Cellular Immunity among HIV Exposed, Uninfected Individuals

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"Nothing worth having comes without some kind of fight.

Gotta kick at the darkness 'till it bleeds daylight."

- Bruce Cockburn, 'Lovers in a Dangerous Time'

"Okay, brain. You don't like me, and I don't like you, but let's get through this thing and then I can continue killing you with beer."

-Homer J. Simpson, 'The Simpsons'

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Abstract:

Two models of HIV infection have been studied extensively with the goal of identifying immune correlate(s) of protection against HIV: 1) the simian immunodeficiency virus (SIV) infection of rhesus macaque monkeys and 2) individuals with repeated exposure to HIV who remain uninfected by the virus (EUs). Both paradigms suggest that T cell-mediated immunity plays an important role in controlling HIV replication. Evidence from the SIV/macaque system, however, predicts that HIV vaccines aimed at eliciting T cell responses will fail to induce sterilizing immunity against HIV. The aim of the work presented in this thesis was to correlate HIV-specific T cell immunity in EUs with protection from HIV infection. The study cohort was comprised of (a) men who have unprotected sexual intercourse with HIV-infected men (MSM), (b) intravenous drug users (IVDU) who share syringes with HIV-infected peers, and (c) heterosexual partners of HIV-infected subjects. EUs were shown to exhibit HIV-specific IFN-γ secretion, IL-2 production, and T cell proliferation, whereas low-risk negative controls did not. Furthermore, HIV-specific IFN-γ secretion and T cell proliferation were not observed among a population of EUs who seroconverted soon after the tested time point. HIV-specific IL-2 secretion and T cell proliferation were shown to be correlated in our cohort of EUs. These effector functions are considered hallmark properties of central memory T cells (T_{CM}), the subset of memory T cells that has been shown to mediate sterilizing immunity in mouse models of viral infection. The presence of T_{CM} in EUs implies the development of immunity against HIV that is either fully protective or partially so, by increasing the

threshold for HIV infection. Since these HIV-specific effector functions were absent in EUs who eventually seroconverted, it can be inferred that EUs in our cohort develop HIV-specific T cell immunity that mediates protection from HIV infection. Thus, our contribution to the EU paradigm is that sterilizing immunity is an attainable goal of prospective HIV vaccines.

Résumé:

Deux modèles d'infection VIH ont été étudiés intensivement afin d'identifier des corrélations immunitaires de la protection contre le VIH: 1) l'infection des singes macaques avec le virus d'immunodéficience simien (VIS) et 2) les personnes exposées au VIH (PEV) qui restent non infectées. Ces deux systèmes suggèrent que l'immunité médiée par les cellules T joue un rôle important dans le contrôle de la réplication du VIH. Cependant, suite à l'application du système VIS/macaque, il est prévu que les vaccins dirigés contre le VIH visant à obtenir des réponses cellulaires T ne produiront pas la protection immunitaire escomptée contre le virus. Le but de la thèse suivante était de trouver des corrélations entre l'immunité cellulaire T spécifique au VIH chez les PEVs et la protection contre l'infection par le VIH. La cohorte d'étude a été composée (a) d'hommes qui ont eu des rapports sexuels non protégés avec des hommes infectés par le VIH, (b) des utilisateurs intraveineux de drogue qui partagent des seringues avec les pairs VIH-infectés, et (c) de conjoints hétérosexuels de sujets séropositifs. Il a été démontré qu'il y a sécrétion d'INF-γ, production d'IL-2 et prolifération cellulaire spécifiques au VIH chez les PEVs, tandis que ces réponses ne sont pas observées chez les contrôles séronégatifs à faible risque. De plus, la sécrétion d'INF-γ et la prolifération cellulaire spécifiques au VIH n'ont pas été observées chez les PEVs dont la séroconversion s'est produite peu après la date de l'échantillon testé. Dans notre cohorte de PEVs, nous avons trouvé une corrélation entre la sécrétion d'IL-2 et la prolifération des cellules T spécifiques au VIH. Ces fonctions effectrices s'avèrent être

caractéristiques aux cellules T mémoire centrales (T_{CM}), celles-ci étant connues pour médier l'immunité protectrice dans les modèles d'infection virale chez la souris. La présence de T_{CM} parmi les PEVs de notre cohorte suggère le développement de l'immunité protectrice contre le VIH. Ainsi, en étudiant les PEVs et leur réponse immune cellulaire spécifique, nous contribuons à l'élaboration d'éventuels vaccins protecteurs dirigés contre le VIH.

Preface:

A manuscript-based thesis is here-in assembled in compliance with the guidelines set by the Faculty of Graduate Studies and Research, McGill University. The introduction includes a comprehensive review of the literature relevant to the elements of science that were investigated. Based on the reviewed body of knowledge, a thesis hypothesis is established and supported by a disclosure of our rationale. The main objectives of the thesis project conclude the introduction. Three manuscripts ensue, bridged by connecting texts that provide a logical progression of the work. The first two documents have been published in *AIDS* [2002 Aug 16; 16(12):1595-602 and 2005 Feb 18; 19(3):251-9], while the third document has been submitted to *AIDS*, as well, for publication. The discussion at the end of the thesis attempts to integrate the findings from this project into the current understanding of HIV infection. The relevance of the work is put into context, and measures needed to further this work are proposed.

I, **George Makedonas,** am the first author on all of the papers, as I contributed significantly to the design of the studies, performed the bulk of the experimentation, and composed the manuscripts. My thesis supervisor, **Dr. Nicole Bernard**, conceived of the projects, developed the design of the studies, supervised the collection of data, and edited the manuscripts. I am grateful to **Kenneth Huang, Marie-Pierre Boisvert, Henry Lin**, and **Yoav Peretz** for assisting in my experimentation, developing analytical protocols, and managing our databases. I am also indebted to **Dr. Christos M. Tsoukas,** who supplied analytical equipment and storage facilities.

Dr. Julie Bruneau deserves outstanding recognition for developing and maintaining the St. Luc cohort of intravenous drug users. I would also like to thank **Martin Rioux** for database management and subject recruitment at St-Luc, as well as all the nurses that provide us with the blood samples. I am grateful to **Dr. Michel Alary** for directing the OMEGA cohort, for patient recruitment, and for providing statistical counsel. I am also thankful for the work performed by **Catherine M. Lowndes,** who was instrumental in the development of the PEV (EU) cohort, subject recruitment, and follow-up at OMEGA. I wish her all the best as a consultant scientist for epidemiology at the Health Protection Agency of the Communicable Disease Surveillance Centre in London, UK. **Dr. Rafick-Pierre Sekaly** deserves honourable mention for providing critical analytical methodology and for furnishing invaluable subject samples from pre-seroconversion time points.

I must also express thanks to the members of my thesis advisory committee, **Dr. Danuta Radzioch, Dr. Julie Bruneau, Dr. Mary Stevenson, Dr. Marianna Newkirk**, and **Dr. Nicole Bernard**, for providing remarkable counsel and insight that undoubtedly shaped my thesis project.

Acknowledgements:

All that I have accomplished has been made possible by my family. My parents, Dimitra and Antonios, have supported my choices in life without prejudice or admonition. Upon completing my Bachelor's of Science degree, I decided to experience life beyond the ivory tower by working as a technician for a manufacturer of dietary supplements. I quickly realized that my need for intellectual stimulation was not being fulfilled, so I decided to pursue higher education. My parents supported my decision to return to academia, despite the fact that graduate study was a foreign concept to them. Over the years, my parents have exhibited tremendous patience with me and have supplied me with a healthy dose of unconditional love. I thank you both, and return the love with all my heart.

I would be remiss if I did not extend my gratitude to my sister, Mary, and her husband, Peter. In addition to their love and support, they have provided me with two profound sources of motivation and joy: my nephew, Petro, and my niece, Dimi. There is more to life than experiments and notebooks; these two little creatures have allowed me to mature as an adult, by assuming a position of responsibility and mentorship, as well as to revert to a state of care-free playfulness. I love you all!

From a professional standpoint, all I am and all I ever will be are directly attributed to Dr. Nicole Bernard, who took a chance on a boy with marginal academic success and virtually no prospects for funding scholarships. I am eternally grateful to you for the opportunity you gave me. Thank you for sharing

your wisdom with me, for mentoring a very raw talent, and for displaying incredible patience with me. You are more than a boss to me; you are my friend. I hope I did not disappoint you. You will forever be with me wherever I go.

I am extremely grateful to have been able to conduct my research in what must have been the greatest laboratory environment in the history of science. I cannot recall a day in which I did not break out in hysterical laughter at least once. Kenneth, Salix, Yoav, Mapi, Nancy, Louise, Enza, Karen, Becky, Alefia, Henry, Wendy, Jenny, Adam, Nektaria, Michel, Emilie, and Lydia, I cherish the times we spent together and will carry the memories with me to my grave. Incidentally, you have all graduated to an inner loop in my circle of friends!

A special thanks to Famane Chung, who took me, a naïve and intimidated boy, under her wing and showed me the patience and encouragement needed to advance my development. Also, I would like to thank Galit Alter, with whom I embarked on the road of scientific research at the same time, for providing me with a friendship beyond the lab, filled with delightful highs and infuriating lows. All the best, my long-lost little sis! Last, but certainly not least, I would like to thank Luis for offering invaluable advice on life and for engaging me in stimulating debates on many a late nights. You were truly my father figure at the MGH!

Despite the findings that I report in this thesis, my greatest discovery in the laboratory was Alison Mockler. I can easily write another thesis on my love for this young woman. Suffice it to say, I consider myself the most fortunate man in the world, for I can lay claim to a profound love that buoys me out of the depths

of frustration and doubt. From the first moment I looked into her eyes, I saw a spring of eternal happiness. You are my one and only, forever.

List of Abbreviations

AIDS- acquired immunodeficiency syndrome

CA- capsid protein

cART- combined antiretroviral therapy

CDC- Centers for Disease Control

CRF- circulating recombinant form

CTL- cytotoxic T lymphocyte

DNA- deoxyribonucleic acid

EU- exposed uninfected individual

EUs- exposed uninfected individuals

EU-SC- exposed uninfected individual who seroconverted

ELISPOT- enzyme-linked immuno-spot assay

Gp- glycoprotein

HIV- human immunodeficiency virus

HIV IIIB- laboratory adapted strain of HIV

ICS- intracellular cytokine staining

IFN- interferon

IL- interleukin

IN- integrase

IVDU- intravenous drug user

Kb- kilobases

kd- kilodaltons

KS- Kaposi's sarcoma

MA- matrix protein

mRNA- messenger RNA

MSM- men having sex with men

NC- nucleocapsid

NIH- National Institute of Health (U.S)

PCP- Pneumocystis carinii pneumonia

PCR- polymerase chain reaction

PR- protease

RNA- ribonucleic acid

RT- reverse transcriptase

SIV- simian immunodeficiency virus

SU- surface protein

T_{CM}- central memory T cells

TCR- T cell receptor

T_{EM}- effector memory T cells

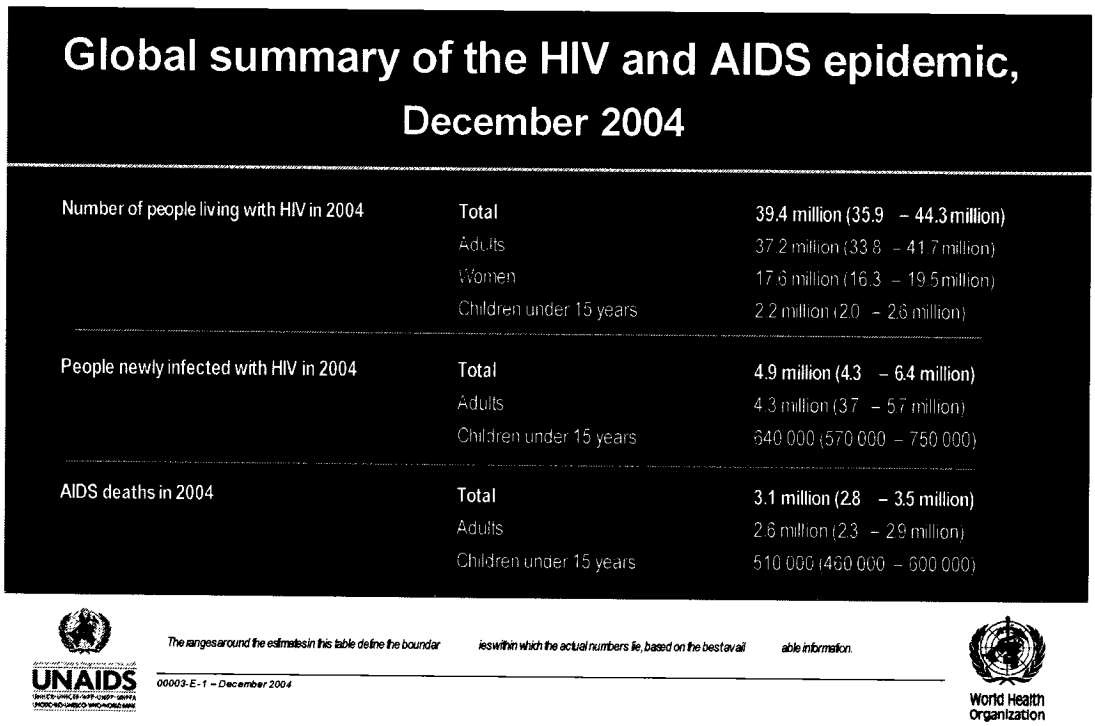
TM- transmembrane

Section 1: Introduction

The Impetus for HIV Vaccine Development

At the end of 2004, 39.4 million people were estimated to be living with the human immunodeficiency virus (HIV), in addition to the 21.8 million who have already died as a result of HIV infection[1]. Despite significant advances in our understanding of the virus and in our abilities to treat HIV infection, there were 3.1 million deaths last year attributed to HIV[1,2]. Despite intense efforts in the domains of HIV prevention and education, there were 4.9 million new HIV infections around the world in 2004[1,2].

Figure 1:



Global summary of the HIV and AIDS epidemic, December 2004

Number of people living with HIV in 2004	Total	39.4 million (35.9 – 44.3 million)
	Adults	37.2 million (33.8 – 41.7 million)
	Women	17.6 million (16.3 – 19.5 million)
	Children under 15 years	2.2 million (2.0 – 2.6 million)
People newly infected with HIV in 2004	Total	4.9 million (4.3 – 6.4 million)
	Adults	4.3 million (3.7 – 5.7 million)
	Children under 15 years	640 000 (570 000 – 750 000)
AIDS deaths in 2004	Total	3.1 million (2.8 – 3.5 million)
	Adults	2.6 million (2.3 – 2.9 million)
	Children under 15 years	510 000 (460 000 – 600 000)

The ranges around the estimates in this table define the boundaries within which the actual numbers lie, based on the best available information.

00003-E-1 – December 2004

UNAIDS

World Health Organization

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Combination antiretroviral therapy (cART) has greatly diminished both mortality and morbidity in HIV-infected patients, but when administered over prolonged periods these drugs induce considerable toxicity. In addition, the

effectiveness of cART is undermined by the emergence of drug-resistant strains of HIV. The relentless evolution of the virus is driven by continuous replication, even during the asymptomatic stage of HIV infection that precedes AIDS[3]. The generation of viral variants is a major mechanism by which HIV not only resists antiretroviral therapy, but also evades host immune responses[3]. Although cART greatly reduces the spread of HIV to susceptible cells, HIV's ability to form latent reservoirs in the blood, spleen, and lymph nodes effectively renders the infection incurable by cART alone[4]. Furthermore, cART does not prevent infection by HIV.

There is, thus, a desperate need for the development of a preventative vaccine against HIV infection. The design of an effective HIV vaccine has been stunted largely by the fact that the immune correlates of protection against HIV are not understood. Individuals who remain uninfected by HIV despite repeated exposure to the virus represent an ideal study cohort, since they may possess the key(s) to HIV resistance.

The Discovery of the Human Immunodeficiency Virus

The acquired immunodeficiency syndrome (AIDS) was first described in 1981, when an article in the Morbidity and Mortality Weekly Report (MMWR) from the Centers for Disease Control (CDC) in the United States (US) described five cases of *Pneumocystis carinii* pneumonia (PCP) in previously healthy men in Los Angeles[5]. Another report released a month later in the MMWR chronicled a similar occurrence of PCP along with Kaposi's sarcoma (KS) in homosexual men in New York and Los Angeles[6]. These findings were alarming because PCP had

been hitherto rarely observed by physicians in the US. By 1984, seminal research by Francoise Barre-Sinoussi at the Pasteur Institute in France and Robert Gallo at the National Institute of Health (NIH) in the US determined the causative agent of this outbreak to be a retrovirus that induces T cell dysfunction by specifically targeting CD4+ T cells[7,8]. This pathogen was later named the human immunodeficiency virus (HIV).

HIV is the etiologic agent of AIDS, as all of Koch's postulates are satisfied:

1) *The microorganism must be found in all cases of the disease.*

2) *It must be isolated from the host and grown in pure culture.*

3) *It must reproduce the original disease when introduced into a susceptible host.*

4) *It must be found in the experimental host so infected.* [9]

Polymerase chain reaction (PCR) technology has been used to demonstrate the presence of HIV in virtually all patients with AIDS[10-12]. HIV from infected patients at all stages of disease has been isolated and propagated using co-culture techniques[13,14]. In the absence of direct inoculation experiments, due to ethical considerations, several case studies from the literature fulfill Koch's postulates:

a) Three laboratory workers developed AIDS after exposure to HIV IIIB and in the absence of any other risk factors. In all three cases, HIV was isolated from the infected persons and shown to be the original HIV IIIB strain by sequencing analysis. All three patients displayed a marked drop in their CD4+ T cell count, and one subject developed PCP[15].

b) Seventeen (17) of 42 health care workers (HCW) who contracted HIV occupationally developed AIDS in the absence of other high risk incidents[16].

c) There are no reports in which clinical AIDS has not been preceded by seroconversion for HIV. This chronological association has been repeatedly observed in studies of haemophilia[17-19], intravenous drug use[20,21], and sexual transmission in which serial blood samples were available to pinpoint the time of seroconversion[22-24]. Mother-to-child transmission cases also strengthen this association[25].

In 1986, a similar retrovirus was isolated from the cells of AIDS patients in West Africa[26]. This second form of HIV (HIV-2) remains localized in West Africa and is not as pathogenic as the originally described virus (HIV-1); individuals infected with HIV-2 can live much longer lives before the development of disease than those infected by HIV-1[27]. In contrast, HIV-1 is prevalent in all regions of the world and is responsible for the AIDS pandemic that has devastated the human race.

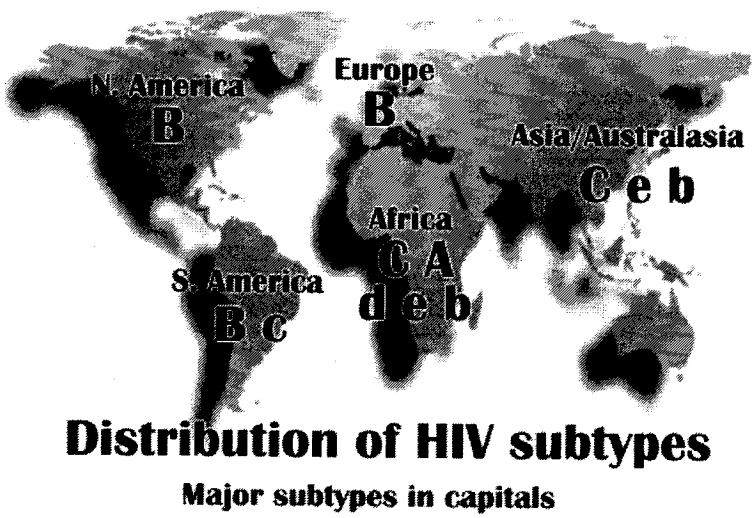
Classification and Origin of HIV

HIV is a primate lentivirus, belonging to the family *retroviridae*[9]. Phylogenetic studies of primate lentiviruses conclude that HIV-1 is closely related to a virus that naturally infects chimpanzees, the simian immunodeficiency virus (SIVcpz)[28], whereas HIV-2 is akin to an SIV strain whose natural host is the sooty

mangabey monkey (SIVsmm)[29]. By extrapolation, it is believed that a zoonotic transfer of these SIVs resulted in the emergence of HIV-1 and HIV-2[28-30].

There are three genetically distinct groups of HIV-1, each thought to represent a separate monkey-to-human transfer of SIV[31-33]. Group M (for majority) consists of eleven distinct clades of HIV-1 (A1, A2, B, C, D, F1, F2, G, H, J, and K)[34,35]. They are all diversifications of an ancestral strain that is estimated to have entered the human population in the 1930's[36]. These subtypes account for over 90% of HIV infections worldwide[34].

Figure 2: Geographic Distribution of the Most Common HIV subtypes



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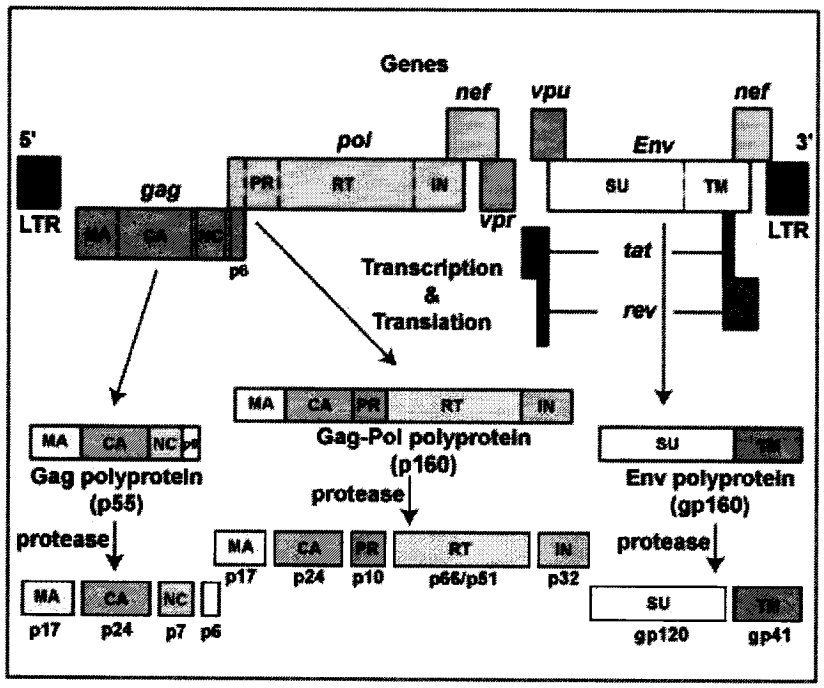
Two different clades of HIV-1 can recombine in a host to produce a hybrid virus. While most of these hybrid viral strains do not propagate efficiently, those that are able to infect other hosts are named circulating recombinant forms (CRF)[37]. The best example is CRF A/E. The viruses that spread rapidly throughout Thailand in the late 1980's were originally classified as Subtype E based on distinct sequences in the extracellular portion of gp120 and gp41[38]. Every subtype E Gag protein that had been sequenced to date, however, belonged to the subtype A radiation[32]. Cloning and analysis of full length proviral sequences of subtype A and E isolates confirmed that the outbreak of HIV-1 in Thailand was caused by a recombinant form, CRF A/E[39]. Phylogenetic analysis, along with the presence of multiple crossovers in the viral genome characteristic of recombination events, eliminated the possibility that subtype E viruses arose from the unusual evolution of subtype A viruses[39].

Group O (for outlier) contains fewer strains than Group M, all of which are largely restricted to Cameroon, Gabon, and Equatorial Guinea[31,32]. Non-M and non-O (Group N) is a very recent designation of a small number of strains found exclusively in Cameroon[33].

