a β-hairpin conformation. These findings suggest that the gain of structure in the V3 makes this region unrecognisable to Nabs leading to escape. Besides the changes in structure, V3 region increases in size due to the insertion of an amino acid in position 24, which might also influence Nab recognition. One important issue is the influence of antiretroviral treatment in disease outcome of these infants, despite the fact that response to antirretroviral treatment is weak in HIV-2 infected patients [411, 423-426]. Child 2 initiated treatment immediately after birth but there were not many therapeutic options at that time (1992). Child 1's antirretroviral treatment started more than 10 years later, when an arsenal of drugs were available, and these most likely controlled viral load and probably also played a role in increasing diversity and evolution rate. More studies with HIV-2 infected patients in early stages of the disease are needed, to confirm our findings and address other unanswered questions. For instance, what is the role of maternal antibodies in HIV-2 MTCT? Are Nabs produced by recently infected adults more broad and/or potent than in children and is the viral evolutionary rate in these adults, with a capable immune system, similar to that of child1? Studying early HIV-2 infection seems a very difficult task, for patients are only diagnosed several years after seroconversion and vertical transmission is a rare event [114, 122-125, 376]. To overcome this obstacle, incidence cohorts should be established in countries with high prevalence of HIV-2, such as Guinea-Bissau and Cape Verde [33, 48, 51, 427], in order to increase the possibility of finding recently infected individuals or even seroconversion.

Memory B cell populations were found to be depleted in HIV-1 and HIV-2 infections, in association with T cell activation, an imbalance not recovered by ART [270, 271, 280, 282]. However, in contrast to HIV-1, HIV-2 patients are able to produce high levels of broadly neutralizing antibodies. Understanding, which subset of B cells is responsible for inducing

and maintaining the high levels of broad and potent Nabs found in HIV-2 infection might be important for the development of a vaccine against both HIV-2 and HIV-1. Hence, the second objective of this work was to investigate the Nab response in HIV-2 patients with different levels of B cell depletion and the relationship between the Nab response and the different subsets of memory B cells. In Chapter 4, it was found that HIV-2 infected individuals produced broad and potent Nabs despite memory B cell disturbances. Nabs were negatively correlated with frequency of both unswitched ($CD19^+CD27^+IgD^+$) and switched ($CD19^+CD27^+IgD^-$) memory B cells in untreated patients which suggests that memory B cell depletion somehow favours Nab production and implies that Nab production is maintained by other B cell subsets. In HIV-1 infected individuals, there is no association between Nab production and any B cell subset [284, 289, 290]. The lack of association between Nabs and memory B cells in HIV-1 is not surprising since Nabs are rare and take many years to develop [306, 307], and hence they are produced in a setting of major B cells imbalances. In HIV-1 infection the levels of terminally differentiated B cells (plasmablasts and plasma cells) are increased very early after transmission leading to hypergamablobulinemia [272, 279, 428-430]. Importantly, the majority of IgGs present in HIV-1 infected patients is not HIV-specific [285, 431]. Since hypergamablobulinemia is also present in HIV-2 infection and memory B cells are depleted [270, 291, 292], it is reasonable to think that plasmablasts and plasma cells might also be increased in HIV-2 infected individuals. In contrast to HIV-1, however, these antibody producing cells are most likely responsible for the production and maintenance of elevated levels of Nabs in HIV-2 infection. Further studies are needed to evaluate if plasmablasts and/or plasma cells are indeed increased in HIV-2 infection and to determine which B cell population is responsible for HIV-2-specific antibody production (binding and neutralizing). With this information one would perceive which subset of B cells is important to stimulate in vaccination in order to continuously produce antibodies capable of neutralizing HIV-1 and HIV-2. It is also crucial to know the epitopes targeted by these Nabs. Hence, another important finding in our study was the association between Nabs and C2V3C3-specific antibodies in patients with high $CD4^+$ T cell counts and with gp36- specific binding antibodies in later disease stages (<350 CD4 T cells/µl). The V3 region of HIV-2 is known to be highly immunogenic [323-326, 368] and neutralizing epitopes have also been

described in gp36 [314, 325, 327]. However, this is the first study to describe a change in Nab recognition from C2V3C3 region to gp36 with disease progression. The results show that most Nabs are directed against a dominant neutralizing epitope in C2V3C3 (probably in V3) but as disease progresses, changes in this region most likely make it unrecognisable to Nabs. These changes also lead to R5-to-X4 tropism switch. Since gp36 ectodomain is a more conserved region [432], probably because of lower Nab pressure, in late disease stages Nabs are still able to recognise the epitopes present and hence are mainly directed to this region.