Molecular Biology of HIV-1

The HIV genome is 9.8 kilobases (kb) in length [see Figure 3]. There are nine genes that encode for fifteen proteins. The unspliced mRNA transcript of the proviral DNA genome serves as the genetic material in newly packaged virions. Typical of all retroviruses, the HIV genome contains Gag and Pol genes.

Figure 3: The HIV genome



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Alternative reading frames within the unspliced genomic mRNA give rise to both Gag and Pol gene products. In most instances, the mRNA is translated to produce a 55 kd polypeptide ($Pr55^{gag}$) that is subsequently cleaved by viral protease (PR) to yield several structural proteins, including matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7), p1, p2, and p6. A short tract of polymeric uracil (US), followed by an RNA hairpin structure, induces a (-1) frameshift in the reading of the mRNA that is responsible for the production of a Gag-Pol precursor protein ($Pr\ 160^{Gag\text{-}Pol}$). The efficiency of this frameshift is only approximately 5%, thus there is a greater concentration of $Pr\ 55^{Gag}$ than Pr

$160^{Gag\text{-}Pol}$. The Gag-Pol fusion protein is then cleaved by cellular enzymes to generate reverse transcriptase (RT; p66), protease (PR; p10), and integrase (IN; p32). RT is further cleaved at the carboxy (C)-terminus to release a 14 kd polypeptide.

The remaining amino (N)-terminal p51 domain associates with an uncleaved p66 protein to form a heterodimer. This complex matures into a functional RT enzyme that possesses polymerase and RNAse H activity. [9]

The envelope (Env) gene is another genetic characteristic of retroviruses[9]. In the case of HIV, an 88 kd precursor protein is translated from a singly spliced RNA transcript[40,41]. This polypeptide becomes glycosylated in the rough endoplasmic reticulum (ER) and assumes an apparent molecular weight (MW) of 160 kd (gp160)[42,43]. Cleavage of this glycoprotein by a cellular enzyme creates two mature gene products that migrate to the plasma membrane[44]: transmembrane envelope (TM) gp41 has a hydrophobic domain which serves to anchor the protein in the plasma membrane[45], and surface (SU) envelope gp120, an extracellular unit that remains associated with the cell surface via a non-covalent interaction with gp41[46].

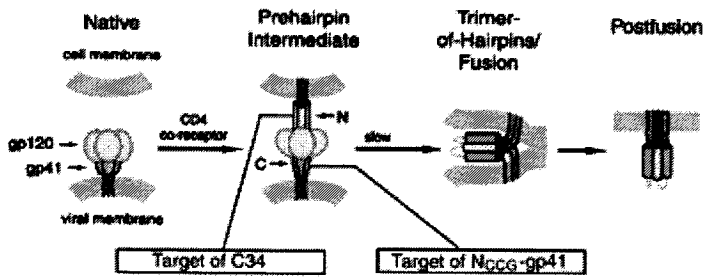
Accessory Proteins and The HIV-1 Life Cycle

1. Entry

HIV infection of a host cell is initiated by the binding of gp120 on the viral membrane to a CD4 receptor on a host T cell or a macrophage[47,48]. This high affinity interaction induces a conformational change in the gp120 structure that

promotes a secondary binding event between gp120 and a chemokine receptor[49,50] [see Figure 4]. Co-culture experiments have revealed that HIV is capable of using up to a dozen different chemokine receptors as co-receptors for entry into target cells[51,52]. The beta chemokine receptor CCR5, however, is the principal co-receptor used in the mucosal spread of HIV[52,53]. Viruses that use CCR5 for entry (R5 viruses) have a tropism for macrophages (M-tropic) and are the dominant viral phenotype found in newly infected individuals[52].

Figure 4: HIV Fusion



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A thirty two base pair deletion in the genetic sequence that codes for the CCR5 receptor (CCR5Δ32) results in a non-functional, truncated protein[54]. Individuals who are homozygous for CCR5Δ32 are thought to be resistant to HIV infection, thereby demonstrating the importance of this receptor for viral infectivity[54,55].

It was originally believed that gp120/gp41 monomers on the viral membrane cluster into symmetrical trimer complexes, but recent imaging evidence suggests that trimer formation succeeds co-receptor engagement[56].

Each of the three associated gp41 proteins spring open and project a peptide fusion domain that effectively harpoons the lipid bilayer of the target cell[57,58]. Subsequent hairpin formation promotes the fusion of virion and target cell membranes, leading to the release of the HIV viral core into the cell interior[57,58].

A contentious area of research is whether or not the mobilization of trimeric gp120-bound CD4 and co-receptor into lipid rafts is necessary for HIV entry into target cells. Some studies have reported that the chemokine receptors that are used by HIV for entry are preferentially located in lipid rafts[59,60]. These cholesterol and sphingolipid enriched microdomains in the plasma membrane mirror the optimal lipid bilayer content of the viral envelope, thereby promoting the fusion of the two membranes[59,60]. Other studies, however, maintain that localization of CD4 and co-receptors into lipid rafts are not required for the fusion process[61]. In either case, cholesterol has been found to be a critical factor in the fusion process, as removal of cholesterol from either the virion or the target cell membrane greatly reduces the infectivity of HIV[62,63].

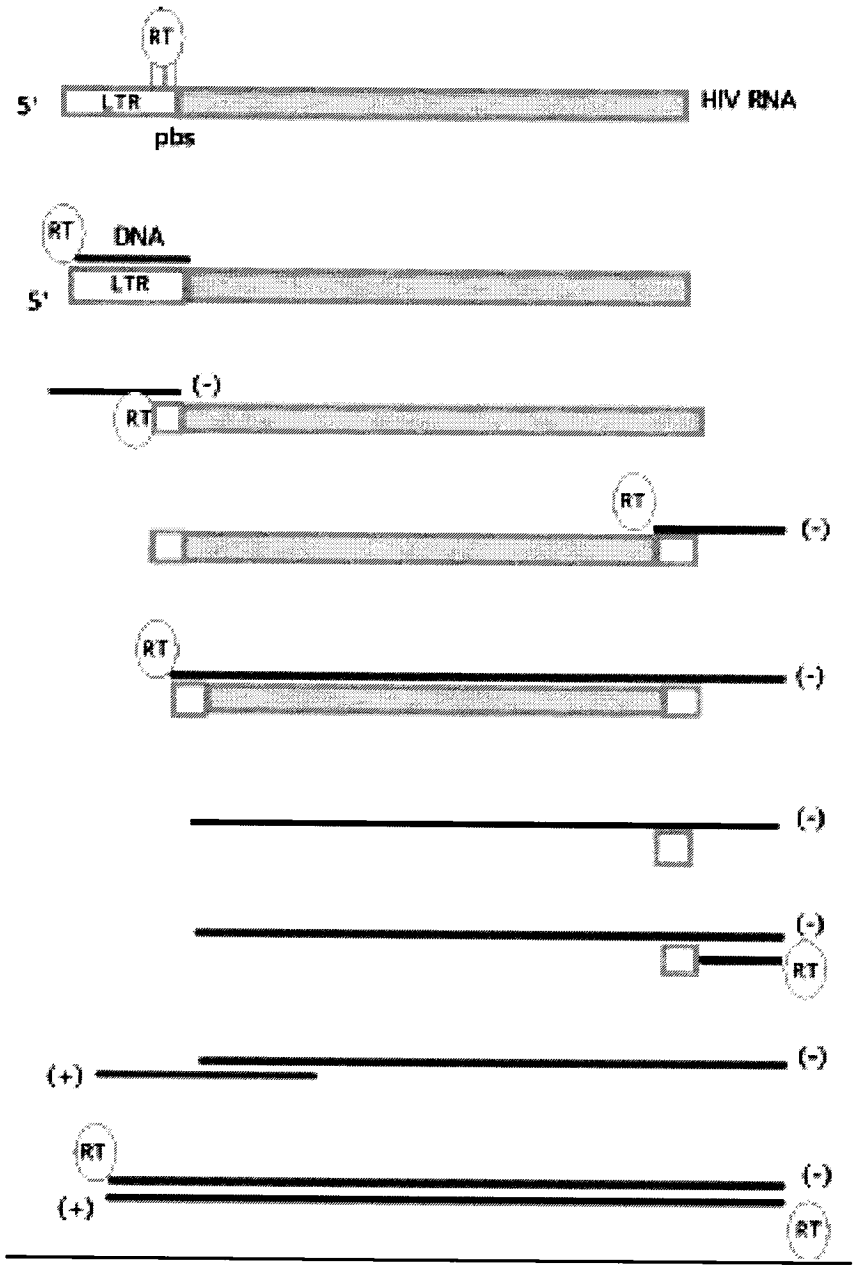
2. Uncoating and Reverse Transcription

Once inside the cell, phosphorylation of the MA proteins by cellular factors is believed to trigger uncoating of the viral core[64]. The phosphorylated MA proteins orient the core to dock with actin microfilaments in the cytoplasm[65]. Reverse transcriptase (RT), which is packaged in the virion, then begins the process of copying genomic viral RNA into DNA [see Figure 5]. Transfer RNA specific for lysine ($tRNA^{lys}$), which is abundantly available in the host cell, primes negative (-)

strand DNA synthesis by hybridizing to one of the viral RNA strands at the primer binding site (pbs)[9]. The resulting partially double-stranded RNA is repaired by RT, such that a complementary DNA copy of the 5' LTR is synthesized[9]. The RNAse H activity of RT then degrades the sequences 5' to the R region, allowing the newly formed DNA strand to reanneal at the 3'R region[9]. DNA synthesis continues from that site, generating a complete complementary DNA copy of the starting material[9]. RT then digests the original genomic RNA, but preserves the RNA portion that hybridizes to a polypurine tract of the (-) strand DNA[9]. This segment serves as the primer for positive (+) strand DNA synthesis[9]. The resulting double stranded DNA, with LTR, U3-R-U5 at both ends, is the HIV provirus that is integrated into the host cell DNA[9].

The generation of HIV proviral DNA from genomic RNA is stabilized by the viral protein Vif. The human protein apolipoprotein B messenger RNA (mRNA) - editing enzyme-catalytic polypeptide-like-3G (APOBEC3G) catalyzes the deamination of deoxycytidine on (-) strand reverse transcripts, yielding deoxyuridine and resulting in cDNA degradation, insertion of hypermutations, and/or defective (+) strand cDNA synthesis[67,68]. This innate resistance to HIV replication is neutralised by the HIV protein Vif, which binds to APOBEC3G and induces its rapid degradation via an ubiquitin-dependent proteosomal pathway[67,68].

Figure 5: HIV Reverse Transcription



3. Integration and Latency

The double stranded viral cDNA combines with RT, integrase (IN), MA, Vpr, and the high-mobility group, DNA-binding protein, HMGI(Y), to form the pre-integration complex (PIC)[69]. Canonical nuclear localization sequences (NLS) in the phosphorylated MA proteins[70,71], along with non-canonical NLS in Vpr[72] and IN[73], have all been proposed to be responsible for the migration of the PIC into the nucleus of the host cell[74,75].

Once inside the nucleus, the viral DNA genome may circularize by ligating its 3' and 5' LTRs and remain as an extrachromosomal body in the nucleus[76]. Alternatively, the proviral DNA may integrate into the host chromosome. Integrase (IN) removes 2 bases from both ends of the linear dsDNA[69], and then catalyzes a joining reaction between the staggered ends of the provirus and cellular DNA that has been cleaved asymmetrically[69]. The HIV provirus is preferentially integrated into activated genes and transcriptional hot spots[77], whereas it is rarely found in the centromeres[78].

As a lentivirus, HIV can remain transcriptionally dormant (latent) for extended periods of time[79]. HIV is capable of lifelong persistence in its host, partly because of its ability to integrate into the chromosome of terminally differentiated, non-dividing cells[80]. Indeed, macrophages and CD4+ T cells serve as the major viral reservoirs during HIV infection[81]. There are significantly more cells in a host that harbour replication-competent, proviral DNA, than cells that are transcriptionally active[81]. In quiescent CD4+ T cells, cellular activation provides an abundance of

resident transcription factors that allow the production of viral proteins to commence[82].

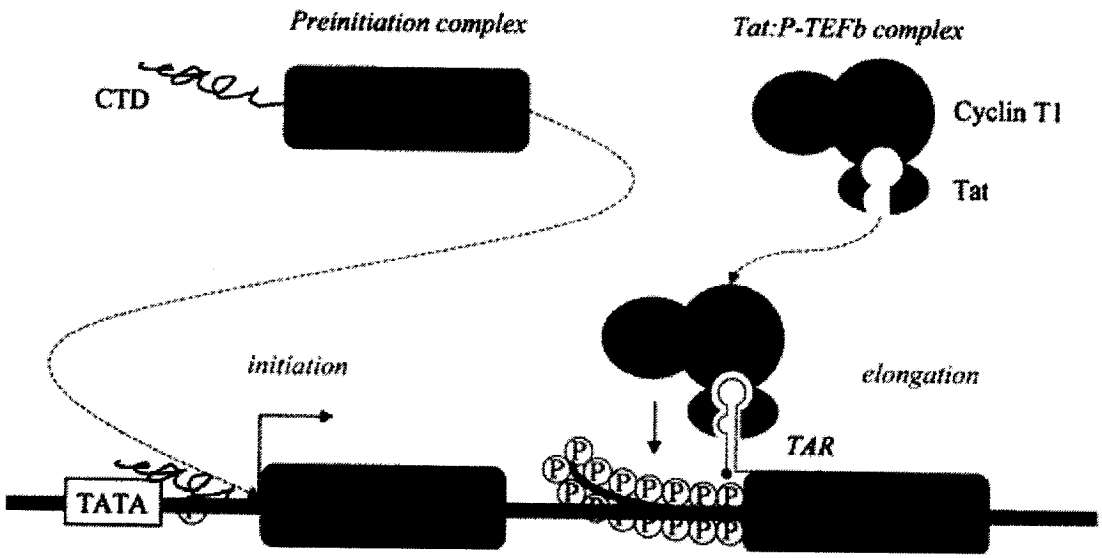
4. HIV Transcription

Transcription of the HIV genome is largely regulated by the viral protein Tat [see Figure 6]. Its importance is highlighted by the fact that HIV virions are not produced when Tat function is impaired[83]. The HIV 5' LTR contains a prototypic promoter for RNA Pol II[9]. There is a consensus TATA motif, immediately upstream of which are binding sites for host cell transcription factors including NFK-B, SP-1, and NFAT[9]. Although transcription is capable of being initiated without the help of Tat, polymerase fails to elongate efficiently along the genome, resulting in the synthesis of a short, non-polyadenylated RNA transcript[84]. The formation of a stem-loop-bulge secondary structure allows the RNA transcript to remain stable in the cell, and is called a transactivation response (TAR) element[84]. Tat possesses an arginine-rich motif (ARM) that binds the bulge region of the nascently transcribed TAR RNA, effectively tethering the viral protein upstream the promoter[85]. Tat also possesses a cysteine–rich domain (cofactor binding domain) that is responsible for dimerization with the cellular protein cyclin T1[85,86]. Tat binds cyclin T1, in effect recruiting it to the promoter as well[86]. Cyclin T1 binds the TAR loop and then recruits the cellular kinase CDK9 to the promoter, which phosphorylates the C-terminal domain (CTD) of RT[87,88]. Phosphorylation of the enzyme significantly enhances its processivity[87,88]. Tat,

therefore, upregulates HIV transcription more than 100-fold by catalyzing the phosphorylation of the CTD of RT[88].

Overlapping reading frames and complicated mRNA splicing schemes are used to generate more than 30 different mRNA species[9]. Small, multiply spliced

Figure 6: Tat Control of HIV Transcription



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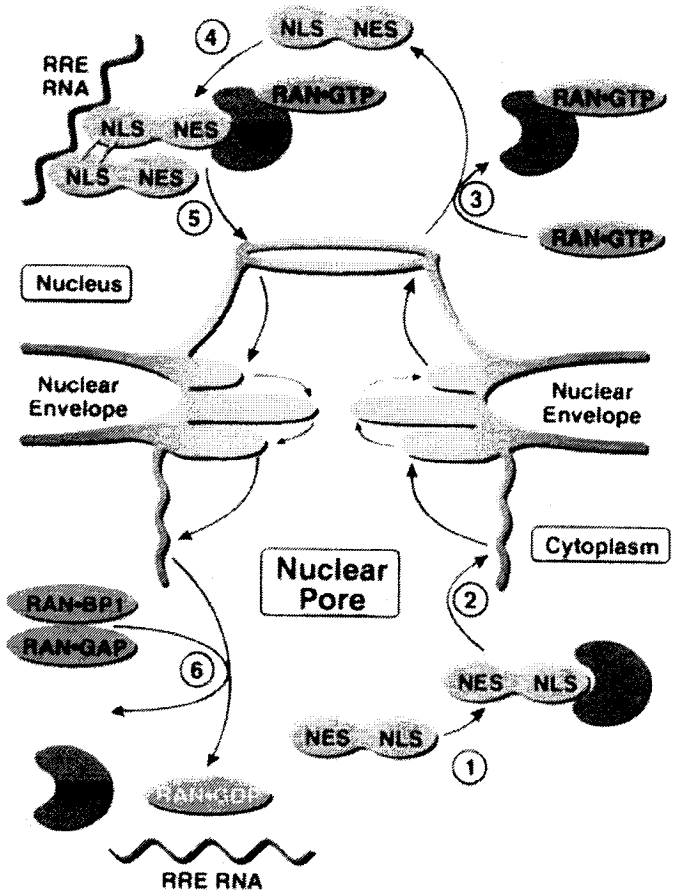
mRNAs encoding Tat, Rev, and Nef predominate in the cytoplasm early after viral transcription[90]. The transport of genomic and singly spliced RNA into the cytoplasm depends on the production of a threshold amount of Rev[90]. The target sequence of this viral protein, the rev responsive element (RRE), is found in bicistronic mRNAs, such as the Gag-Pol transcript [91]. Rev contains two domains: an ARM domain that binds RNA and functions as a nuclear localization sequence

(NLS), and a leucine-rich domain known to function as a nuclear export signal (NES)[92].

Rev transports mRNA transcripts out of the nucleus and into the cytoplasm for translation [see Figure 7]. This shuttle service of Rev is driven by a concentration gradient of a host factor Ran. The enzyme RCC1 in the nucleus maintains Ran in a triphosphorylated state (RanGTP) whereas RanGAP in the cytoplasm dephosphorylates the protein to produce RanGDP. Briefly, Rev binds an RRE in an mRNA transcript via its ARM. Multimerization of Rev on the mRNA induces the binding of RanGTP to the NES of Rev. RanGTP guides the protein complex down its concentration gradient and across the nuclear membrane. In the cytoplasm, dephosphorylation of RanGTP by RanGAP initiates the dissolution of the protein-RNA association. Once the mRNA transcript is released, osmotic flow returns RanGDP-bound Rev to the nucleus. Mutations in the NES or NLS of Rev abolish its function and confer a dominant trans-negative phenotype to the virion. [90]

Figure 7: Shuttling of mRNA into the Cytoplasm by Rev



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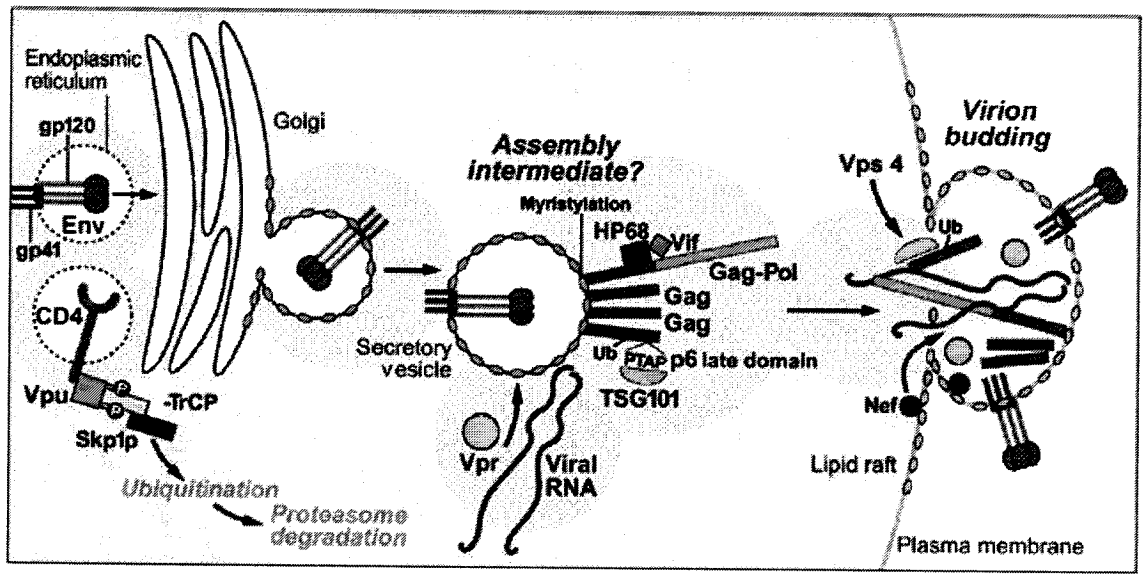
5. Budding and Virion Maturation

In the cytoplasm, P7 gag binds a packaging sequence (Ψ) in the 5' LTR of the unspliced viral RNA genome, a sequence that is excised during all splicing events[9]. P24 stabilizes the core structure by binding to it and precipitating a tubular shape[9]. The nucleoprotein migrates to the plasma membrane and assembles with the viral envelope coat. The terminal step in the budding reaction necessarily involves a second membrane fusion event. Myristylated Gag p17 mediates the alignment of the viral core and gp41[94]. Myristylation and

Figure 8: HIV Budding



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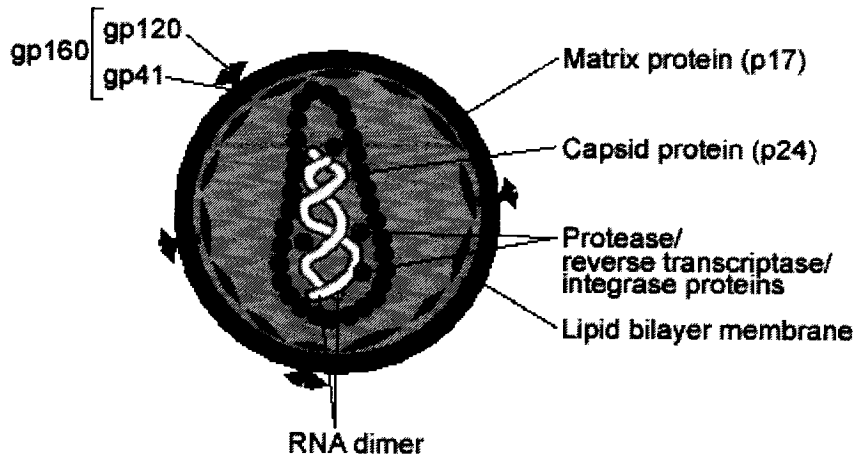
palmitoylation confers hydrophobic properties to the protein, which enable it to associate preferentially with cholesterol and glycolipid rich membrane microdomains[94,95]. Virion budding occurs through these specialized domains in the lipid bilayer, resulting in virions that are coated in a cholesterol-rich membrane[95]. Budding depends critically on a late domain sequence (PTAP) present in the p6 portion of Gag[96], which is bound by Tsg101, an ubiquitin-binding protein[97,98]. Tsg101 usually associates with other cellular proteins in the vacuolar protein sorting pathway that selects cargo for exportation[97,98].

The processing of Gag and Gag-Pol precursors occurs late in the budding progression, although the mechanism by which the precursor Pr160gag-pol is

cleaved is not completely understood. It has been hypothesized that protease p10, which mediates the cleavage reaction, is poorly active until the terminal stages of nucleoprotein complex formation, when a high local concentration of p10 is achieved. Once the precursor protein is cleaved, the individual products coalesce and the core condenses into an electron-dense cylindrical structure. [99]

A mature HIV virion is a spherically shaped structure of approximately 100 nanometers (nm) in diameter [see Figure 9]. Its core is composed of nucleoproteins complexed to two genomic ribonucleic acid (RNA) molecules. This core is embedded in a protein sheath and enveloped by a lipid bilayer spiked with seventy-two Env glycoproteins. [9]

Figure 9: Simplified HIV Virion



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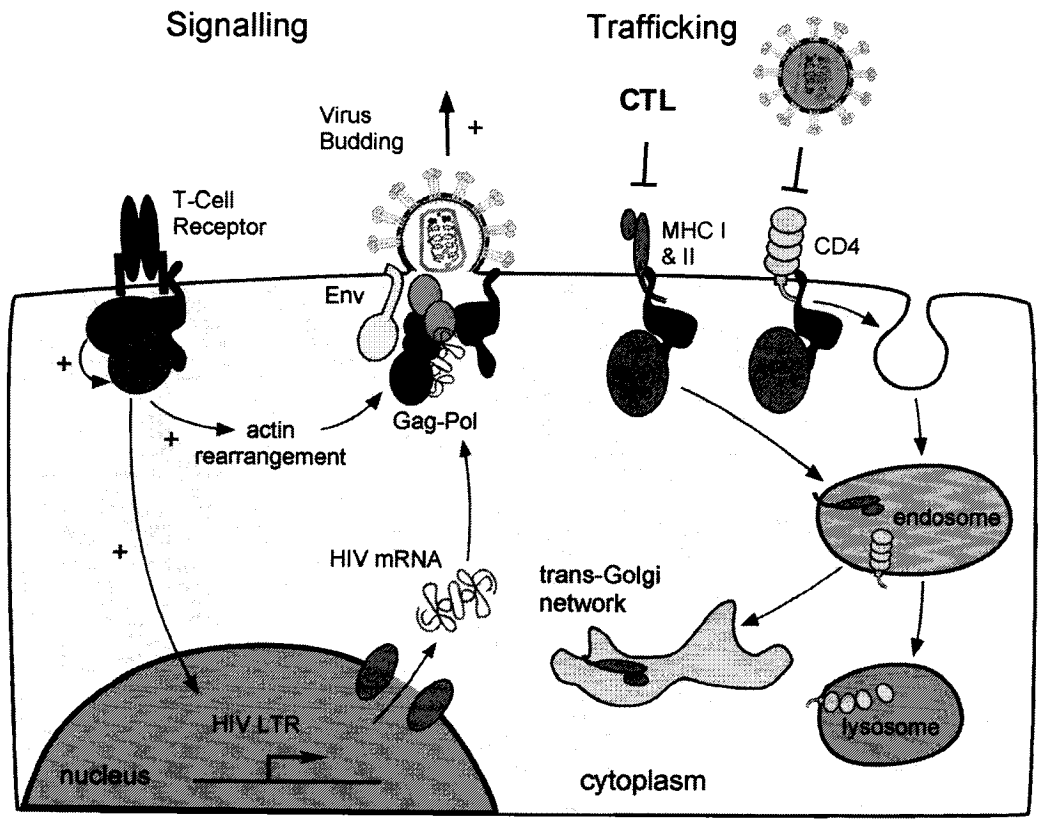
6. Accessory Proteins Nef, Vpr, and Vpu

The well conserved gene Nef encodes for a 27 kd protein that promotes the production and release of more infectious virions[9]. Inactivation of the Nef gene, from both SIV[100,101] and HIV[102,103], results in a slow progression towards end-stage disease. Nef enhances virion infectivity during viral assembly by affecting cellular signalling, causing profound cytoskeletal rearrangements, and altering

Figure 10: Functions of Nef



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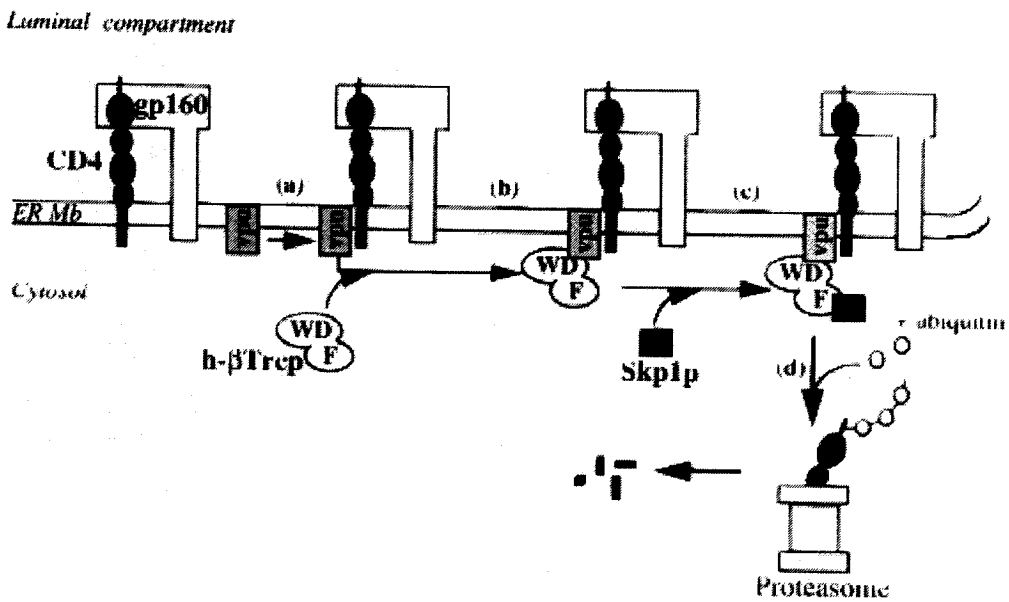
downstream effector functions[101,103] [see Figure 10]. Nef downregulates the cell surface expression of CD4 receptors by binding to the cytoplasmic tail of CD4 and to the host adaptor protein AP-2[104]. A signalling sequence is thus triggered by Nef that culminates in the internalization of CD4 into clathrin-coated pits[104]. Furthermore, Nef mediates the elimination of major histocompatibility complex (MHC) class I molecules in the cytoplasm, thereby allowing virions to evade antiviral immunity from cytotoxic T lymphocytes (CTL)[92,105]. Nef also blocks host immunity by blocking the induction of apoptosis within an infected cell. Nef binds to and inhibits the apoptosis signal regulating kinase (ASK)-1, an intermediate protein of the TNFR and Fas signalling pathways[106]. Similarly, Nef can bind p53, another important protein in the apoptosis signalling pathway, and inhibit its function[107]. Alternatively, Nef can activate the expression of Fas ligand (FasL), which induces apoptosis in bystander cells that express Fas (for example CTL)[108]. Nef, therefore, is critical in allowing newly formed virions to propagate by disarming the host immune system.

Vpr can transactivate many different viral and cellular promoters[9]. Though not as seminal as Tat, it is believed that Vpr synergizes with Tat to boost HIV transcription[109]. Its presence in virions suggests an important function early in infection. Since Vpr has an NLS and has been shown to interact with nucleoporins, it has been postulated that Vpr is important for the nuclear import of the pre-integration complex[92,110]. Another theory proposes that Vpr can induce cell cycle arrest in the G2 phase, during which the HIV-1 promoter is most active[111].

Vpu is an integral membrane protein that enhances viral infectivity by facilitating the budding of virions from the plasma membrane[112]. Cells infected by HIV strains defective in Vpu function show rapid syncytia formation and cytopathic effects, but progeny virions stay in the cytoplasm[112]. The mechanism lacks specificity and is not restricted to HIV[112]. The N-terminal transmembrane domain of the protein appears to be responsible for increased viral shedding[112]. In addition, Vpu is involved in the selective degradation of CD4 in the ER [see Figure 11]. The mechanism is highly specific, as Vpu recruits the cellular factor h-bTrCP, to the ER membrane while binding the cytoplasmic tail of CD4[113]. H-bTrCP recruits Skp1, triggering the ubiquitin-mediated lysis of CD4 by the proteasome[113]. The ability of Vpu to induce CD4 degradation is lost if the C-terminal domain of the protein is truncated[112].

Figure 11: Vpu-mediated Degradation of CD4



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HIV Tropism

HIV isolates that engage the chemokine receptor CCR5 as a co-receptor during viral entry into target host cells (R5 viruses) are primarily responsible for the establishment of HIV infection[115]. In addition to CD4+ T cells, R5 viruses have a tropism for macrophages (M-tropic)[115]. Viruses that use CXCR4 (X4 viruses) only infect CD4+ T cells (T-tropic), induce syncytia in vitro, and are generally observed in the later stages of HIV infection[115]. Although over ten G protein-coupled receptors have been ascribed co-receptor function in vitro, all primary HIV isolates use either CCR5 or CXCR4 or both (R5x4 viruses) to mediate entry into target cells[116,117]. Furthermore, infections performed on peripheral blood lymphocytes demonstrated viral isolates that can use multiple receptors beyond CCR5 or CXCR4 are completely inhibited by CCR5 and/or CXCR4 specific antagonists[117].

Many HIV isolates that primarily use one co-receptor can be induced to use another if the other receptor is over-expressed[118,119]. In fact, co-receptor switching from R5 to X4 usually occurs 8-10 years after HIV infection and is a harbinger of accelerated clinical disease progression[120]. Factors that mediate evolutionary pressure on the virus include the host target cell prevalence, chemokine levels, and susceptibility to neutralizing antibodies (nAb) or CTL killing[121].

In contrast, intrinsic properties of the virus resist the switch. An increased susceptibility to co-receptor inhibitors during the transition implies suboptimal co-receptor usage. Also, diminished replication compared to the parental R5 isolate

implies a loss of competitive fitness. Lastly, there are only a limited number of molecular transitions that will effectuate co-receptor switching. The V3 loop of gp120 is the most important determinant in viral tropism. The amino acid charge in a few specific positions to a large extent determines the virus' ability to bind the host cell chemokine receptor. Silent mutations are rare, and there is an inherently strong bias in favor of a G to A substitution rather than random mutations. [122]

HIV Evolution

Viral replication is responsible for the generation of sequence variation, and is a function of the selective pressure for structural change imposed by the host immune system[123-125], antiretroviral drugs[126-128], and/or the availability of preferential target cells[129]. In early HIV infection, most individuals possess a viral population that is fairly homogeneous in the Env gene sequence[130-132], although some people have relatively diverse HIV-1 populations at the time of seroconversion[133]. There is a low level of variation in the other genes, such as Gag p17, gp41, and Nef[130]. After seroconversion, HIV variants with different, but related, gene sequences emerge, and by the chronic stage of infection, HIV patients normally have developed a heterogeneous population of viral quasi-species[123,134].

Cellular compartmentalization plays a significant role in the evolution of HIV quasispecies over the course of infection. Genetic analysis of HIV Env sequences in serodiscordant heterosexual couples revealed genetic distinctions among viral populations in PBMC, plasma, seminal cells, and seminal fluid[133].

Viral phenotyping has also shown that drug resistance mutations in HIV populations are unequally distributed in the blood and semen[135]. Recently it was shown that HIV in CD14+ blood monocytes could evolve in parallel to viral quasi-species in CD4+ T cells in the lymphoid tissue[136].

HIV Epidemiology

There are numerous variables that affect the infectivity of HIV, but the concentration of HIV in a person's blood and/or genital secretions is the strongest predictor of HIV transmission[137,138]. Thus, individuals are most likely to transmit HIV soon after they are infected and when they have progressed to AIDS. The amount of HIV in the serum of men with CD4+ T cell counts below 200/µl plasma or with an AIDS defining illness is greater than that in men with higher CD4+ T cell counts or at earlier stages of infection[139,140]. Although the probability of HIV transmission is high at end-stage disease, recent epidemiological evidence suggests that HIV is preferentially transmitted during primary HIV infection, when robust viral replication yields a large amount of infectious HIV virions in the serum of acutely infected patients[141].

There are also host factors that influence an individual's susceptibility to HIV infection. The presence of reproductive tract infections, including genital ulcer disease (GUD), gonorrhea, clamydia, and trichomonas, is strongly associated with susceptibility to HIV infection[137,142]. Sexual promiscuity and certain sexual practices are also conducive to HIV infection. For example, there is a greater risk of HIV acquisition associated with receptive anal intercourse than

with receptive vaginal intercourse[142]. Alternatively, male circumcision is associated with a reduced risk of HIV infection[143]. The foreskin of uncircumcised males is enriched with Langerhans cells, which serve as primary cellular targets for HIV entry[143]. While strong epidemiological data supports the notion that circumcised males are less likely to seroconvert than uncircumcised men, data also exists to the contrary[144].

Environmental factors also play a role in HIV transmission. The length of time the epidemic has been present in a community dictates the local prevalence rate and the probability of exposure to the virus. In parallel, the number of afflicted people within a community raises awareness and influences behavioural and social responses. The risk of contracting HIV is increased in certain environmental settings, such as commercial sex facilities, crack/cocaine houses, and bathhouses. [142]

Lastly, properties inherent to the infecting viral strain determine the level of transmission as well. HIV subtypes with distinct geographical distributions have varied degrees of pathogenic potential. For example, CRF A/E has a greater tropism for Langerhans cells than HIV subtype B[145]. The relatively high concentration of HIV in the semen from infected patients in sub-Saharan Africa may reflect differences among clades in their ability to replicate in vivo[146]. Alternatively, viral subtypes harbouring drug resistant mutations may be preferentially transmitted[147-150]. These viruses possess an adaptive advantage over wild-type strains, thereby increasing the risk of HIV acquisition in the endemic region.

Although unprotected sexual intercourse is the mode by which HIV is transmitted in the vast majority of cases worldwide, anal intercourse is associated with the greatest risk for HIV acquisition[142]. The estimated per contact risk of HIV acquisition from unprotected receptive anal intercourse is 0.0082 when the partner is HIV seropositive, and 0.0027 when partners of unknown HIV serostatus were included in the calculations[151]. The probability of contracting HIV via vaginal intercourse is between 0.0003 and 0.0015[137,152,153], roughly ten times less than that by anal intercourse.

Mother to infant transmission represents another major modality that accounts for a large number of HIV infections around the world. HIV may be transmitted while the fetus is in utero, during the delivery of the baby, or during breast feeding. In the absence of antiretroviral treatment, the estimated rate of mother to child HIV transmission ranges from 0.14 to 0.25 in developed countries and between 0.13 and 0.42 in the developing world[154]. HIV transmission via breast milk is greatly dependent on the maternal viral load in the plasma and on her CD4+ T cell counts[155,156]. The probability of transmission via breast milk is 0.00064 per liter of consumption, or alternatively 0.00028 per day of breast-feeding[155]. It is approximately equivalent to the probability of acquiring HIV during heterosexual intercourse.

Intravenous Drug Use

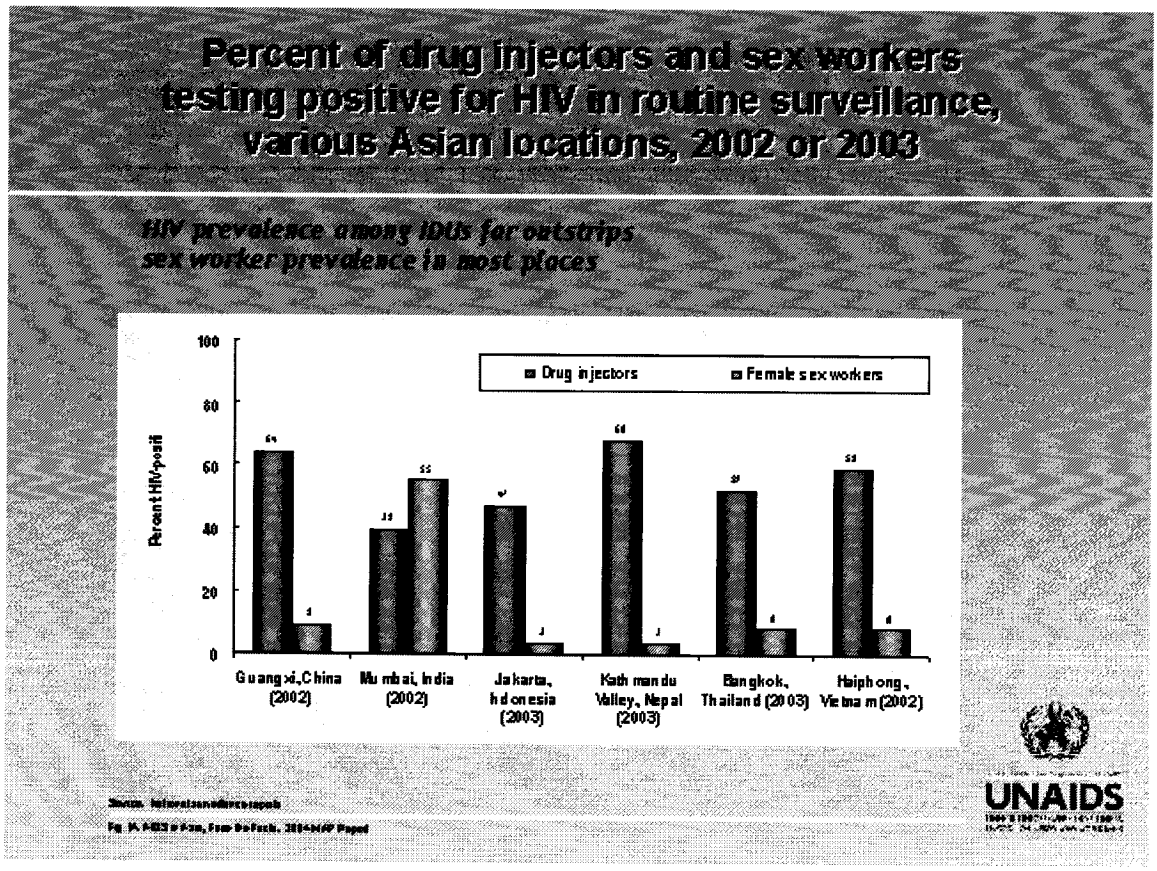
Although sexual contact accounts for the vast majority of HIV transmissions around the world, the World Health Organization (WHO) estimates

that unsafe injections result in 80,000-160,000 new HIV infections globally every year[1]. Through needle and syringe sharing, intravenous drug abuse (IDA) remains a major path of HIV transmission in China, Malaysia and Viet Nam[157] [see Figure 12]. In addition, it is responsible for regional outbreaks of HIV-1 in Russia, Ukraine, India, Pakistan, and Indonesia[157]. In the United States (US), IDA directly and indirectly accounts for nearly half the annual HIV infections[158].

Intravenous drug users (IVDUs) become infected with HIV because they inject themselves with contaminated syringes. An intravenous drug user will insert the needle of a syringe loaded with drugs into a vein and draw the plunger back until blood appears in the barrel of the syringe. Once the needle is registered, the drugs are injected. Many injectors pull up on the plunger again to ensure that the all the drug remnants are adequately mixed with the blood, and then re-inject. This process is called "booting" and is characteristic of cocaine IVDU. By sharing a syringe with a partner(s), an IVDU exposes himself to the micro-organisms present in his partner's blood, including HIV. The probability of HIV infection as a result of a parenteral exposure is estimated to be 0.003 per exposure event, which is on par with the probability associated with an exposure to HIV by unprotected anal intercourse. [159]

Younger age is an independent predictor of HIV seroconversion for both men and women IVDUs[158]. New injectors share syringes more frequently than older IVDU, and are often associated with social networks, for example crack/cocaine houses, that are more conducive to HIV infection than those of

Figure 12:



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more experienced IVDU[160]. In addition, injectors addicted to cocaine are more prone to HIV infection[158,160]. A high frequency of injections, attendance at shooting galleries, and trading sex for money and drugs are all associated with an elevated risk for HIV infection[158,160].

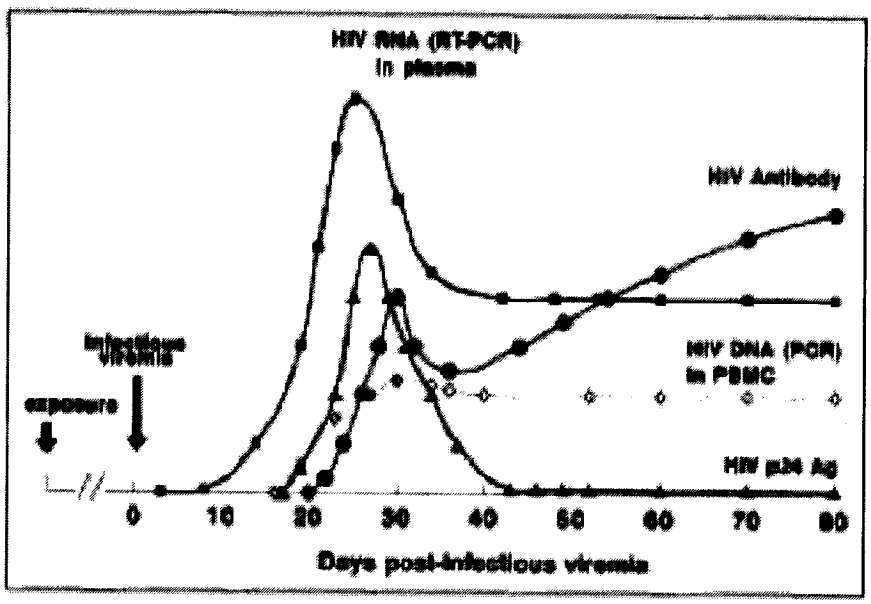
Clinical Course of HIV Infection

Primary HIV infection is characterized by an initial burst of rapid viral replication and dissemination, leading to the presence of extremely high levels of

viral RNA in the blood of the infected patient[161,162] [see Figure 13]. Patients in primary infection often experience a brief illness immediately before antibody seroconversion resembling mononucleosis, with fever, pharyngitis, adenopathy, and rash[163].

The time from HIV exposure to viral dissemination and the development of infectious viremia in the blood is highly variable between hosts, generally requiring 1-2 weeks, but occasionally up to six months. The surge of HIV RNA in the plasma is first detectable by reverse transcription (RT)-PCR approximately ten days after its appearance in the blood (day 0), and peaks between days 20 and 30. Peripheral blood mononuclear cells (PBMC) infected with proviral DNA are recognized by DNA PCR at day 15, and remain present at stable, albeit low, levels. HIV p24 antigens can be first identified by Enzyme-linked immunoassays (EIA) in the blood on day 15, but then disappear by day 40. [164]

Figure 13: Time Course of HIV infection



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Host antibodies specific for HIV appear in the blood approximately 30 days post-viremia, with an early IgM spike followed by the progressive development of HIV-specific IgG antibodies over several months[163]. During seroconversion, the development of a cellular immune response coincides with a 2-3 log unit decrease in viral RNA levels[165]. Generally by day 60, plasma viremia falls to a steady-state level[163,164]. The viral set point correlates with long-term outcome of disease, as well as with the probability of secondary transmission of the virus[140].

Although PCR and EIA for p24 antigenemia consistently detect infections 1-2 weeks earlier than the Western Blot assay for antibody detection, they do not report positive results until weeks or months post exposure, and the results may revert to negative following antibody seroconversion[164]. Consequently, a negative p24 antigen or RNA result is not a basis for excluding HIV infection. HIV antibody testing performed at regular intervals, and for at least 6 months following exposure, represents the gold standard for diagnosing HIV infection[164].