Recent studies found that X4 tropic HIV-2 viruses isolated from patients with advanced disease are resistant to neutralization [97]. We also detected Nab resistance by X4 strains isolated from infants (Chapter 3). These findings strongly suggest an association between Nab escape and R5-to-X4 tropism switch. However, it remains to be determined if resistance to neutralization is an intrinsic characteristic of X4 tropic HIV-2 strains. Hence, the third objective of this work was to characterize the neutralization phenotype of an extended set of primary X4 isolates obtained from HIV-2 patients in diverse disease stages in order to establish if all X4 strains are resistant to Nabs regardless of disease stage. To address this problem we compared Nab sensitivity of X4 viruses from patients with recent infection (perinatally infected children) and with chronic infection in late stages the disease ($CD4^+$ T cell counts < 350 cell/µl) in comparison with R5 variants from chronically infected individuals (Chapter 5). The results showed that X4 viruses are significantly more resistant to Nabs than R5 isolates independently of disease stage, confirming that neutralization resistance is an intrinsic characteristic of X4 tropic strains [97]. We also found significant differences between X4 variants from early and late infections, with viruses from chronically infected patients (late infection) being more resistant to Nabs compared to vertically infected children (early infection), suggesting that resistance to neutralization is a gradual process that occurs through infection. Analyses of the V3 sequences showed that all X4 strains were longer (by the insertion of 1 amino acid in position 24) and had a higher mean global net charge compared to R5 tropic viruses. Additionally, despite all X4 viruses sharing the amino acid changes that lead to tropism switch, the mean global net charge of the V3 region was lower in viruses from early infection compared to late infection. By homology modelling it

was shown that R5 strains from early (Chapter 3) and chronic infections [97] are characterized by the absence of a regular secondary structure in the V3 loop. The secondary structure of the V3 region of X4 tropic strains from early infection is composed of a β-hairpin (Chapter 3) and X4 viruses from chronically infected individuals have a very regular secondary structure characterized by either a β-α-β motif or a helix-loop-helix conformation [97]. Taken together these findings suggest that amino acid mutations in V3 region have a major impact in the secondary structure and conformation of this region. Furthermore, these changes most likely prevent antibody recognition leading to Nab escape. Following all these transitions in V3 conformation, we proposed a model of V3 evolution as a consequence of Nab pressure, in which as disease progresses and Nabs exert pressure over this region, it acquires an increasingly more rigid conformation, and this process changes the epitope making it unrecognisable to Nabs. As a consequence, X4 strains become resistant to antibodies directed to V3. This model fuscous exclusively on the V3 region and, although this region comprises the major determinants for coreceptor usage, changes in other regions might also influence coreceptor switch as seen in HIV-1 infection where changes the glycosylation pattern and length of the V1/V2 loop have been associated with differences in coreceptor phenotype [106, 107]. The role of changes in length and glycosylation pattern of V1/V2 region in coreceptor usage during HIV-2 infection is still elusive [84] and further studies are needed before discarding the influence of other regions in tropism switch. Another important finding was the confirmation that Nab escape occurs in advanced stages of HIV-2 infection. Previous authors failed to detect the presence of Nab escape mutants [180] or found that these were not predominant in the individuals' viral population [109], however these studies, contrarily to ours, did not include patients in more advanced disease stages infected predominantly with X4 tropic viruses. To definitely prove that HIV-2 X4 variants are intrinsically resistant to neutralization it is necessary to revert X4 tropic strains into R5 variants by directed mutagenesis and evaluate if Nab resistance is lost. Using the same approach, converting R5 into X4 strains should increase resistance to neutralization by the initially R5 viruses.

In conclusion, these studies have shown that Nab responses emerge very early in HIV-2 infection, persist through infection despite memory B cell imbalances, and drive the viral molecular evolution, structural changes in V3 and tropism switch that ultimately leads to resistance to neutralization. The results from this work provide new and potentially important information for the HIV vaccine field. First, and most importantly, Nab escape exists in HIV-2 infection, in association with X4 tropism, and therefore a vaccine most likely needs to elicit responses against both R5 and X4 strains. Nabs seem to target mainly the V3 region in the early and chronic phases of HIV-2 infection. In late stages of the disease response against the V3 region is weaker but Nabs targeting the gp36 arise. Taken together these results confirm that V3 is a good immunogen for vaccine design but highlight the fact that gp36 contains important epitopes to include in a vaccine. Another important finding was that a sustained Nab response is possible to be maintained by B cell populations other than the memory B cell subset. The frequency of memory B cells is directly associated with the levels of protective antibodies against the majority of viral infections [415]. However, in infections with viruses capable of maintaining latent reservoirs, such as varicella-zoster virus or Epstein-Barr virus, antibody levels do not correlate with the frequency of memory B cells [415]. Most likely, with HIV, because it also maintains latent reservoirs, memory B cells are a poor predictor of antibody levels. Consistent with this, strong and potent Nabs persist in HIV-2 infection despite memory B cell depletion. Our results show that other B cell subsets can maintain the antibody response needed for vaccination. The fact that, in HIV-1 infected individuals, plasmablasts are responsible for the production of elevated levels of IgGs, is in agreement with this line of thinking. Therefore, a vaccine against both HIV-1 and HIV-2 would ideally stimulate responses from plasmablasts and/or long lived plasma cells. The difficult part is finding the right immunogen or group of immunogens that will elicit these responses. From our studies, immunogens based on HIV-2 V3 region in association with gp36 seem good candidates for vaccine design.

CHAPTER 7

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