Primary infection is followed by an asymptomatic phase called chronic infection.The average length of the asymptomatic phase is approximately 8 years. Although patients do not suffer with any clinical symptoms during this stage of HIV infection, the virus is continuously replicating vigorously and producing copious amounts of infectious virions[166]. The total daily production of HIV-1 is estimated at 10.3 x 10(9) virions[167]. The average amount of time required for a newly released virion to infect another cell and cause the release of a new generation of viral particles is 2.6 days[167]. Consequently, CD4+ T cells are

rapidly eliminated, as the average life-span of productively infected cells has been estimated to be 2.2 days (half-life t 1/2 = 1.6 days)[167].

AIDS is diagnosed clinically when AIDS defining opportunistic tumours and infections, such as PCP and KS, develop [see Table 1], or when CD4 T cell numbers fall below 200 cells/mm^3[3,168]. The rate of CD4 T cell decline and the amount of viral RNA in the bloodstream are surrogate markers of a patient's progression to AIDS and, ultimately, death[140].

HIV Pathogenesis

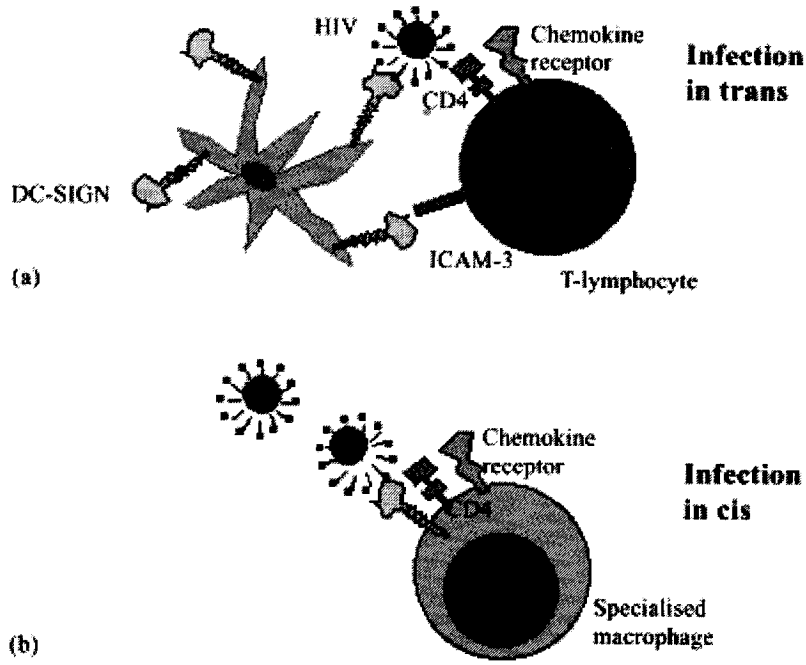
Dendritic cells are the first cell types encountered by HIV during infection and are prominent factors in the transmission of HIV[169]. DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3) grabbing non-integrin) is a type II transmembrane protein with a C-type lectin extracellular domain, expressed exclusively on the surface of epithelial dendritic cells[170]. It normally functions to initiate an adaptive immune response by binding ICAM-3 on resting T cells in the secondary lymphoid organs[170]. In the setting of HIV infection, however, DC-SIGN binds HIV gp120 and transfers the virus to proximal T cells, either via de novo infection (*cis* transfer) or without infection (in *trans*, or trans-infection)[171] [see Figure 14]. Such transfer may occur locally in the inflamed mucosa, or after dendritic cells have matured and migrated to the local draining lymph nodes[171]. DC-SIGN augments HIV's pathogenic potential by preserving HIV virions in an infectious state, thereby extending the period of time (by several days) during which HIV may efficiently infect CD4+ T cells[170,172]. Other C-type

Table 1: AIDS defining opportunistic tumours and infections

- *Pneumocystis jiroveci pneumonia (PCP)*
- *Toxoplasma gondii encephalitis*
- *Cryptosporidiosis*
- *Microsporidiosis*
- *Mycobacterium tuberculosis (MTB)*
- *Mycobacterium avium complex disease*
- *Bacterial pneumonia*
- *Salmonellosis*
- *Campylobacter jejuni infections*
- *Shigellosis*
- *Bartonella infections*
- *Treponema pallidum infection (syphilis)*
- *Candidiasis (mucosal)*
- *Cryptococcus neoformans meningitis*
- *Histoplasma capaulatum infections*
- *coccidiodomycosis*
- *invasive aspergillosis*
- *cytomegalovirus (CMV) disease*
- *herpes simplex virus (HSV) disease*
- *varicella zoster virus (VZV) disease*
- *human papillomavirus disease*
- *hepatitis virus C (HCV) disease*
- *hepatitis virus B (HBV) disease*
- *penicilliosis*
- *leishmaniasis*
- *paracoccidiodomycosis*
- *isospora belli infection*
- *Chagas disease (American trypanosomiasis)*

lectin receptors, such as Langerin on Langerhan cells and mannose receptors on dermal DCs, are equally adept at binding gp120 and mediating transfer to CD4+ T cells[173].

Figure 14: DC-SIGN Mediated HIV Infection



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The lymphoid tissue has been identified as a major site of HIV replication in vivo[4,81]. Based on studies of vaginal SIV infection in macaques that demonstrated a preferential infection of CD4+ T cells at the portal of entry[175,176], it is now understood that CD4+ T cells in the mucosal-associated lymphoid tissue (MALT) are selectively infected by HIV during acute infection[177,178]. CD4+ T cells reside largely within the gastrointestinal (GI) tract, the lymph nodes (LN), and the other lymphatic tissues rather than in peripheral blood[4]. The GI tract contains the greatest quantity of CD4+ T cells that express CCR5[179], the co-receptor favored

by HIV for entry into target host cells. It was, thus, found that there exists, at all stages of HIV disease, a preferential and substantial depletion of GI tract CCR5+ CD4+ T cells[180]. This finding was supported by the SIV/macaque model of HIV infection[181], as a complete depletion of CD4+ T cells in the GI tract of SIV-infected macaques was observed as early as two weeks after infection[175,176]. Furthermore, 30-60% of total body CD4+ memory T cells are infected by SIV during the acute phase of SIV infection, the majority of which are eliminated within four days of virus appearing in the bloodstream[182].

CCR5+ CD4+ T cells from mucosal surfaces are most likely killed directly by either the cytopathic effects of HIV itself or by HIV-specific CD8+ cytotoxic T lymphocytes (CTL)[178,180]. Conversely, CCR5+ CD4+ T cells in the lymph nodes and the peripheral blood are not devastated by HIV, as HIV-infected individuals maintain quantities that are not significantly different than those in HIV-uninfected individuals[180]. The substantial depletion of GI tract $CCR5^+$ $CD4^+$ T cells very early after HIV infection imposes a considerable homeostatic strain on the immune system to maintain the effector and memory $CD4^+$ T cell pools[180]. Failure to reconstitute the CCR5+ CD4+ T cell compartment in the GI tract, resulting in impaired host immune responses against HIV, likely accelerates the host's progression to AIDS[180]. Such a phenomenon has already been observed in rapidly progressing SIV-infected macaques[183].

Activated CD4+ T cells are the principal hosts of viral replication throughout the course of HIV infection, and it is well established that HIV-infected individuals contain higher percentages of activated T cells compared with

uninfected individuals[184]. HIV-associated immune activation results in the abnormal accumulation of effector T cells within the lymph nodes[184]. One of the manifestations of the resulting inflammation is the deposition of collagen in the lymph nodes, which leads to the destruction of lymph node architecture[180,185]. Since the lymphoid environment is critical in supporting normal lymphocyte homeostasis, inflammation and concomitant tissue remodeling of the lymph nodes contribute significantly to the decreased survival and depletion of the CD4+ T cell compartment in HIV-infected patients[180,185].

HIV Latency and Viral Reservoirs

The lymphoid tissues represent major reservoirs of HIV during chronic infection[4]. The size of HIV reservoirs can be estimated by the combination of in situ amplification techniques sensitive enough to detect a single copy of a lentivirus genome, and immunohistochemical staining with antibodies against cell type specific markers[81,186]. The quantity of productively infected cells in the lymph tissues is commensurate with progression to AIDS[187].

Latent HIV reservoirs are established extremely quickly during primary HIV infection. In a study of ten acutely infected HIV patients, the frequencies of resting CD4+ T cells carrying either integrated HIV-1 DNA or infectious virus were not correlated to the time between the onset of clinical symptoms of primary HIV infection and the time of initiation of antiretroviral therapy (range: 0.3 to 4 months)[188]. Integrated proviral DNA, as well as infectious HIV virions, were detectable in all subjects who were started on cART as early as ten days after

presenting with symptoms of primary HIV infection, despite the successful reduction of plasma viremia to undetectable levels shortly after the commencement of cART[188].

There are approximately $1x10^{11}$ HIV infected CD4+ T cells in the lymph tissue during the latent phase of infection, of which 1:100 to 1:400 possess transcriptionally active genomes[81]. There are $5x10^6$ latently infected CD4+ T cells with replication competent, but transcriptionally silent genomes at any given point in chronic infection[81,186]. The majority of these cells are resting, long-lived memory CD4+ T cells[189], although replication-competent HIV can also be detected within naive CD4+ T cells[190]. The latently infected resting memory CD4+ T cell pool is strikingly stable, with a half-life ($t_{1/2}$) of 44 months[191]. This reservoir does not decay significantly, even in patients who have exhibited marked suppression of viremia (<50 copies for 6-7 years), as viral RNA quantities rebound to wild-type levels within 2 weeks of cART cessation[192,193].

Follicular denditic cells (FDC) serve as another important storage site of HIV-infected cells. The level of HIV RNA in virions associated with FDC is greater than that in the plasma by two orders of magnitude[194]. Thus, plasma levels greatly underestimate viral burdens in the host. In the first weeks of infection, substantial quantities of HIV ($>10^{10}$ virions) have already accumulated in the FDC pool[195]. The rapidity with which the FDC pool and latently infected pools is established in early infection underscores the difficulties in purging viral reservoirs with cART, even if treatment is started early. Although it is possible to effectuate a 2,500-fold reduction in the FDC pool of virions after 6 months of

treatment, and to suppress viral RNA levels below the limit of detection (about 10^4 RNA copies/gram lymph tissue) after one year of treatment, a substantial pool of viral DNA remains detectable in lymphoid tissues, even after 2.5 years of effective suppression of viral replication[195].

The virus produced in the lymphoid tissues is sufficient to account for the entire virus population in the body (> 10^9 to >10^{10} virions/day)[4]. By end stage disease, productively infected cells are found in nearly every host organ, including the liver, kidney, lung, central nervous system (CNS), adrenal and other endocrine organs[4]. The persistence and resiliency of HIV likely precludes any possibility of eradicating HIV from infected hosts by standard antiretroviral therapy. As a result, treatment strategies have changed, from aggressive and early intervention, to delaying the onset of cART until later stages of HIV infection[196].

Adaptive Host Immunity

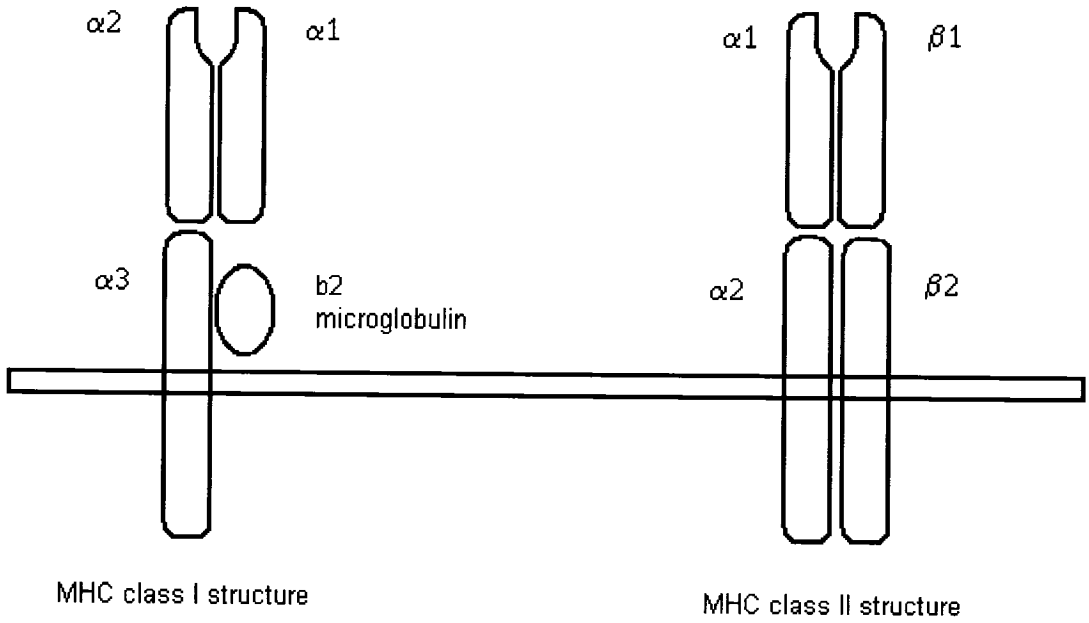
1. Antigen Processing and Presentation

The major histocompatibility complex (MHC) in humans is located on chromosome 6 and contains more than 100 genes. There are three MHC class I genes that encode for human leukocyte antigens (HLA-A, -B, and -C) and three MHC class II genes that yield HLA-DP, -DQ, and –DR. The MHC class I and II genes encode for cellular glycoproteins that are specialized in their ability to present foreign peptides to T lymphocytes. They are the most polymorphic genes known, as some have over 100 alleles that are each present at a relatively high

frequency in the human gene pool. The combination of specific MHC alleles on an individual chromosome is called an MHC haplotype. Most individuals carry different alleles of the same MHC gene on each chromosome. Since MHC genes are co-dominantly expressed, individuals heterozygous at MHC loci have the potential to bind and present a broader array of antigenic determinants on their cell surface. On a population scale, the polygenic and polymorphic nature of the MHC ensures that a single pathogen will not be able to decimate the human population. [197]

MHC class I molecules are expressed on all nucleated cells[198]. Their responsibility is to present peptides derived from intracellular pathogens (mainly viruses) to CD8+ cytotoxic T lymphocytes[198,199]. A MHC class I molecule consists of an α heavy chain that is encoded by the MHC, and a smaller β2 microglobulin chain that is not encoded by the MHC[198,199] [see Figure 15]. The latter is non-covalently linked to the α3 domain of the heavy chain, which serves to anchor the molecule into the plasma membrane of the cell[198]. The domains α1 and α2 of the heavy chain form the peptide-binding cleft on the surface of the molecule[198,199]. MHC I molecules accommodate peptides that are typically 8 to 10 amino acids in length[198,199]. The peptide lies in an elongated conformation along the peptide-binding groove, and is stabilized at its ends by contacts between the atoms in the free N- and C-terminus and the invariant sites of the MHC cleft[199,200]. Class I molecules have been shown to be able to bind larger peptides, by kinking in the peptide backbone and by extending the peptide out of the groove at the C-terminus[200].

Figure 15: MHC class I and II Molecules



MHC class II molecules are normally only expressed on B cells, macrophages, and thymic stromal cells. Dendritic cells significantly enhance their MHC II expression as they mature in response to danger signals. Interferon-γ produced during an antigenic insult activates the MHC class II transactivator gene CIITA, which effectively controls the level of MHC II molecules expressed on the cell surface. Peptides presented by MHC II molecules are recognized by CD4+ T helper cells that initiate a concomitant immune response. CD4+ T cells prime both virus-specific CTL responses and antibody production from B cells. [201]

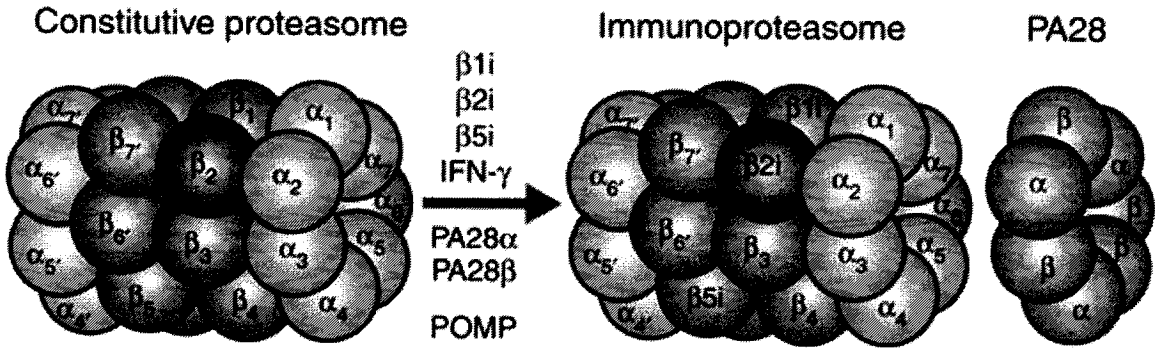
A MHC class II molecule consists of an MHC encoded α and β polypeptide chain that remain non-covalently associated[200] [see Figure 16]. The transmembrane α2 and β2 domains of each heavy chain anchor the complex

onto the cell surface, while the α1 and β1 domains form the peptide-binding cleft[202]. Peptides of at least 13 amino acids lie in an extended conformation along the peptide-binding groove[203]. Although the ends of the cognate peptide are not bound by the MHC II molecule, interactions between the amino acid side chains of the peptide and the variable amino acid residues that line the shallow and deep pockets of the cleft stabilize the association[203].

Both MHC class I and II molecules are highly polymorphic at certain sites in the peptide-binding cleft[197]. The specific interactions between these polymorphic amino acid residues and the amino acid side chains of the peptide define the peptide binding specificity of each allelic variant of MHC molecules[197]. Several peptides that bind to a given allelic form share the same, or biochemically related, amino acid residue at two or three precise positions along the peptide sequence[204]. These anchor residues forge the important interactions with the variable amino acids of the MHC molecule in the deep pockets of the groove[204]. Most peptides of correct length that contain the appropriate anchor residues will bind the cognate MHC irrespective of the rest of the amino acid sequence[204].

The proteasome, a multicatalytic protease complex composed of 28 subunits, is responsible for the degradation of cytosolic proteins tagged by ubiquitin[205]. It was discovered that inhibitors of the proteolytic activity of the proteasome also inhibit the presentation of antigens by MHC class I molecules[206,207]. When IFN-γ is produced during inflammation, new catalytic

Figure 16: The Immunoproteasome



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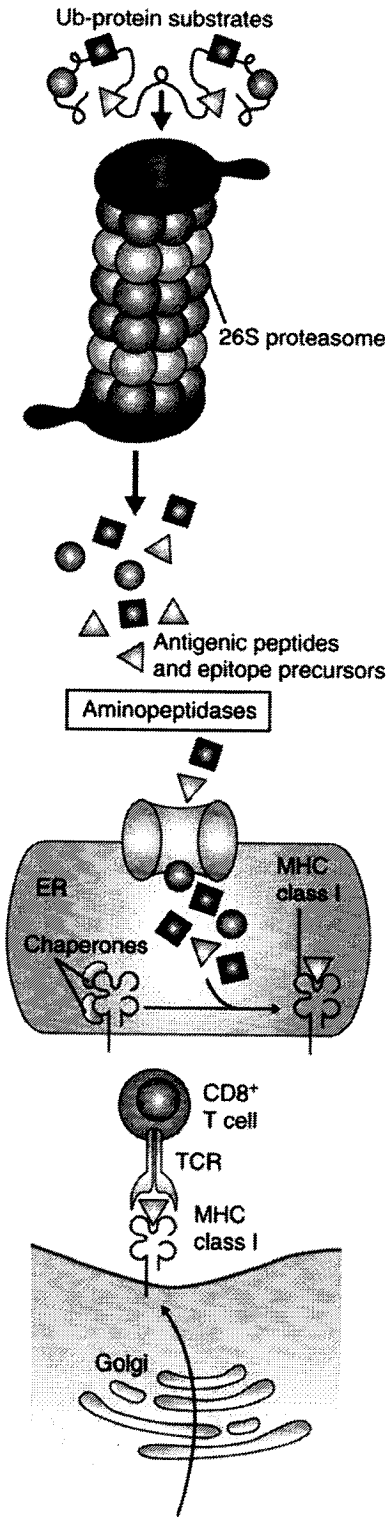
subunits are recruited to form the immunoproteasome[207] [see Figure 16]. The main products of immunoproteasomal processing are epitopes with an extended N-terminal sequence[207]. Aminopeptidases in the cytosol and the endoplasmic reticulum (ER) trim the precursor peptides to generate the epitopes that are eventually bound and presented by MHC class I molecules[208].

Proteins synthesized in the cytosol are translocated into the lumen of the endoplasmic reticulum (ER) by ATP-dependent binding proteins[209] [see Figure 17]. Transporters associated with antigen processing (TAP) 1 and 2 are encoded by genes of the MHC and form a heterodimer that is associated with the ER membrane[209]. In the presence of interferon, Tap-1 and Tap-2 shuttle proteins into the lumen of the ER where they must fold correctly with MHC class I molecules[210]. Newly generated MHC class I α chains are retained in a partially folded state in the ER as a result of their association with the membrane bound protein calnexin[211]. Once β2 microglobulin binds the α chain, calnexin is



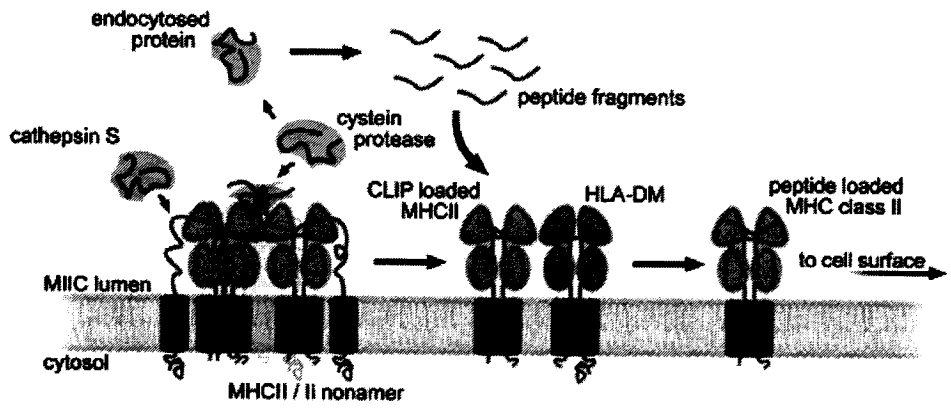
displaced in favour of a complex of proteins that includes calreticulin and tapasin[212]. The former serves as a chaperone whereas the latter binds to TAP-1, allowing the MHC/β2 microglobulin heterodimer to sample the imported peptides[212]. Binding to a cognate peptide releases the partially folded heterodimer from TAP and allows the properly folded MHC class I molecule (with peptide) to be exported to the cell surface[212].

In contrast, pathogens that replicate in intracellular vesicles, such as Leishmania, are degraded into peptide fragments by vesicular proteases and presented by MHC class II molecules on the cell surface. Pathogenic antigens that are internalized by phagocytosis, pinocytosis, or receptor-mediated endocytosis become enclosed in endosomes that become increasingly acidic as they progress to the interior of the cell. Vesicles of the endosomal pathway contain acid proteases which are activated at low pH to degrade the pathogenic antigens into peptide determinants. [213]

Figure 17: MHC class I Presentation *(Reproduced from [207] with kind permission from Dr. Kloetzel and Nature Publishing Group)*

In the ER lumen, nascent MHC class II α and β chains are prevented from binding intracellular peptides that should be presented on MHC class I molecules, as well as misfolded proteins, by their association with invariant chain (Ii)[213] [see Figure 18]. It is not clearly understood how the MHC II: Ii heterodimers are delivered to appropriate low pH endosomal compartments that contain antigenic peptides, but upon arrival the invariant chain is sequentially cleaved by cysteine proteases[214]. Cathepsin S appears to be the most critical protease, since its inhibition leads to the accumulation of a processing intermediate and a dearth in MHC class II end products[214]. The final portion of the invariant chain that remains bound to the MHC class II molecule after processing is CLIP, which occupies the peptide binding groove. The MHC class II homologue DM mediates the exchange of CLIP for antigenic peptides[210-212]. Once the MHC class II molecules bind their cognate peptides, they are exported to the cell surface for presentation to CD4+ helper T cells [215-217].

Figure 18: MHC class II Presentation



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2. The Antibody Response to HIV

The envelope of HIV virions is composed of gp120-gp41 heterodimers that assemble as trimers[219]. Interactions between conserved regions in gp120 that are not exposed on the virion surface are responsible for maintaining the integrity of each trimer[220]. During viral entry into target cells and concomitant gp120 shedding, these conserved domains of gp120 stimulate the production of antibodies that is characteristic of seroconversion[220]. The antibodies that are produced, however, are ineffective at controlling viral replication, since the conserved gp120 receptor and co-receptor binding sites that they recognize are buried in thermostable crypts[221]. Furthermore, loops of heavily glycosylated variable sequences line the surface of the viral envelope, impeding the ability of the antibodies to neutralize their targets[219,222].

Antibody-producing B cells often recognize the variable loops of gp120, but the high error rate of RT, combined with the rapid turnover of plasma virions, provides a broad base of variants that escape from humoral immune responses[223]. Neutralizing antibodies directed at the envelope are rendered irrelevant due to the rapid appearance of viral escape mutants[224]. Even within a subtype, antibodies that are specific for the variable loops of isolates from one patient typically do not recognize the variable loops of isolates from other patients[223]. In fact, viral escape is facilitated by changes in cell surface glycosylation[219]. Macaques challenged with a strain of SIV that had been manipulated to deliberately remove glycosylation around the V1 hypervariable loop resulted in an effective antibody response that controlled the virus[225].

Neutralization was lost, however, as compensatory mutations that repaired the glycosylation defects quickly appeared[225].

The sera from HIV infected individuals have been analysed extensively for the presence of neutralizing antibodies. Several human monoclonal antibodies have been isolated that exhibit neutralizing activity against a broad range of primary clade B HIV isolates, including IgG1b12, 2G12, 2F5, 4E10, and Fab Z13[226]. Although it is not the most potent of the neutralizing antibodies, 4E10 is the most broadly neutralizing antibody[227]. It has been shown to neutralize primary isolates from clades A, B, C, D, and CRF01 (A/E)[227]. Three of the antibodies (2F5, 4E10, and Fab Z13) bind epitopes in gp 41 that are exposed upon CD4 engagement by gp120, thereby arresting the process of fusion[226]. The other antibodies, IgG1b12 and 2G12, bind to the CD4 binding site of gp120[226]. The latter antibodies, however, require an unusually long complementarity-determining region (CDR)-3 loop to access the deeply recessed site[226]. IgG1b12 and 2G12 are also capable of recognizing a complex polymannose epitope, but this is due to extraordinary structural properties that are rarely seen on other antibody molecules[226].

SCID mice that have been reconstituted with human lymphoid cells are protected from challenge with HIV when a combination of the neutralizing antibodies is passively infused into the hosts[228]. The antibodies have also been shown to protect monkeys against challenge with a SHIV virus, although very high titres were required to observe the protection[229]. Such titres of antibody might be difficult to achieve by active immunization.

Studies of vaccines that protect macaques against SIV infection indicate that antibody-mediated protection is possible. It has been repeatedly shown that vaccines based on the recognition of viral envelope domains can protect non-human primates when challenged with a homologous virus[230-232]. The results from these small studies, however, may have limited relevance to humans. Furthermore, primary HIV isolates from infected patients were shown to be resistant to neutralization by antibodies raised against viruses adapted to laboratory culture[233]. The challenge in antibody research is, thus, to design approaches that will reliably induce truly neutralizing antibodies in sufficiently high titres.

3. The CD8+ T cell Response to HIV

During primary HIV infection, there is a strong temporal relationship between the appearance of HIV-specific CD8+ T cells in the blood and the decline of viral RNA copies in the plasma of HIV infected patients[165,234]. Despite the fact that the HIV-specific CD8+ T cell response is limited in breadth[235-237], likely a reflection of the relatively homogeneous population of the infecting viral strain, the relationship suggests a degree of control exerted by CD8+ T cells over HIV replication during acute HIV infection. In the chronic phase of HIV infection, however, HIV replicates copiously in the presence of apparently robust and polyclonal CTL responses[238-240], implying that HIV-specific CD8+ T cells have become functionally impaired.

The strongest evidence supporting a role for CTL as an immune correlate of protection against HIV infection comes from the SIV/macaque model of HIV infection and pathogenesis. In 1999, Jin et al used an anti-CD8 monoclonal antibody, OKT8F, to deplete the CD8+ T lymphocytes from the peripheral blood of six SIV-infected macaques[241]. A concomitant rise in plasma viremia of one to three orders of magnitude was noted in five of the six animals[241]. Schmitz extended the finding that elimination of CD8+ lymphocytes from monkeys during chronic SIV infection results in a rapid and marked increase in viremia by demonstrating that control of viral replication was restored with the reappearance of SIV-specific CD8+ T cells[242]. These results provided in vivo confirmation of the importance of cell-mediated immunity in controlling HIV-1 infection.

The appearance of viral mutations in both HIV[243-247] and SIV[248-252] CTL epitopes that result in escape from CTL recognition is a manifestation of the tremendous immune pressure exerted by CTL on these viruses. Within a few weeks of HIV infection, mutations appear in viral epitope sequences that result in diminished recognition of peptides by virus-specific CTL[253]. For example, the frequency of CTL escape mutations within previously recognized viral sequences ranged from 1/21 in a subject who maintained CTL control over viral replication, to 5/7 in a subject who did not[253]. In addition, mutations in the viral sequences flanking CTL epitopes contribute to viral escape in HIV-infected individuals by reducing the efficiency with which these sequences are processed and presented to virus-specific CTL[254,255]. For example, in chronically-infected HIV persons expressing the HLA-B57 allele, an alanine to proline transition in the sequence

immediately upstream of the dominant HLA-B57-restricted epitope, ISPRTLNAW, prevented proper trimming of the peptide to yield the optimal B57 epitope[255]. As a result, CTL clones specific for the optimal epitope did not recognize cellular targets transfected with the mutant viral sequence[255].

4. The CD4+ T cell Response to HIV

CD4+ T helper lymphocytes are critical in the maintenance of effective immunity against several viral infections. Virus-specific CD4+ T cells proliferate and secrete cytokines, for example IL-2, that promote antiviral functions from other arms of the immune system, especially CTL[256]. In mice infected with lymphocytic choriomeningitis virus (LCMV), depletion of CD4+ T cells by the administration of a CD4+ T cell-specific monoclonal antibody results in the abrogation of CTL control of viral replication during the chronic stage of infection[257,258]. CD4+ T cell-depleted mice experience persistent high viral loads and are unable to sustain effective CTL responses to the virus[257,258].

In the setting of HIV infection, CD4+ T cell proliferation in response to stimulation with p24 antigen is inversely related to the plasma viral load in patients with chronic infection[259]. Individuals who display long-term control of viremia in the absence of antiviral therapy maintain polyclonal HIV-1-specific CD4+ T cell proliferative responses throughout the course of HIV infection, whereas this effector function is absent in individuals with persistent viral loads[259]. The selective depletion of activated CD4+ T helper cells during early HIV infection is considered to be responsible for the impairment of CTL function

during chronic infection. It has been shown that aberrant HIV-specific CD8+ T cell proliferation can be restored, in vitro and in vivo, by the addition of fully competent autologous HIV-specific CD4+ T cells that secrete IL-2[260]. Thus, CD4+ T lymphocytes appear to be an important correlate of immunity against HIV infection.

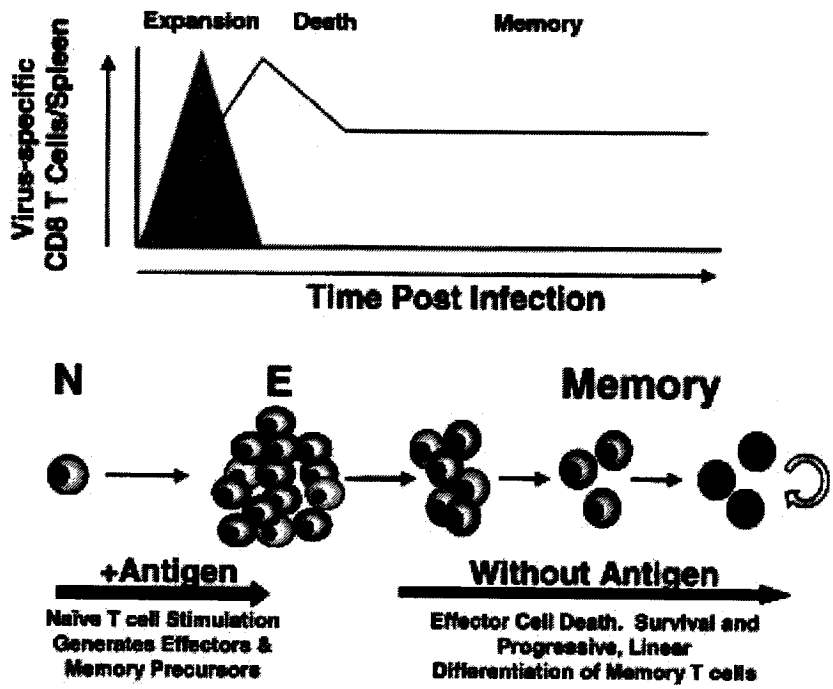
5. T Cell Memory

As naïve T cells transit through secondary lymphoid organs, such as the spleen and lymph nodes, they sample the environment for foreign antigens[261]. The engagement of the T cell receptor (TCR) to a foreign peptide/MHC complex on the surface of an APC induces the autocrine production of IL-2[262]. Co-stimulatory signals, arising from the interaction between CD28 on T cells and B7.1 or B7.2 molecules on APCs, stimulate the expression of CD40 ligand (CD40L) on T cells[262,263]. Binding of CD40L by CD40, which is present on the surface of APCs, transmits additional signals that serve to amplify co-stimulation of T cells[263].

It is believed that TCR ligation and co-stimulation trigger a programmed series of events that command the dramatic proliferation and differentiation of the responding T cell population[264,265] [see Figure 19]. During this expansion phase, a complex pattern of genetic regulation and expression dictates the acquisition of antiviral effector functions, including rapid IFN-γ and TNF-α secretion, and, in the case of CD8+ T cells, the expression of granzymes and perforin that mediate cytotoxicity[266]. The cytokine milieu contributes to the T cell signaling patterns that

induce the coordinated expression of genes controlling tissue homing and effector functions. For instance, T cells primed in the presence of IL-12 will activate the transcription factor T-bet[267], which induces histone modifications and DNA methylation of the IFN-γ genes, resulting in a T helper cell 1 (Th1) - mediated immune response[268]. In contrast, IL-4 stimulates GATA-3, which favours the development of a Th2 response[269,270].

Figure 19: Memory T cell Development



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Following the peak of the primary immune response, a contraction phase ensues in which 90-95% of the activated T cells die by apoptosis[272] [see Figure 19]. A subpopulation of the responding T cells that expresses the IL-7 receptor (IL-7R) survives the contraction phase to constitute the memory T cell pool[273].

The adoptive transfer of IL-7R^{hi} T cells from the expansion phase of acutely infected mice into naïve mice gave rise to antigen-specific memory T cells, whereas adoptive transfer of IL-7R^{lo} T cells did not[273].

There are two main subsets of memory T cells that have been characterized: effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). They were originally distinguished based on the expression of chemokine receptors and adhesion molecules required for homing to various anatomical locations[274]. T_{CM} were postulated to reside exclusively in lymphoid tissues based on their expression of CCR7 and CD62L, whereas T_{EM} that lack these surface antigens were hypothesized to home to inflamed tissues where they can mediate immediate effector functions such as rapid antiviral cytokine secretion and cytotoxicity[274]. T_{CM} were thought to provide a second wave of effector cells, in response to the IL-2 that they are adept at producing[274].

It is now understood, however, that both subsets are equally good at mediating rapid effector functions in response to re-stimulation with cognate antigen. The latest studies in mice[275,276] and humans[277] indicate that, on a per cell basis, T_{CM} confer more effective protective immunity during recall challenge than T_{EM}. This is attributed to the enhanced proliferative and IL-2 secreting capacities of T_{CM} relative to T_{EM}[275].

The development of memory T cells is a gradual process, as determined by gene expression profiling and functional characterization of antigen-specific T cells in murine models of viral infections[266]. Activated T cells that emerge from the contraction phase continue to differentiate; trading effector traits for memory

attributes[266]. An extensive set of experiments has been dedicated to elucidating the lineage relationship between the T_{EM} and T_{CM} memory cell subsets. Are T_{EM} the final result of the developmental pathway, or do T_{EM} give rise to T_{CM} as end products? Despite severe controversy, it appears now that the latter is true. It has been shown in vivo, in both humans and mice, that, in the absence of antigen, CCR7+ CD62L+ T_{CM} cells are quite stable, whereas a significant portion of CCR7- CD62L- T_{EM} cells either die by apoptosis or convert to CCR7+ CD62L+ T_{CM} cells[275,278].

In addition to CCR7 and CD62L expression, a plethora of cell surface antigens have been used to further delineate T cell subsets along the memory T cell differentiation pathway[279] [see Figure 20]. For example, the co-stimulatory protein antigen CD27 modulates the signaling threshold of T cells and is differentially expressed throughout the different stages of T cell maturation[280]. Some cells within the human T_{EM} population express CD27 and display phenotypic and functional features that are intermediate between naive and effector T cells[281].

Chemokine receptors can also be used to discriminate functional groups within the T_{CM} and T_{EM} subsets, although most of these markers are transiently modulated upon cellular activation. Upon antigenic stimulation, for example, T_{EM} upregulate CCR7 and CXCR5 while downregulating CCR5 expression[282]. Also, CD62L is rapidly shed after TCR triggering and lymph node immigration[283]. Given the complexity of memory T cell nomenclature, the new focus of researchers is to correlate defined memory phenotypes with antigen-specific IL-2 secretion and

proliferation, effector functions that are accepted to be important in the control of viral infections[284-286].

Figure 20: Memory T cell Markers

Antigen Experienced T Cells					
CD44	Adhesion Molecule	Lo	Hi	Hi	Hi
CD11a	LFA1, α integrin; Adhesion Molecule	Lo	Hi	Hi	Hi
Ly6C	GPI-linked; unknown function	Lo	Hi	Hi	Hi
CD122	IL-2/IL-15 receptor β chain	Lo	Hi	Hi	Hi
IL-15Rα	IL-15 receptor α chain	Lo	Hi	Hi	Hi
Associated with Effector T Cells					
CCR5	Chemokine Receptor	Lo	Hi		Lo
Perforin	Cytotoxic granule protein	Lo	Hi		Lo
Granzyme B	Cytotoxic granule protein	Lo	Hi	Int	Lo
Dynamically Regulated during Memory T Cell Development					
CD62L	Lymph node homing receptor	Hi	Lo	Lo	Hi
CCR7	Lymph node homing chemokine receptor	Hi	Lo	Lo	Hi
CD27	TNF receptor superfamily; costimulation	Hi	Lo/Int	Lo/Int	Hi
CD127	IL-7 receptor α chain	Hi	Lo	Lo/Int	Hi
Bcl-2	Anti-apoptotic/cell survival	Int	Lo	Int	Hi

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$CD4^+$ T cell help is critically important for the development of $CD8^+$ T cell memory. Although effective $CD8^+$ T cell responses to various stimuli are generated in the absence of $CD4^+$ T cell help during priming, such "unhelped" memory $CD8^+$ T cells are functionally impaired during secondary immune responses to recall antigens[287-289]. Unhelped memory $CD8^+$ T cells are unable to undergo clonal expansion, they experience defects in their ability to produce IFN-γ and IL-2, and they do not persist long-term[287-289]. A recent study, however, showed that defective $CD8^+$ T cell responses can be recovered during secondary antigenic challenge with recovery of $CD4^+$ T cell help[290]. Thus, $CD4^+$ T cells

support the persistence and functional viability of the memory $CD8^+$ T cell pool by influencing the lymphoid environment[290]. The mechanism by which $CD4^+$ T cells prevent the attrition of $CD8^+$ memory T cells is not understood. $CD8^+$ T cells are, therefore, not irreversibly programmed by $CD4^+$ T cell help during the primary immune response[290].

It is not surprising, therefore, that chronically infected HIV patients exhibit impaired CD8+ memory T cell function, since the IL-2-producing, HIV-specific CD4+ T cells that are capable of rapid proliferation during recall stimulation are functionally impaired in these individuals[291]. HIV-specific CD8+ T cells from HIV infected patients with persistent viremia are able to secrete IFN-γ, but they are low in perforin expression, poorly cytotoxic, and incapable of antigen-specific proliferation[292]. In mice chronically infected with the lymphocytic choriomeningitis virus (LCMV), virus-specific CD8+ memory T cells progressively lose their effector capabilities[285]. IL-2 secretion appears to be the most sensitive to persistently high viral loads, followed by TNF-α production, and finally IFN-γ secretion[285]. Functional exhaustion and deletion of antigen-specific CD8+ memory T cells has also been observed in primates infected with SIV[293,294], in humans infected with hepatitis B virus (HBV)[295] and hepatitis C virus (HCV)[296], and during malignant melanomas[297].

Exposed Uninfected Individuals

Individuals who remain uninfected by HIV despite repeated exposure to the virus represent an important study group for natural resistance to HIV

infection. In theory, a person with an isolated experience at high-risk for HIV infection could be considered an EU subject, however enrolment into most EU study cohorts requires a minimum duration of seronegativity in the face of repeated exposure to HIV. Alternatively, a minimum frequency of exposure is needed for inclusion into other EU studies.

There are several different types of EU cohorts that have been reported. Commercial sex workers, men who have sex with men (MSM), and heterosexual couples discordant in HIV serostatus are all EU study populations that represent mucosal exposure to HIV by means of unprotected sexual intercourse[298]. Parenterally exposed study subjects include healthcare workers (HCW) who experienced needle-stick injuries, and intravenous drug users (IVDU) who share syringes with HIV-infected partners.

The phenomenon of natural resistance to HIV gained considerable attention when a cohort of commercial sex workers (CSW) in Kenya was identified as being resistant to HIV infection. In the Punwami district of Nairobi, Kenya, a community of prostitutes was studied for risk factors associated with HIV seroconversion. From 1985 to 1994, 1666 women were recruited, of which 1046 (62.8%) were HIV-1 positive at the time of enrolment. Four hundred and twenty four of the seronegative women were followed for 1 to 10 years. Every six months, information on sexual behaviour, contraception, and condom use during the previous six months was collected, as well as samples of peripheral blood and endocervical swabs for laboratory tests. Forty three (43) women remained persistently seronegative for HIV-specific antibodies despite over three years of

commercial sex work. The apparent resistance to HIV infection was verified by HIV PCR using Env, Nef, and Vif gene primers. [299]

The study of EUs has revealed several potential mechanisms by which these individuals remain protected from HIV infection.

1. Genetic Resistance to HIV infection

A thirty two base pair deletion in the CCR5 gene (CCR5Δ32) results in the expression of an improperly folded protein that is not expressed on the cell surface[54]. In 1996, CD4+ T lymphocytes from two persistently seronegative homosexual men, who reported sexual encounters with multiple HIV-1 infected partners, were resistant to infection by primary HIV-1 isolates, but not by laboratory adapted T-tropic strains[300]. The observed resistance was later attributed to the discovery that both men were homozygous for the CCR5Δ32 mutant allele[54]. In a subsequent cohort study of homosexual men, none of the HIV seropositive subjects were found to be homozygous for the CCR5Δ32 genotype, whereas 3.6% of the at-risk, but uninfected Caucasian participants possessed the mutation[301]. As a result, the CCR5Δ32 homozygous phenotype was associated with resistance to HIV infection.

Subsequent surveys revealed that approximately 21% and 1% of Caucasians are heterozygous and homozygous, respectively, for the CCR5Δ32 mutant allele[55]. Although heterozygotes are not protected against HIV transmission, they do exhibit delayed HIV-1 disease progression and prolonged survival. This association is based on a cohort studies in which the frequency of

CCR5Δ32 heterozygosity was found to be elevated among white, heterosexual men who have been infected with HIV for over ten years compared to infected men who did not survive as long[302]. Patients heterozygous for the CCR5 Δ32 phenotype were also found to have lower viral loads than those measured in individuals who express wild-type CCR5 receptors[302]. It is likely that a reduced number of viable CCR5 co-receptors hinder the ability of HIV to spread, effectively lowering the concentration of HIV in the plasma.

The CCR5Δ32 homozygous phenotype, however, does not confer absolute protection against HIV infection, as several reports have described cases of HIV infection in subjects who are homozygous for the mutant CCR5 allele[303-305]. In most of these instances, the HIV quasi-species isolated from the infected patients have been phenotypically characterized as being T cell-tropic, syncytium inducing, CXCR4 viruses that are able to thrive in CCR5 -/- primary T cells[304,306,307]. Thus, HIV is capable of using alternative co-receptors for entry when its ability to use CCR5 is compromised.

The mutant CCR5 allele is virtually absent in African and Asian populations[55], in whom HIV is most rampant. Since many of the EU cohorts are composed largely of non-white participants, it is not surprising that only a small proportion of EUs studied to date are homozygous for the mutant allele. The frequency of the homozygous phenotype in EU cohorts ranges from 1.7% to 9%[308-313]. Several EU studies did not even detect the mutant allele at all[314-317]. Thus, although the homozygous CCR5Δ32 phenotype is strongly associated with resistance to HIV infection, it is not a prevalent factor in EUs.

In the Nairobi cohort of commercial sex workers, an association between the expression of certain (HLA) alleles and HIV resistance was established. Polymorphisms in HIV co-receptors or chemokine receptors that alter cellular susceptibility to HIV infection were not identified, and thus not responsible for the apparent resistance of the CSW[318]. The HLA class II allele DRB1*01 was associated with protection from HIV infection[319,320]. In addition, alleles of the A2/6802 supertype were found to be associated with a significantly decreased rate of HIV transmission in this cohort of female CSW[319,320], as well as in a prospective cohort of HIV-infected mothers and children in Kenya[321]. The link between the HLA-A2/6802 supertype and resistance to HIV-1 infection was supported by a recent study that compared the HLA alleles expressed by 100 EU men to those expressed by 184 HIV seropositive men[322]. It was determined, however, that the A*0205 subgroup alleles were largely responsible for the observed association with resistance[322].

A HLA supertype is a functional grouping of HLA alleles that share similar peptide-binding motifs[323]. A peptide that can bind to one allele within a supertype can, theoretically, bind to all alleles within that supertype. Consequently, all of the HLA-A alleles can be grouped into four HLA-A supertypes, while all of the HLA-B alleles can be organized into five HLA-B supertypes[323,324]. Thus, the identification of a HLA supertype that is associated with protection from HIV infection, and that encompasses a significant proportion of all alleles expressed in the human population, would significantly advance the design of a HIV vaccine.

An analysis of the relationship between the frequency of HLA supertypes and viral loads in HIV infected men, however, found that HIV adapts to the most frequent alleles in the population. The most common supertype, HLA-B7, was linked with rapid progression to AIDS, whereas the least frequent supertypes, B27 and B58, were associated with protection against disease progression. The transmission of escape variants may occur more frequently between individuals who share alleles belonging to the more common supertypes, thereby conferring a selective advantage to individuals who express rare alleles. [325]

Cross-sectional studies of EUs have reported associations between other HLA alleles and HIV resistance[326-328], but the correlations are inconsistent. Most of the studies have performed multiple comparisons of various HLA alleles between a small number of EUs, HIV-infected patients, and/or low-risk seronegative controls. The diversity of the study populations, in terms of ethnicity, route of exposure, and amount of exposure, further weakens the associations. Without corroboration from molecular and T cell studies, the significance of these proposed associations with HIV protection remain in doubt.

2. Innate Resistance to HIV

Chemokines were optimistically thought to serve as antagonists to HIV infection. The natural ligands of CCR5 (RANTES, MIP-1α, MIP-1β) and CXCR4 (SDF-1) were shown to be able to block viral entry in vitro, presumably by competing with HIV gp120 for binding sites on the receptors[329,330]. Alternatively, receptor down-regulation as a result of cognate chemokine binding could,

potentially, retard HIV infection. The regions in CCR5 required for ligand binding and HIV co-receptor activity, however, are not identical and only partially overlap[331]. Not all primary isolates use the same structural elements of CCR5 to gain entry[331]. Furthermore, in vitro studies show that MIP-1α and MIP-1β are selectively secreted by HIV-1 infected monocytes, and elevated β-chemokine secretion is detected in the microglia and astrocytes from patients with AIDS dementia[331]. Up-regulated chemokine production by HIV-1 infected cells of the monocyte lineage may recruit uninfected, immune competent T cells to sites of active viral replication in order to serve as new targets of infection.

In accordance, the EU literature does not support a role for enhanced chemokine secretion in protection from HIV infection. A significantly greater level of production of chemokines in HIV EUs compared to low-risk negative controls has only been demonstrated in a few studies[300,317,332]. The reported results lack statistical power as a result of small sample sizes and/or missing relevant control populations. It is therefore unlikely that unique patterns of chemokine secretion contribute to the natural resistance against HIV infection.

The role of natural killer (NK) cells in resistance to HIV infection was recently explored in a cohort of 37 Vietnamese IVDU who remained HIV seronegative despite many years of high-risk needle-sharing[333]. NK cell lytic activity in vitro was significantly elevated in EU IVDU compared to both low-risk controls and IVDU who had seroconverted[333]. NK cells isolated from the EUs also produced significantly more IFN-γ and TNF-α after in vitro stimulation[333].

Further studies will be required to confirm a protective role for NK cells against HIV infection.

3. Humoral Immunity against HIV

Antibody immunity is another potential immune correlate of protection against HIV infection. The genital mucosa is enriched with lymphoid follicles specialized for the development of immunoglobulin-producing plasma cells. Since HIV is primarily transmitted by sexual contact, immunoglobulin A (IgA) antibodies localized in the mucosa likely represent the first line of defence against HIV penetration. In fact, the development of mucosal SIV-specific IgA in SIV-vaccinated macaques was associated with the observed resistance to intra-rectal challenge with live infectious SIV[334].

Although the presence of HIV-specific IgA antibodies in the genital mucosa of EUs has been demonstrated in some studies[316,335-337], other reports do not support the notion that HIV-specific IgA contribute to the observed resistance to HIV infection in these individuals[317,338,339]. A common caveat to all of these studies is that a proportion of the low-risk negative controls also exhibit detectable IgA levels. The lack of specificity of the assays suggests that the IgA antibodies in the genital mucosa of these women may simply serve as a marker for high-risk sexual activity. Women infected with Chlamydia have been shown to develop IgA antibodies in their cervix as well.

In 1999 Mazzoli attempted to validate the hypothesis that HIV-specific IgA antibodies in the genital mucosa can mediate protection against HIV infection by

testing the sera of fifteen EUs from serodiscordant couples for neutralizing activity. Sera from five of the EUs exhibited neutralizing activity against two primary isolates of HIV-1[340]. The observed reduction of infectivity, however, was only attributed to the IgA fraction of the serum in two of the five cases[340].

4. Cellular Immunity against HIV

There is a wealth of EU literature that suggests CD8+ T cell lymphocytes are immune correlates of protection against HIV infection. Experimental evidence for acquired cellular immunity against HIV in EUs comes largely from cohort studies that report the presence of HIV-specific CD8+ T cell activity in a significantly higher proportion of EUs than in low-risk negative controls. Cytotoxic T lymphocyte (CTL) responses directed against Env, Nef, Gag, and Pol epitopes have been demonstrated in EU commercial sex workers[328,341-344], healthcare workers[345], uninfected sexual partners of seropositive individuals[310,314,317,346], intravenous drug users[347], and children born to HIV-1 infected mothers[348,349]. Despite variation in sample sizes and criteria for a positive response, the consistent detection of HIV-specific CD8+ T cell function across diverse EU study populations strongly supports an association with resistance to HIV infection.

The Nairobi cohort of CSW has provided the most compelling evidence from the EU literature for a correlation between CD8+ T cell immunity and protection from HIV-1 infection. Eleven resistant female sex workers seroconverted after an average follow-up period of 6.9 years as a result of waning CTL responses. It was deduced that a reduction in sex work, due to an

extended vacation, led to the loss of pre-existing HIV-specific CTL. Infection by CTL escape variants was unlikely, since five of the six viral epitope sequences from the seroconverters matched those recognized by pre-seroconversion CTL.[350]

HIV-specific CD4+ T cell responses have also been identified in EUs. Originally, a panel of peptides corresponding to sequences from within the gp120 envelope of laboratory adapted HIV-1 strains was used to stimulate the PBMC from EUs[351]. Several studies reported the production of HIV-specific IL-2 in the supernatant of the cultures as a correlate of CD4+ T cell function[316,337,341,351], whereas others relied on proliferation measured by tritiated (^{3}H) thymidine incorporation[317,352]. While these assays did demonstrate HIV-specific CD4+ T cell function in a greater proportion of EUs than controls, the results were largely qualitative. The fact that, in some cases, a small proportion of the negative control populations responded to the peptide stimulation suggests that these assays also lacked an appreciable amount of specificity.

Recent experimental protocols that measure HIV-specific IFN-γ secretion from CD4+ T lymphocytes by flow cytometry analysis and/or the ELISPOT assay have significantly improved the veracity of the claim that CD4+ T cells contribute anti-HIV immunity in EUs. CD4+ T cell responses in EU study subjects have been elicited using whole protein antigens from various clades of HIV, including Gag p24 and gp120 envelope proteins, as well as overlapping peptide sets that span all the gene products expressed by HIV[353-356]. The frequency of HIV-specific

CD4+ T helper responses in EUs is often variable, reflecting heterogeneity in study populations and thresholds defining a positive response.

The EU studies on systemic HIV-specific T cell responses, both CD4+ and CD8+, support the design of HIV vaccines aimed at stimulating T cell immunity. An important finding from the experimental literature is that EUs recognize different HIV targets than do HIV seropositive patients. Kaul compared the specificity of CD8+ T cell responses to a panel of 54 known CTL epitopes, as measured by the IFN-γ ELISPOT assay, in 91 EU and 87 seropositive women from the Nairobi CSW cohort. Immunodominant epitopes restricted by HLA-A2, A*6802, A24, B14, and B18 were more frequently recognized by EU CSW than by seropositive women of the same cohort. These specificities are likely biologically relevant, since all of the MHC class I alleles are associated with protection from HIV infection in the Nairobi cohort of CSW. [357]

Differences in the patterns of T cell recognition of HIV epitopes were also noted by Kebba in a cohort of 28 HIV-1 serodiscordant couples. Using overlapping peptide sets that span the sequences of all expressed HIV gene products in the IFN-γ ELISPOT assay, it was determined that EUs tended to recognize peptides that were largely ignored by their seropositive partners. For example, EUs mostly recognized peptides from Vif and Vpu, whereas the infected partners mainly responded to stimulation by Gag, Nef, and Vpr peptides. [358]

Differential epitope recognition has also been observed between HIV infected patients in acute and chronic infection, where CTL epitopes that

dominate in chronic infection are largely irrelevant during the antiviral CTL response in primary HIV infection that is associated with the initial clearance of the virus[359]. As a result, information regarding HIV-specific CTL specificities from studies of EUs are likely more relevant to vaccine design than data obtained from studies of HIV-infected persons.

Thesis Hypothesis:

Individuals who remain uninfected by HIV despite repeated exposure to the virus possess an HIV-specific T cell response that mediates protection against HIV infection. The frequent detection of HIV-specific T cell responses among EUs suggests that these individuals first encountered virus in a manner that promoted the development of a T cell response that cleared the virus before a productive infection could become established. A resulting HIV-specific, memory T cell population mediates protection against HIV infection at subsequent encounters with the virus. Since EUs do not incur CD4+ T lymphocyte damage as a result of persistent viral replication, HIV-specific immunity is not expected to become compromised in these subjects.

A relevant precedent for this hypothesis is the murine LCMV model of acute viral infection, in which a potent CD8+ T cell response controls and clears primary viremia. The maintenance of virus-specific CD4+ T lymphocytes in a viable state promotes the development of a memory T cell population that is capable of rapid proliferation and the secretion of IFN-γ, TNF-α, and IL-2 upon re-exposure to the virus[256,258].

Thesis Objectives:

1. To identify HIV-specific T cell responses in a population of HIV seronegative individuals with multiple exposures to the virus.
2. To assess the influence of route of HIV exposure on the development of HIV-specific T cell responses in EUs.
3. To correlate the presence of HIV-specific T cell activity with protection against HIV infection.
4. To characterize the effector function of HIV-specific T cells in EUs.

Section 2: Results

Chapter 1:

HIV-specific IFN-γ secretion in EU IVDUs

Identifying Antigen-specific T cell Responses:

CD8+ T cell responses have traditionally been measured by the chromium (^{51}Cr)-release cytotoxic T lymphocyte (CTL) assay. PBMC are stimulated with antigen(s) and then incubated for 10-14 days. CD8+ T cells that have expanded during culture are assayed for their ability to lyse ^{51}Cr-labelled autologous target cells that express the antigen with which they were stimulated. The amount of ^{51}Cr released from the lysed cells is correlated to the specific lysis activity of the CTL in the bulk PBMC culture. The results from the CTL assay, however, are largely qualitative and do not permit the phenotypic characterization of the responding T cell subsets.

Recently, alternative immunoassays have been developed to offer a quantitative assessment of ex vivo T cell function with a high degree of sensitivity and specificity. The enzyme-linked immuno-spot (ELISPOT) assay is performed in a 96 well plate. The membrane of every well is coated with a capture antibody specific for a cytokine of interest. PBMC are layered in the wells and stimulated with antigens. In response to stimulation, the T cells secrete the cytokine for which the capture antibody is specific. The response is amplified by a series of conjugate antibodies, yielding a spot in a well for every T cell that has responded to the stimuli. By counting the number of spots produced by the antigenic stimulation, an accurate quantitative assessment of the frequency of antigen-specific T cells can be deduced.

In the intracellular cytokine staining (ICS) assay, cytokine(s) produced by T cells in response to antigenic stimulation are retained in the cytoplasm and

stained for visualization with a specific antibody. The responding cells are detected individually with the help of a flow cytometry apparatus. The advantage of this assay over the ELISPOT assay is that multiple antibodies can be employed simultaneously to yield a highly descriptive functional and phenotypic profile of the responding T cell population. The ELISPOT assay, however, can reliably detect 50 antigen-specific T cells/10^6 PBMC, whereas the limit of detection of the basic 4-colour ICS assay is 300 antigen-specific T cells/10^6 PBMC.

Both the ELISPOT and ICS assays are now considered superior to the ^{51}Cr-release CTL assay in terms of specificity and sensitivity. Whereas the latter assay measures effector T cell populations that have been expanded in culture, the cytokine detection-based assays measure true memory T cell responses ex vivo, thereby offering a realistic depiction of in vivo immunity. Furthermore, the ELISPOT and ICS immunoassays can be adapted to detect more than one cytokine produced by activated T cells, allowing the evaluation of T cell subsets.

Interferon-γ

The production of interferon (IFN)-γ is routinely used as a marker for antigen-specific T cell activity. IFN- γ is the only member of the type II class of interferons, a family of proteins that was originally discovered to interfere with viral replication[360]. IFN- γ is structurally unrelated to type I interferons (multiple α subtypes, β, ω, and τ), and is bound by a receptor that is distinct from that used by the type I IFNs[361]. Mice with mutations in either the gene that encodes IFN- γ

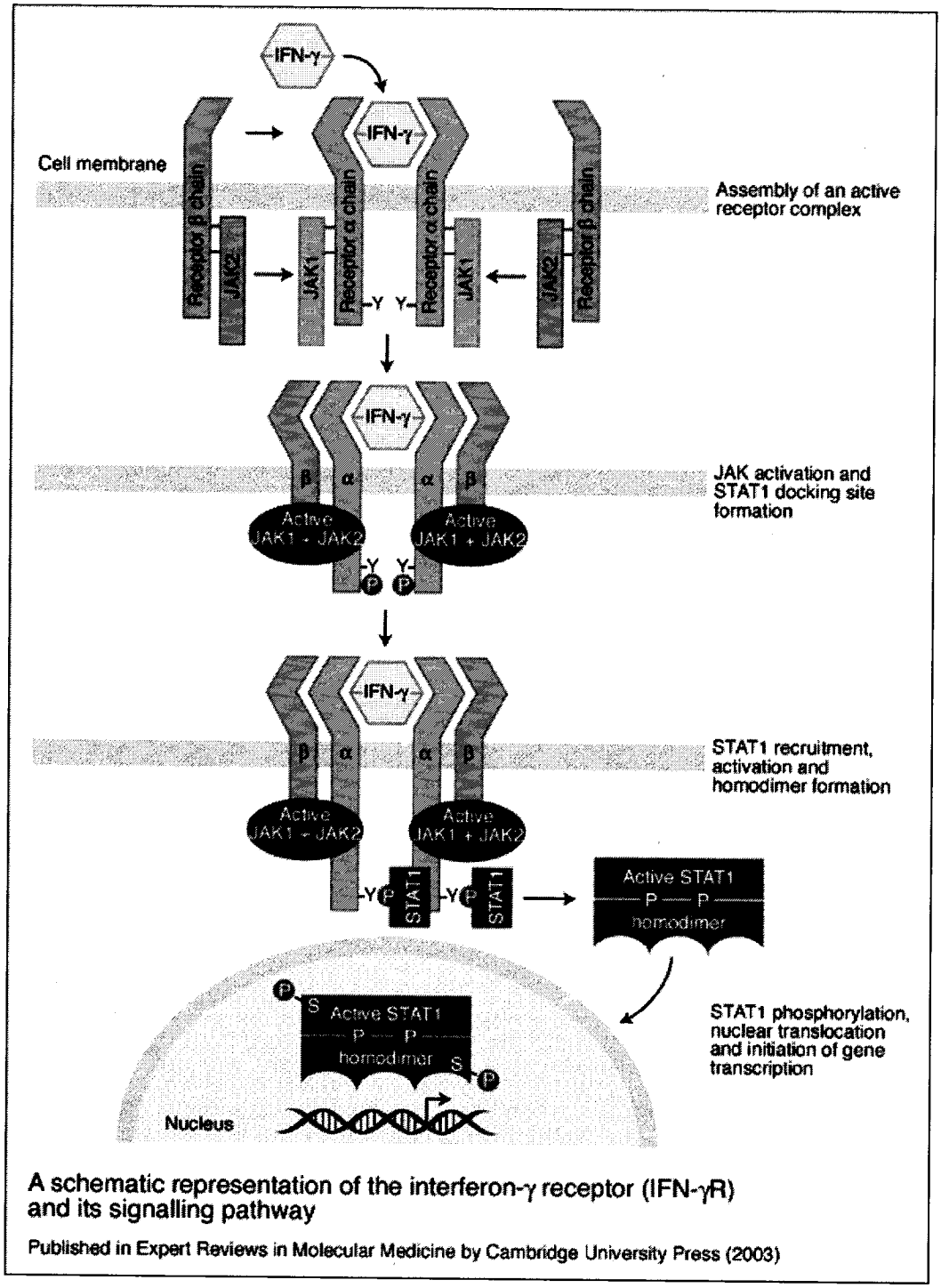
or the receptor through which it signals demonstrate deficiencies in natural resistance to bacterial, parasitic, and viral infections such as vaccinia virus, Theiler's murine encephalomyelitis virus, *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, and several poorly virulent mycobacteria species[362-367].

The receptor for IFN-γ is a heterodimer composed of two IFNγR1 chains and two IFNγR2 subunits. The former are usually found in excess, whereas the latter represent the limiting factor for IFN-γ responsiveness. Although IFNγR2 chains are constitutively expressed, it is the activation state of the cell that determines the quantity of the receptor chain that is found on the cell surface. Immunoregulators secreted by APCs, for example IL-12 and IL-18, influence both the expression of IFNγ and IFNγR2 chains. [368]

The two IFNγR1 chains form the ligand-binding domain of the receptor[368] [see Figure 21]. The two IFNγR2 chains associated with the IFNγR1 chains mediate the signal-transduction function of the receptor, although the IFNγR1 chains also participate in the signalling process[368]. The intracellular domain of IFNγR1 contains binding motifs for the Janus tyrosine kinase (Jak)-1[369-371] and the signal transducer and activator of transcription (Stat)-1[372,373], both of which are required for receptor phosphorylation and signal transduction. The cytoplasmic domain of the IFNγR2 subunit contains a non-contiguous-binding motif that is recognized by Jak-2[374,375].

IFN-γ induced signalling regulates the expression of transcription factors that, in turn, regulate the expression of key antiviral enzymes, such as protein kinase R (PKR)[376-379]. The activation of PKR by dsRNA following IFN-γ induced

Figure 21: IFN-γ Signalling Pathway



A schematic representation of the interferon-γ receptor (IFN-γR) and its signalling pathway

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expression leads to the inhibition of viral protein synthesis[376-379]. In addition, IFN-γ signalling increases the sensitivity of virally infected cells to apoptotic mechanisms, by promoting the expression of the TNF-α receptor on the cell surface[381] and/or by inducing the cellular expression of Fas and Fas ligand[382,383]. As a result, several viruses encode proteins designed to specifically interfere with IFN-γ receptor signalling[384,385].

In addition to promoting an antiviral environment in an infected cell, IFN- γ induces many functions that, collectively, endorse the generation of an adaptive immune response against pathogens:

- IFN-γ activates the immunoproteasome[386,387], the TAP transporter proteins[388-390], and the synthesis of MHC class I molecules[391,392], all of which favour efficient processing and presentation of viral antigens.
- IFN-γ favours Th1 cell lineage commitment and inhibits Th2 cell differentiation[393,394].
- Gene chip analyses of antigen-specific CD8+ T cells from the acute phase of a response reveal the activation of multiple genes by IFN-γ[266].

IFN- γ secretion constitutes a first line of antiviral defense, as T cells release IFN- γ immediately following stimulation with antigen[395]. Furthermore, IFN-γ serves as an autoregulator of CD8+ T cell development during acute viral infections. It was recently shown in mice that cells which rapidly secrete IFN-γ in response to antigenic stimulation become more numerous than those that slowly produce the cytokine[396]. IFN-$\gamma^{-/-}$ or IFN$\gamma R^{-/-}$ mice generate 5 to 10-fold fewer

activated antigen-specific CD8+ T cells than wild type strains, indicating that the cytokine acts directly upon CD8+ T cells to increase their abundance during acute viral infection[397].

Hypothesis: EU IVDU subjects develop an HIV-specific T cell response that mediates protection from HIV infection.

Rationale: Direct challenge studies are not ethically feasible to conduct in humans. As an indirect demonstration of protection, a comparative study between EUs who remain seronegative and EU's who seroconvert was conducted. If the development of an immune response to HIV in EUs plays a role in preventing progressive HIV infection, then one would expect a higher proportion of EUs who remain seronegative than those who seroconvert to possess HIV-specific effector activity. Since CD8+ T cells only synthesize IFN-γ when in contact with cognate antigen[398], the amount of IFN-γ detected by immunoassays is thought to reflect the magnitude of the responding T cell population.

At the time of this work, no study had reported HIV-specific IFN-γ secretion in EU IVDU. Since intravenous drug use is responsible for an increasing frequency of global HIV infections, it is important to search for HIV-specific immunity in this fresh resource of HIV resistance.

Objective: To detect HIV-specific T cell activity in a population of EU IVDU using the IFN-γ ELISPOT assay.

HIV-SPECIFIC EFFECTOR ACTIVITY IN HIV EXPOSED UNINFECTED INJECTION DRUG USERS IS ASSOCIATED WITH MAINTENANCE OF SERONEGATIVITY

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Author Contribution:

1) George Makedonas: Chief experimenter, primary author, study design.

2) Julie Bruneau: Director of St. Luc IVDU cohort, development and subject recruitment.

3) Henry Lin: Assisted in the standardization of the proviral PCR assay.

4) Rafick-Pierre Sekaly: Provided PBMC samples from pre-seroconversion time points.

5) Dr. Francois Lamothe: patient recruitment at the St-Luc cohort

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ABSTRACT

Repeated exposure to HIV does not always result in seroconversion. Understanding the conditions that permit, or protect against, the establishment of a progressive HIV infection is important for vaccine development. Twenty-eight injection drug user subjects, with documented risk for HIV infection over a one-year period, were screened for the presence of HIV-specific effector responses by Interferon-γ ELISPOT assays. Eighteen remained seronegative after the sampling date while ten became HIV seropositive within 7 months of screening. Twelve of the 18 (66.7%) persistently seronegative subjects exhibited HIV-specific effector activity while none of the 10 seroconverters had detectable HIV-specific effector responses at the sampling date ($p<0.001$; Fisher's exact test). This represents an odds ratio of 40.38 (95% confidence intervals 2.95 to >3000). The association between maintenance of seronegativity and presence of HIV-specific effector activity in exposed seronegative subjects supports the hypothesis that this response is a correlate of immune protection from HIV infection in this population of injection drug users.

INTRODUCTION

Cohorts of human immunodeficiency virus (HIV) exposed, uninfected subjects (EUs) represent a potentially important resource for the design of protective vaccines because they may have acquired protective immunity to HIV. Maintenance of seronegativity despite exposure to HIV has been observed in sexual partners of HIV infected persons [1-7], infants born to HIV-infected mothers [8-10], commercial sex workers [11-15] and health care workers occupationally exposed to HIV-contaminated body fluids [16, 17].

Parenteral exposure is an important route of HIV transmission as well as a portal of entry into the heterosexual community in several populations worldwide. Injection drug use has directly and indirectly accounted for 36% of AIDS cases in the United States [18], and is reported to be the basis of epidemics in Thailand, certain areas of South East Asia and Central Europe [19]. There is little information available on HIV-specific immunity in injection drug user (IDU) EUs as most of the research published to date has been done on mucosally or vertically exposed individuals.

Immune reconstitution and CD8+ T cell depletion experiments show that HIV- or simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocytes (CTL) control viral replication in infected subjects [20-22]. To address whether CTLs also play a role in protection from infection, we measured HIV-specific effector responses in EU IDU with well-documented HIV exposure. All subjects studied were exposed to HIV infected blood through needle sharing with seropositive partners over a period of 1 year prior

to screening for HIV-specific effector activity by the Interferon γ (IFN-γ) enzyme linked immunospot (ELISPOT) assay. The proportion of EUs having such activity that remained seronegative was compared with the proportion of EUs with HIV-specific effector responses that seroconverted within 7 months after sampling. We report here that the presence of HIV-specific effector activity in EUs is associated with a reduced risk of seroconversion.

METHODS

Study population

The study population is composed of 28 EUs selected from the Hôpital Saint Luc cohort of IDUs followed in Montreal, Quebec, Canada. At each visit a blood sample was drawn for serological HIV testing and isolation of peripheral blood mononuclear cells (PBMC) and plasma for cryopreservation and storage. Questionnaires detailing drug use, health, service utilization, injection, and sexual risk behaviors were administered at all visits by research nurses trained to conduct interviews with IDUs. Participants were eligible for this study if they were HIV seronegative at the time of interview and if they reported needle-sharing activity with known HIV-infected partners in the past 12 months. EUs were separated into 2 groups, depending on whether or not they seroconverted by their next study visit. Group I (n=18) included subjects who remained seronegative at the next follow-up visit. Group II (n=10) included those who had seroconverted at the next follow-up visit. The possibility that some of the study subjects were in the acute or early phase of infection prior to seroconversion was ruled out in all Group I and 4 of 10 Group II individuals using a nested PCR that detects proviral HIV-1 Nef, Vif, and Pol sequences [23]. The remaining 6 study subjects in Group II were all negative, at the time they were screened for HIV-specific immunity, for plasma HIV by an ultrasensitive b-DNA assay (Quantiplex HIV RNA version 3 assay, Chiron Emeryville, CA; detection limit 50 copies/ml). Undetectable viral load would be unlikely in individuals in the acute

phase of infection [24]. For comparison, 18 individuals at low risk for HIV exposure were also screened by ELISPOT assay and found to be negative for HIV-specific immunity. Four of these individuals were IDU with no history of needle-sharing with HIV-infected partners.

Sample processing

PBMC were isolated from blood collected in EDTA anticoagulant by density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsala, Sweden) and frozen in 90% fetal calf serum (FCS, GIBCO BRL Life Technologies, Burlington, Ontario), 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis MO).

HLA typing

Genomic DNA for molecular HLA typing was prepared from either fresh blood or Epstein-Barr virus transformed B cell lines using the QIAamp DNA blood kit (Qiagen Inc. Mississauga, Ontario). HLA typing was performed by standard molecular methods using 95 primer sets amplifying defined major histocompatibility complex (MHC) class I alleles (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI) [25].

Synthetic HIV peptides

The sequences of the HIV-1 peptides used for PBMC stimulation are available at the Los Alamos HIV Molecular Immunology Database [26].

Peptides of 9-, 15-, or 20-amino acids in length containing these sequences were obtained from the Medical Research Council AIDS Reagent Project (Hertz UK), the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD) or synthesized to greater than 85% purity by solid phase synthesis using F-MOC chemistry (Sheldon Biotechnology Center, Montreal, Quebec, Canada).

The ELISPOT assay

Cryopreserved PBMC were thawed and stimulated individually with a panel of HIV-1 peptides each at a concentration of 20 μM for 3 hrs at 37°C. Stimulating peptides were chosen on the basis of their having amino acid sequence binding motifs for ≥1 of the MHC class I alleles expressed by the person being tested. PBMC were seeded in duplicate at 2 x 10^5 cells/per well into 96-well polyvinylidene difluoride-backed plate(s) (MAIPS 45; Millipore, Bedford MA) pre-coated with 5 μg/ml of anti-human IFN-γ monoclonal antibody (mAb) (1-D1K, Mabtech, Stockholm, Sweden). Positive and negative control stimuli for these experiments were anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) and no peptide, respectively. After an overnight incubation PBMC were washed away. IFN-γ secreting cells were detected as spots by the sequential addition, followed by washing, of a second biotin-conjugated mAb to IFN-γ (7-B6-1, Mabtech), diluted 1:2000, streptavidin-alkaline phosphatase (Mabtech), diluted 1:2000, and p-nitro blue tetrazoliumchloride 5-bromo-4-chloro-3-indolyl phosphate toluidine substrate (Bio-Rad Laboratories,

Richmond, CA). Spots were counted under a stereomicroscope (Carl Zeiss, London, Ontario, Canada) and expressed as the number of spot forming cells (SFC)/10^6 PBMC. Negative control stimulation produced less than 5 spots per well. Wells with greater than 50 SFC/10^6 PBMC were considered positive. The identity of IFN-γ secreting cells as CD8+ was confirmed by the reduction of SFC numbers following depletion of CD8+ cells with magnetic beads (Dynal, Lake Success, NY).

RESULTS:

Groups I and II were similar in age, sex ratio and CCR5 genotype. The 2 groups were similar with respect to the number of time they had had a documented HIV exposure whether all subjects or only those who were homozygous wild type for CCR5 were included in the analysis (Table 1). None of the study subjects was homozygous for the Δ32CCR5 deletion mutant that confers resistance to HIV infection. Low risk controls (8 males and 10 females) were similar in age to Groups I and II.

HIV-specific IFN-γ secreting cells.

Despite there being no between-group differences in the number of HIV peptides used to stimulate PBMC, a greater proportion of subjects in Group I responded to HIV peptides than did individuals in Group II (12 of 18 [66.7%] Group I subjects and none of the 10 Group II subjects had detectable HIV-specific effector activity, $p<0.001$; Fisher exact test; OR 40.38 [95% CI 2.95 to >3000]). None of the 18 low risk controls responded above background to any of the HIV peptides they were screened with.

Figure 1 displays ELISPOT assay results for the 12 Group I individuals who had HIV-specific effector activity. HIV-specific responses present in Group I individuals recognized multiple peptides and HIV gene products. Of those with positive responses, 7 subjects recognized a single HIV peptide, 1 subject recognized 2, 1 subject recognized 3, 1 subject recognized 4, 1 subject recognized 5, and 1 subject recognized 6 peptides with which they were

screened. None of the 9 HIV-1 Env peptides stimulated a positive response in PBMC from EUs. Eleven of 55 (20.0%) Gag peptides, 5 of 46 (10.9%) Reverse Transcriptase (RT) peptides, and 7 of 35 (20.0%) Nef peptides elicited above background reactivity in Group I EUs. Responses were restricted to multiple MHC class I alleles.

The magnitude of effector responses to individual peptides ranged between 50 and 105 SFC/10^6 PBMC. This magnitude is approximately 2 logs lower than that seen in antiretroviral drug naïve HIV infected individuals in the chronic phase of infection tested in our laboratory [27].

DISCUSSION

HIV seronegative IDUs with documented exposure to HIV via needle sharing were screened for the presence of HIV-specific CD8+ effector activity. EU IDUs having HIV-specific effector activity were found to be at a reduced risk for seroconversion compared with EU IDUs not having these responses, as 12 of 18 (66.7%) Group I subjects versus none of 10 Group II subjects exhibited HIV-specific effector activity. Maintenance of seronegativity in this population cannot be explained by either heterozygosity or homozygosity for the 32 base pair deletion mutant of CCR5 [28,29]. Nor would mucosal factors such as the presence of HIV-specific mucosal IgA and cervical HIV-specific CTL, which have been reported to be present more frequently in mucosally exposed EUs than controls, be expected to play a role in resistance to HIV infection in this parenterally exposed population [4, 14, 15].

Several explanations may account for the absence of detectable HIV recognition in some EUs besides the conclusion that no HIV-specific immunity was induced in these individuals. Some EUs may have responses that are undetectable because they are directed at HIV peptides other than those included in the screening panel. The strategy for choosing HIV peptide stimuli relied on information from the Los Alamos HIV Molecular Immunology database, which lists HIV epitopes and MHC class I restriction specificity frequently recognized by HIV-infected individuals in the chronic phase of infection. Several investigators have reported differential recognition of HIV epitopes depending on the stage of HIV disease and exposure status [30-31].

Furthermore, as no information was available on the sequence of the viral isolate(s) to which each EU was exposed there is the possibility that EU IDU mounted effector responses to HIV peptides that did not cross react with the corresponding peptide used in the screening panel. While these explanations for the lack of detectable HIV-specific responses in some EU IDU could lead to an underestimation of HIV-specific effector responses, they are unlikely to account for the differences in the proportion of subjects in Groups I and II since a similar strategy was used to select HIV peptides as stimuli for PBMC from both groups of EUs.

In summary we have shown that IDU EUs exposed parenterally to HIV mount virus-specific immune responses. Those subjects having these responses appear to be at a reduced risk for seroconversion compared to those who have undetectable HIV-specific effector activity.

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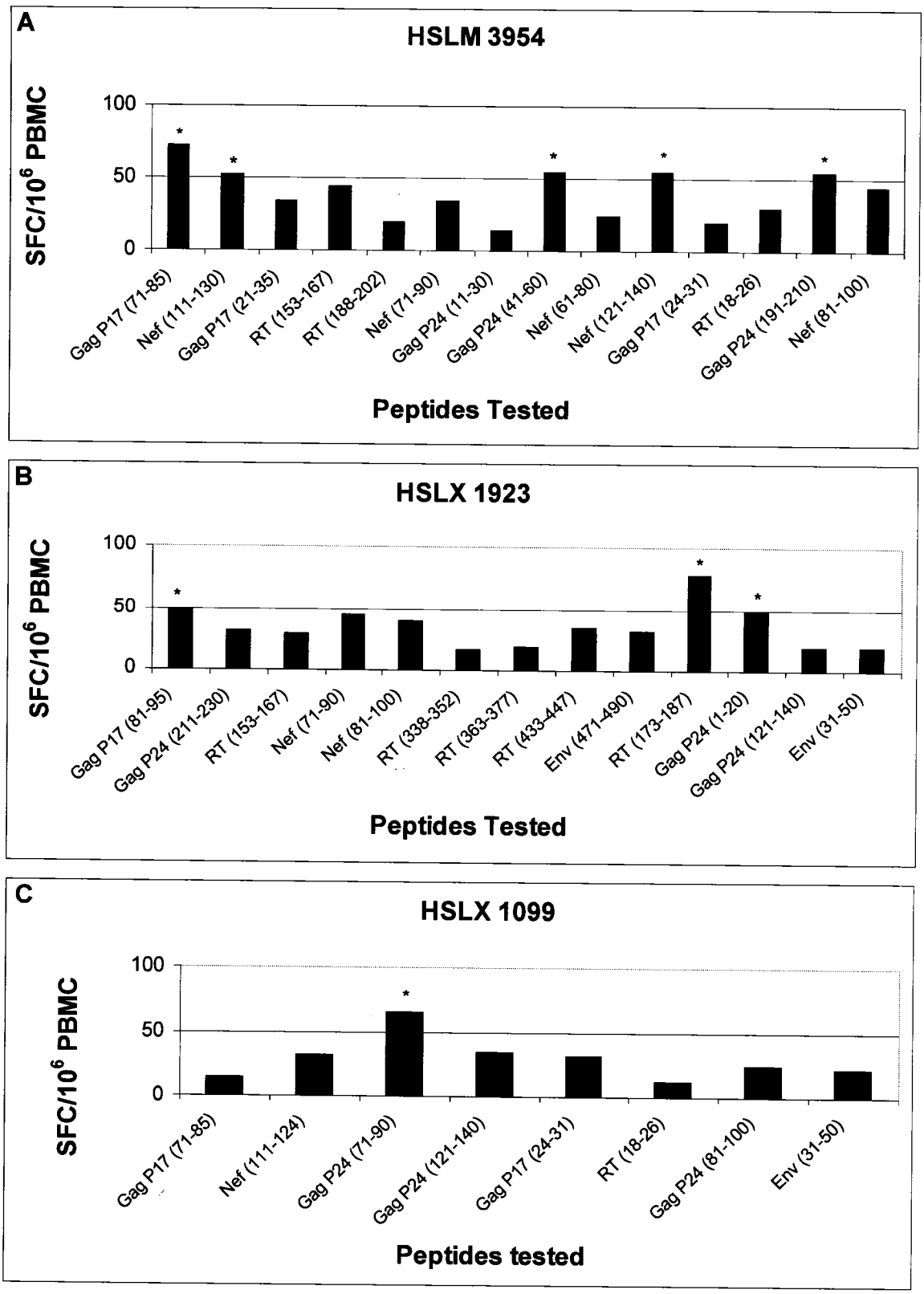
LEGEND

Figure 1: Specificity of HIV recognition of 12 Group I injection drug user exposed uninfected subjects exhibiting HIV-specific effector activity. Peripheral blood mononuclear cells (PBMC) were stimulated individually with the HIV peptides listed on the x-axis of each bar graph. The number of spot forming cells per million indicates the number of PBMC or CD8+ T cell depleted PBMC per million that secreted Interferon-γ in response to stimulation with each peptide. An asterisk (*) over a bar indicates a response over background. The horizontal line indicates the threshold for a positive response (twice the number of SFC generated in response to no peptide). Panels K and L show the number of SFC generated following HIV peptide stimulation of PBMC that had been subjected to immunomagnetic bead depletion with an irrelevant antibody or an anti-CD8+ T cell specific antibody.

Figure 1: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50
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Nef (111-130)
Gag P17 (21-35)
RT (153-167)
RT (188-202)
Nef (71-90)
Gag P24 (11-30)
Gag P24 (41-60)
Nef (61-80)
Nef (121-140)
Gag P17 (24-31)
RT (18-26)
Gag P24 (191-210)
Nef (81-100)
Peptides Tested
B
HSLX 1923
SFC/10^6 PBMC
100
50
0
Gag P17 (81-95)
Gag P24 (211-230)
RT (153-167)
Nef (71-90)
Nef (81-100)
RT (338-352)
RT (363-377)
RT (433-447)
Env (471-490)
RT (173-187)
Gag P24 (1-20)
Gag P24 (121-140)
Env (31-50)
Peptides Tested
C
HSLX 1099
SFC/10^6 PBMC
100
50
0
Gag P17 (71-85)
Nef (111-124)
Gag P24 (71-90)
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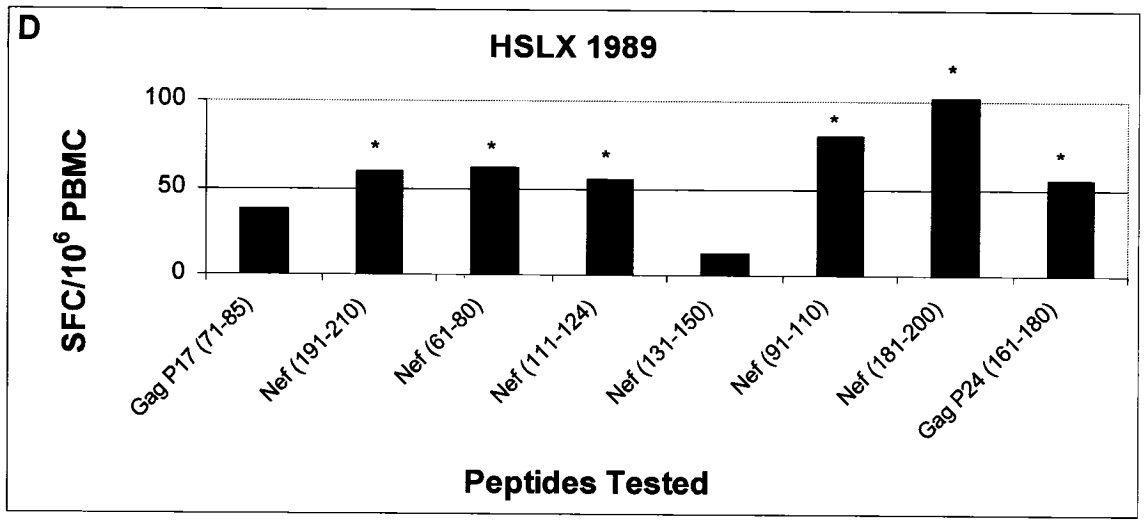
Gag P17 (24-31)
RT (18-26)
Gag P24 (81-100)
Env (31-50)
Peptides tested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SFC/10^6 PBMC
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50
0
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Nef (191-210)
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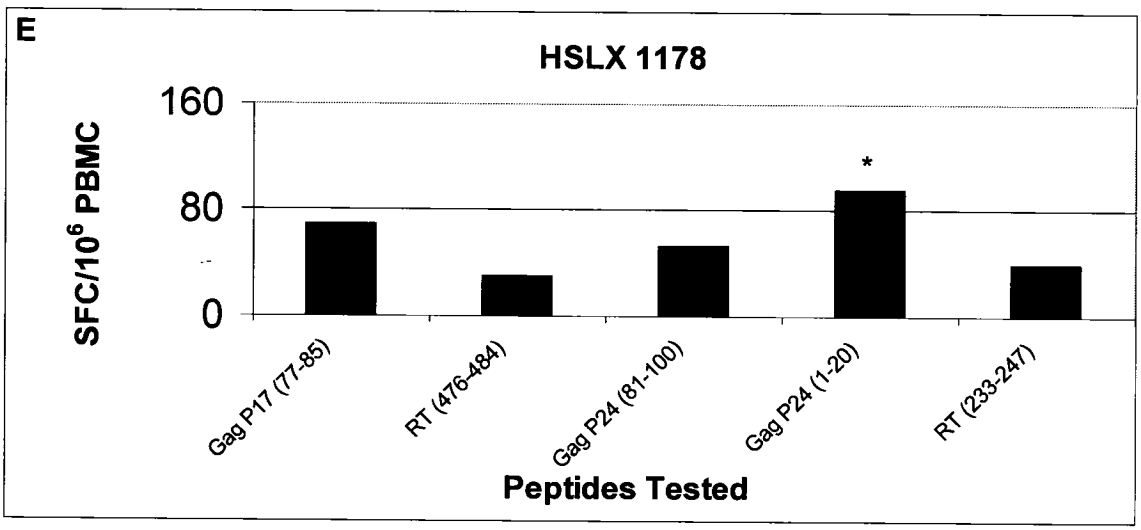
Nef (111-124)
Nef (131-150)
Nef (91-110)
Nef (181-200)
Gag P24 (161-180)
Peptides Tested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80
0
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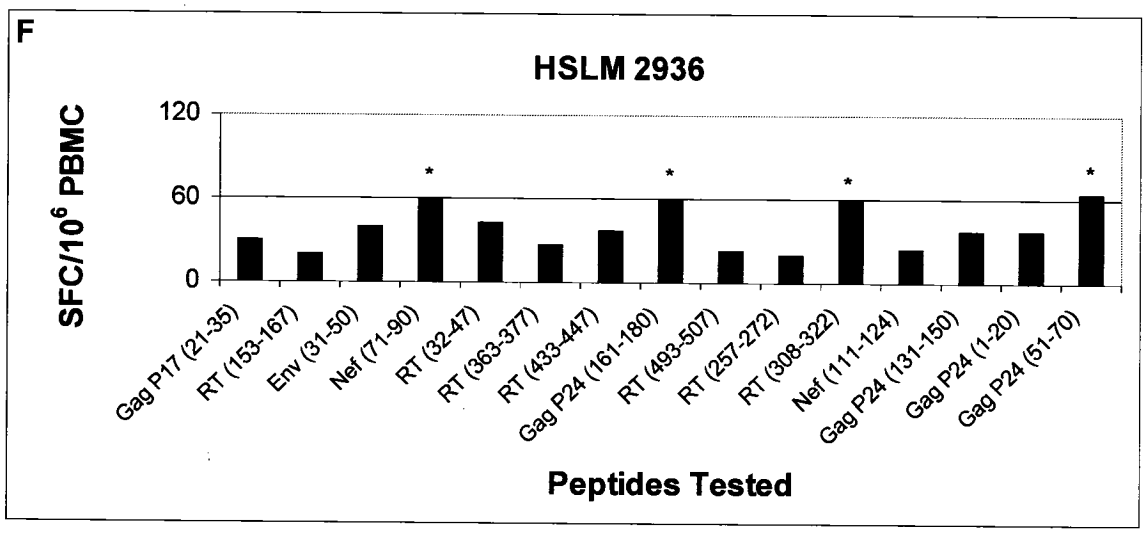
RT (476-484)
Gag P24 (81-100)
Gag P24 (1-20)
RT (233-247)
Peptides Tested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SFC/10^6 PBMC
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60
0
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RT (153-167)
Env (31-50)
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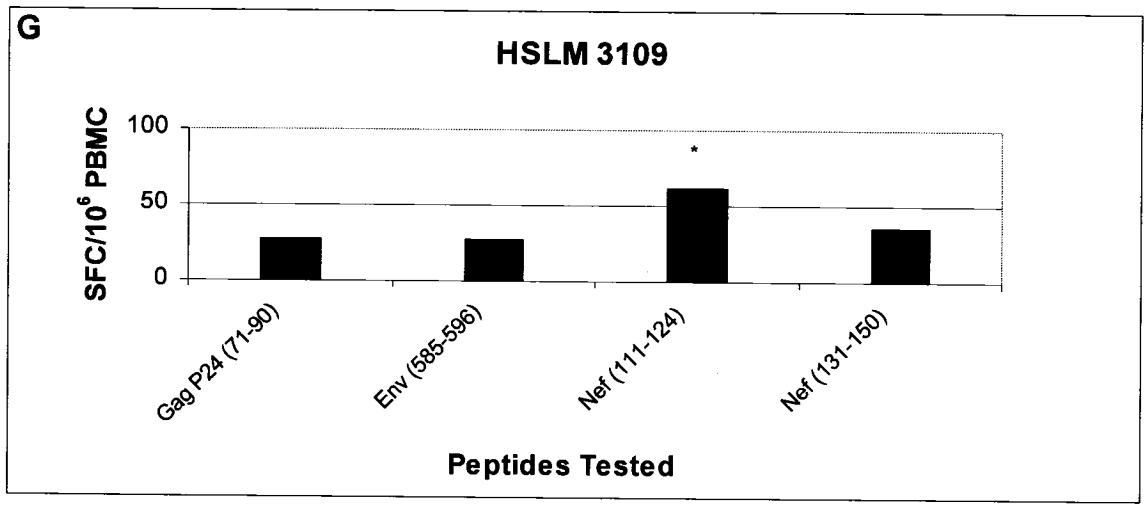
RT (32-47)
RT (363-377)
RT (433-447)
Gag P24 (161-180)
RT (493-507)
RT (257-272)
RT (308-322)
Nef (111-124)
Gag P24 (131-150)
Gag P24 (1-20)
Gag P24 (51-70)
Peptides Tested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0
*
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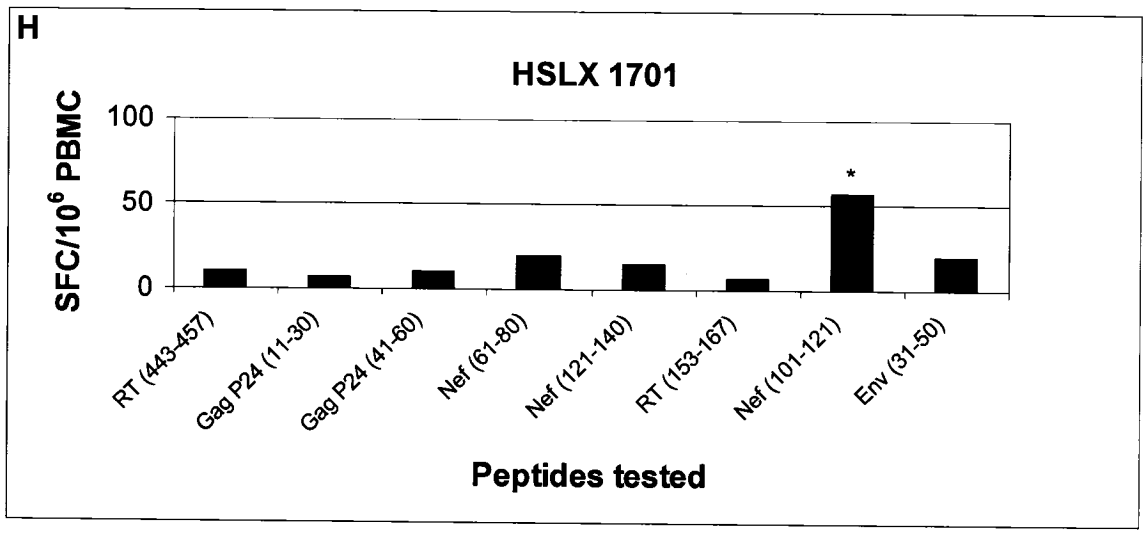
Env (585-596)
Nef (111-124)
Nef (131-150)
Peptides Tested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50
0
*
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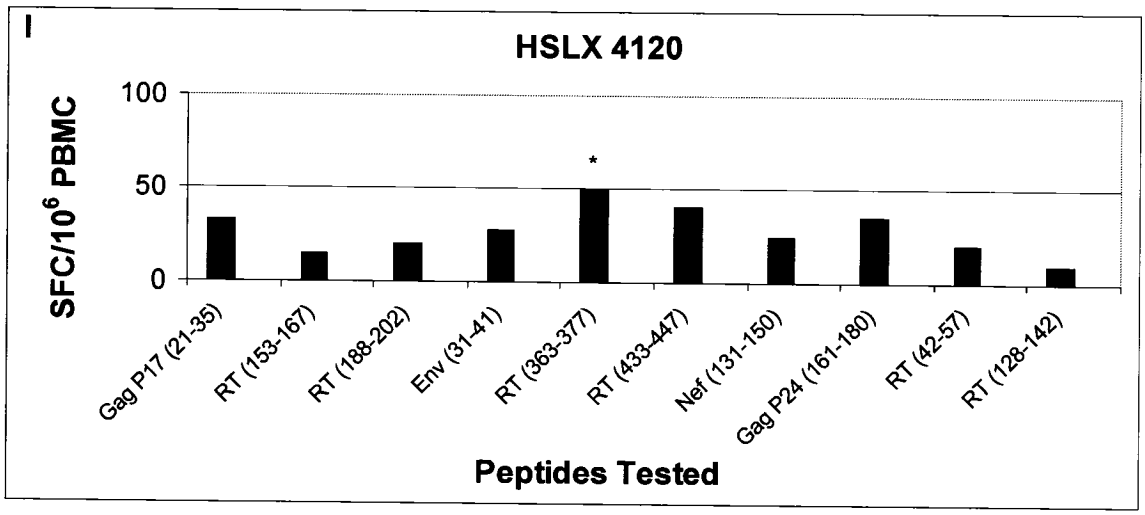
Nef (61-80)
Nef (121-140)
RT (153-167)
Nef (101-121)
Env (31-50)
Peptides tested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50
0
*
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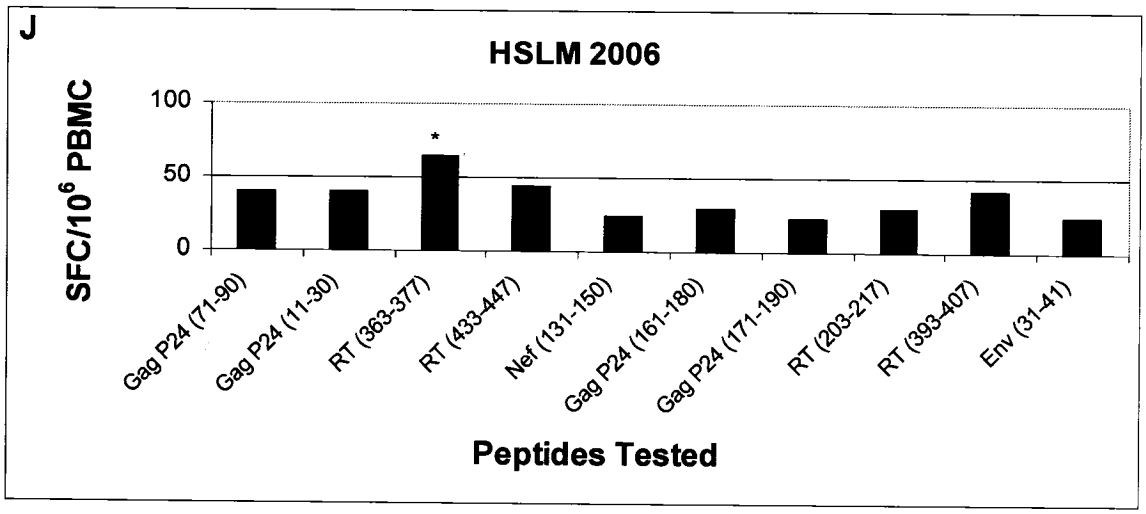
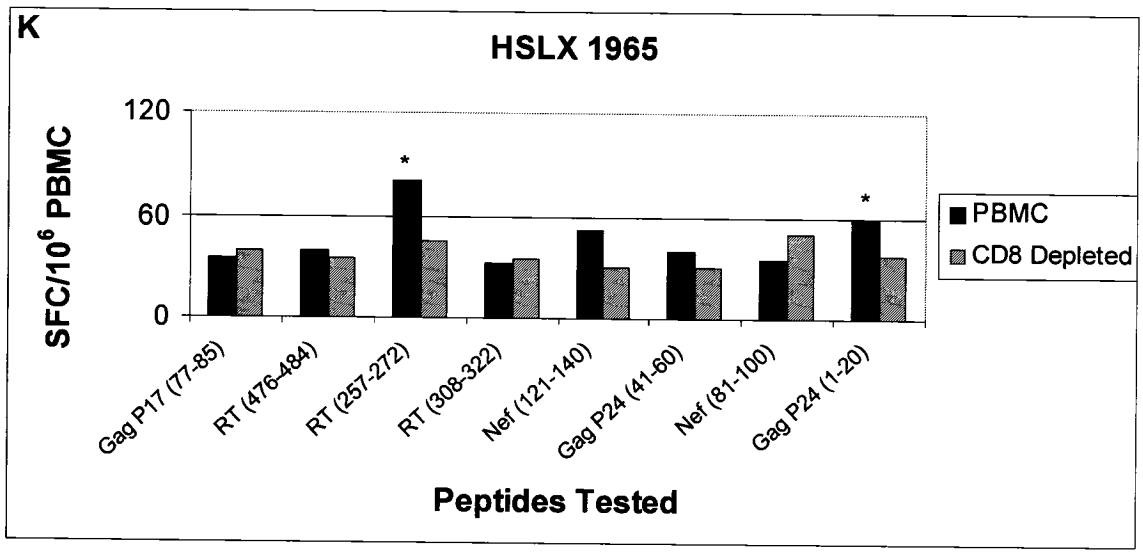
RT (188-202)
Env (31-41)
RT (363-377)
RT (433-447)
Nef (131-150)
Gag P24 (161-180)
RT (42-57)
RT (128-142)
Peptides Tested


J
HSLM 2006
SFC/10^6 PBMC
100
50
0
*
Gag P24 (71-90)
Gag P24 (11-30)
RT (363-377)
RT (433-447)
Nef (131-150)
Gag P24 (161-180)
Gag P24 (171-190)
RT (203-217)
RT (393-407)
Env (31-41)
Peptides Tested


K
HSLX 1965
SFC/10^6 PBMC
120
60
0
*
*
PBMC
CD8 Depleted
Gag P17 (77-85)
RT (476-484)
RT (257-272)
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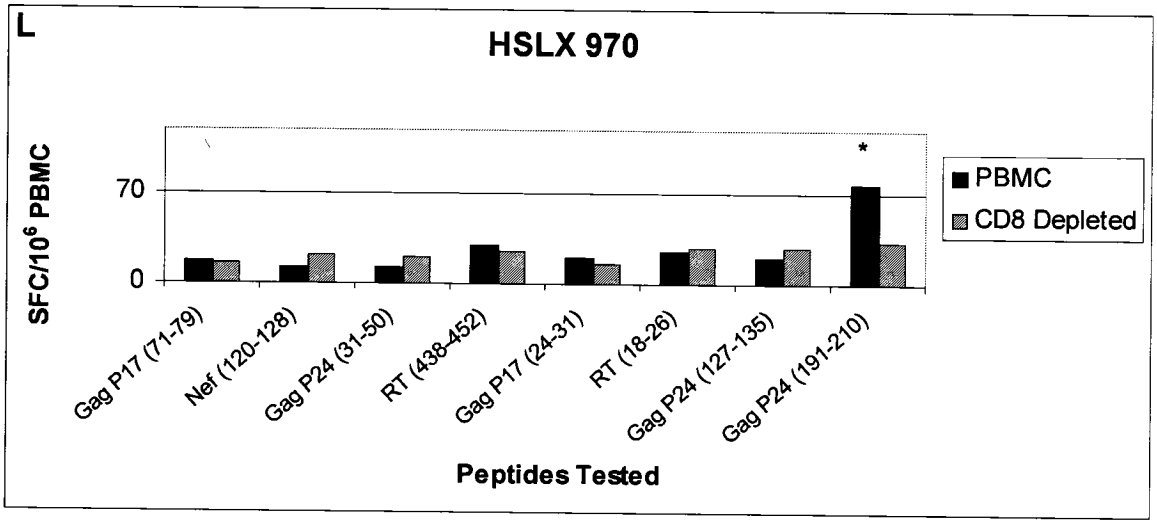
Nef (121-140)
Gag P24 (41-60)
Nef (81-100)
Gag P24 (1-20)
Peptides Tested


L
HSLX 970
SFC/10^6 PBMC
70
0
*
PBMC
CD8 Depleted
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Nef (120-128)
Gag P24 (31-50)
RT (438-452)
Gag P17 (24-31)
RT (18-26)
Gag P24 (127-135)
Gag P24 (191-210)
Peptides Tested

Table 1. Study Population

		Group I (n=18)	Group II (n=10)	P value
Age[a]		**38.5** (25-54)	**35** (22-49)	**0.32**[b]
Sex[c]	M	**17 (94)**	**8 (80)**	**0.24**[d]
	F	**1 (5)**	**2 (20)**	
CCR5 genotype[c]	Homozygous wild type	**13 (72)**	**9 (90)**	**0.27**[d]
	Heterozygous	**5 (28)**	**1 (10)**	
Exposures[e]	All	**4.5** (1-100)	**16.5** (3-300)	**0.29**[b]
	Only CCR5 homozygous wild type subject	**5** (1-70)	**20** (3-300)	**0.24**[b]
No. Peptides tested[f]		**8** (3-15)	**8.5** (3-14)	**0.82**[b]

[a] Median age in years, range in parentheses

[b] Unpaired t-test

[c] Number, percent in parentheses

[d] Chi^2 test

[e] Number of documented exposure events

[f] Median number of peptides included in a screening panel, range in parentheses

Chapter 2:

Does the Route of Exposure Influence the Development of HIV-Specific T Cell Immunity?

Background:

Considerable research efforts have been dedicated to optimizing vaccination protocols. Since most HIV vaccines are formulated for parenteral administration, it will be important to confirm whether or not intravenous inoculation efficiently stimulates protective HIV-specific T cell immunity. Vaccine studies in macaques show that the route of challenge with pathogenic SIVmac251 significantly affects vaccine efficacy[399]. In contrast, macaques vaccinated intranasally, intravaginally, and intravenously developed similar immune responses and were similarly protected when challenged with pathogenic SHIV[400].

While the nature of the pathogen influences the development of specific immunological responses, the immunological compartment that contains the foreign insult is also an important determinant of immunity. Intravenous exposure to HIV results in the deposition of the virus into the bloodstream. Since only 0.1-1% of circulating PBMCs are DC, antigen processing and presentation to T cells is not expected until the virus reaches the local draining lymph node. Conversely, the genital and anal mucosae are highly enriched in immature dendritic cells that are adept at capturing and processing foreign antigens. In addition, HIV is immediately faced with virus-specific and non-specific immune mechanisms in the mucosa, including secretory antibodies, γδ T cells, and a high concentration of chemokines, type I IFNs, and complement. There is also a large quantity of resident CD4+ T lymphocytes that can mediate potent Th1 immunity, despite serving as primary cellular targets for HIV.

Hypothesis:

Given the disparate immunological compartments HIV enters upon parenteral and mucosal contact, along with the differential kinetics with which HIV is likely processed by local immune mechanisms, we hypothesized that intravenously exposed and sexually exposed subjects do not develop the same immunity against HIV.

Objectives:

1) To compare the frequency of HIV-specific T cell responses, as assessed by the IFN-γ ELISPOT, between two groups of parenteral and mucosal EUs.

2) To compare the magnitude and breadth of the HIV-specific T cell responses between the two groups.

3) To identify between-group differences in the MHC-restriction of the HIV-specific T cell responses.

Comparison of HIV-Specific $CD8^+$ T cell Responses among Uninfected Individuals Exposed to HIV parenterally and mucosally

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Running Head: Influence of Route of Exposure on HIV Immunity

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Word Count: 3479

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Author Contribution:

1) George Makedonas: Chief experimenter, primary author, study design.

2) Julie Bruneau: Director of St. Luc IVDU cohort, development and subject recruitment.

3) Michel Alary: Director of OMEGA cohort of MSMs, development and subject recruitment, statistical consultant.

4) Christos M Tsoukas: subject recruitment, provided analytical equipment and storage facilities.

5) Catherine M. Lowndes: development of and subject recruitment for OMEGA cohort.

6) Dr. Francois Lamothe: patient recruitment for the St-Luc cohort.

7) Dr. Nicole F. Bernard: Primary investigator, study design and supervision, manuscript editing

ABSTRACT

Objective: To assess the influence of route of HIV exposure on the development of HIV-specific $CD8^+$ T cell responses in exposed, uninfected (EU) individuals.

Design: Two groups of EU exposed to virus through either sexual or intravenous contact were studied. Group I included subjects (n=20) who had unprotected sexual contact with known HIV infected partners and no intravenous HIV exposure; Group II included individuals (n=27) who had shared needles with HIV-infected partners and had no sexual exposure to this virus. Between-group comparisons were made for the proportion of responders, breadth, magnitude, and specificity of HIV-specific responses.

Methods: The Interferon-γ ELISPOT assay was used to detect HIV-specific effector activity. PBMC from each subject were stimulated with a panel of HIV peptides restricted to the MHC class I alleles expressed by the individual.

Results: A similar proportion of EU tested from each group (35.0% Group I vs. 22.2% Group II) recognized at least 1 HIV peptide. Group I and II subjects recognized HIV peptides with a similar cumulative intensity of 130 ± 67.5 and 182.9 ± 184.2 spot forming cells/million PBMC, respectively, and similar magnitude per stimulatory peptide of 82.7 and 78.4 SFC/million PBMC, respectively. The proportion of stimulatory peptides derived from HIV Gag, reverse transcriptase, Env, and Nef was not significantly different between the two EU groups. HLA-A*0201 restricted HIV epitopes immunodominant in infected individuals are rarely stimulatory in EUs.

Conclusions: Both mucosal and parenteral exposure to HIV can elicit HIV-specific $CD8^+$ T cell responses with similar characteristics.

Key Words: HIV, Exposed Uninfected Subjects, HLA Supertypes, Route of Exposure, Cellular Immunity, Interferon-γ ELISPOT Assay

INTRODUCTION

Individuals who have been repeatedly exposed to HIV, yet remain uninfected, are an important study population because their persistent seronegativity may be due to having acquired a natural resistance to HIV. Genetic factors, such as homozygosity for a 32 base-pair deletion mutation in the CCR5 receptor that is required for HIV entry into target cells [1,2] and expression of certain major histocompatibility complex (MHC) class I alleles [3-6], have been implicated as factors contributing to resistance to HIV infection. However, genetic factors reported to be associated with resistance are observed in only a small proportion of exposed, uninfected (EU) subjects [7-10]. There is evidence that the presence of HIV-specific CD8+ T cells in EUs is associated with protection. Kaul et al. reported that interruption of commercial sex work led to a waning of HIV-specific effector activity and increased susceptibility to infection [11]. We reported a 40-fold reduced risk of seroconversion in EUs having HIV-specific effector activity compared with similarly exposed subjects with no detectable responses [8].

Several populations of exposed, uninfected (EU) subjects have been studied including sex workers [12-16], men having sex with men (MSM) [7,17], perinatally exposed infants [18-20], and HIV discordant heterosexual couples [7,21-24] to identify the immune mechanisms that may account for their remaining uninfected. In contrast to the several cohorts of mucosally exposed individuals that have been studied to date, there is a relative paucity of information on HIV-specific cellular responses in EUs exposed to HIV

parenterally. Although HIV-specific immunity has been reported in health care workers exposed to HIV via needle-stick injuries [25,26], there are few reports of immunity among injection drug users (IDU) [8,27,28]. Injection drug use has emerged as a major risk factor for HIV infection in Southeast Asia, Eastern Europe, and in several urban centres across North America [29-33].

In this report, we used the IFN-γ ELISPOT assay to compare HIV-specific T cell responses between an uninfected group of IDU exposed to HIV by needle-sharing and a group of uninfected subjects exposed to HIV sexually in order to determine if the route of exposure influences the development and quality of HIV-specific T cell immunity.

METHODS

Study Population

Individuals at high risk for HIV infection were identified from 3 sources: the Omega Cohort of men having sex with men (MSM) [34], the Saint Luc Cohort of injection drug users (IDU) [35], and the Immune Deficiency Treatment Centre (IDTC) clinic of the McGill University Health Centre, Montreal General Hospital Pavilion (Montreal, Quebec, Canada). EU subjects were recruited to the clinics every 3-6 months in order to monitor their HIV serostatus and to document their exposure history. For these studies we chose a single time point on which to perform ELISPOT assays. The time point selected for testing was determined by reviewing questionnaires filled out at each clinic visit addressing high-risk injection drug use activity and sexual behaviour since the last clinic visit. At the time point selected for testing, all participants reported at least one prior exposure to HIV and were seronegative for HIV antibodies using a standard HIV-1 antibody enzyme immunoassay (HIV-1 EIA). The possibility that some of the study subjects were in the acute or early phase of infection prior to seroconversion was ruled out using a nested PCR designed to detect proviral HIV-1 Nef, Vif, and Pol sequences [36].

Two study groups of EU persons matched for age and sex were constituted based on reported high-risk behaviour for HIV acquisition. Group I (n=20) included participants who admitted to having unprotected sexual intercourse with a known HIV infected partner(s) since the last clinic visit, and who reported no intravenous exposure to HIV at any prior time. Group II (n=27)

was composed of IDUs who reported needle sharing with an HIV infected partner(s) in the time period since the last visit to the clinic. None of the participants in Group II had been mucosally exposed to HIV either through unprotected (or protected) sex with commercial sex workers, IDU partners, HIV infected partners, or partners of unknown serostatus at any time prior to testing.

Nineteen subjects at low-risk for HIV infection were recruited to serve as negative controls. Fourteen of these individuals (ten females, four males) were heterosexual adults with no mucosal or parenteral exposure to HIV. The remaining five controls (all males) were IDUs who had never shared needles with HIV infected individuals or with partners of unknown serostatus. They also had never engaged in high-risk sexual relations.

Sample Processing

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected in ethylenediamine tetra-acetic acid (EDTA) anticoagulant by density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsala, Sweden) and frozen in 90% fetal calf serum (GIBCO BRL Life Technologies, Burlington, Ontario), 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis MO).

HLA typing

Genomic DNA for molecular HLA typing was prepared from either fresh blood or Epstein-Barr virus transformed B cell lines using the QIAamp DNA

blood kit (Qiagen Inc. Mississauga, Ontario). Each individual's MHC class I type was determined by the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) using 95 primer sets amplifying defined MHC class I alleles (ABC SSP Unitray, Pel-Freez Clinical Systems, Brown Deer, WI) [37].

Synthetic HIV Peptides

Sets of overlapping peptides were obtained from the Medical Research Council AIDS Reagent Project (Herts, UK) and the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD). Peptide panels selected for this study were selected from sets corresponding to Nef from HIV-1_{BRU} (20-mers with 10aa overlaps), Env from HIV-1_{MN} (20-mers with 10 aa overlaps, Pol from HIV-1_{LAI} (15-mers with a 10 aa overlaps, Gag p17 and p24 from HIV-1_{SF2} (15-mers with 5 aa overlaps and 20-mers with a 10 amino acid overlaps, respectively). A set of peptides corresponding to described optimal HIV-1 CTL epitopes 9-11 amino acids in length were also synthesized (Sheldon Biotechnology Centre, Montreal, Quebec, Canada). Peptides were prepared to greater than 85% purity by solid phase synthesis using F-MOC chemistry. Lyophilized peptides were diluted to 1 mg/ml in Hank's buffered saline solution (HBSS); 10% DMSO and stored at –20°C.

The ELISPOT Assay

Frozen PBMC samples were thawed and stimulated individually in microfuge tubes with a panel of HIV-1 peptides each at a concentration of 20

μM for 3 hrs at 37°C. The peptides included in the panel used for screening were selected because they contained amino acid sequence binding motifs for at least one of the MHC class I alleles expressed by the subject being tested. For all experiments, optimal MHC class I restricted peptides were used as stimuli whenever possible. When optimal peptides were not available, however, 20-mer or 15-mer peptides containing these sequences were used as stimuli.

After antigen pulsing, PBMC were seeded in duplicate at 2 x 10^5 cells per well into 96-well polyvinylidene difluoride-backed plate(s) (MAIPS 45; Millipore, Bedford MA) pre-coated with 5 μg/ml of anti-IFN-γ monoclonal antibody (mAb) (1-D1K, Mabtech AB, Stockholm, Sweden). Positive and negative control stimuli for these experiments were anti-CD3 antibody (Research Diagnostics Inc, Flanders N.J.) and no peptide, respectively. After an incubation of 16 to 20 hr, PBMC were washed away and IFN-γ secreting cells were detected as spots by the sequential addition of a biotin-conjugated mAb to IFN-γ (7-B6-1, Mabtech), diluted 1:2000 in phosphate buffered saline (PBS), streptavidin-alkaline phosphatase (Mabtech), diluted 1:2000 in PBS, and p-nitro blue tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine substrate (Bio-Rad Laboratories, Richmond, CA). The resulting spots were counted under a stereomicroscope (Carl Zeiss, London, Ontario, Canada). Results were expressed as spot forming cells (SFC) per 10^6 PBMC.

The cut off for a positive response was determined by screening the 19 low risk controls with peptide panels that were similar in size to those used to screen the EU subjects. The MHC-restricted peptide panels used to screen the

low risk controls produced a mean of 15.1 ± 10.4 SFC/10^6 PBMCs. The threshold for a positive response was thus defined as 3 standard deviations above the average peptide specific responses generated by PBMC from low risk controls, i.e at least 46.3 SFC/10^6 PBMCs and at least 3 times the average of the autologous negative control wells for each person being tested.

Statistical Analyses

The unpaired t-test was used to assess the significance of between-group differences in age, the size of the peptide panels, the sum of the magnitude in individual HIV-specific responses, and the per peptide responses. The significance of proportional between group differences in reactivity to all HIV peptides tested and peptides categorized by the HIV gene products from which they were derived was assessed using the Fisher's exact test. All statistical analyses were performed on Graphpad software.

RESULTS

Population Characteristics

Table 1 provides information on the two groups of EU studied cross-sectionally in this report. Group I (mucosally exposed EU) included 17 males and 3 females aged a mean of 39.9 years (range 23 to 55), while Group II (parenterally exposed EU) consisted of 26 males and 1 female aged a median of 38.9 years (range 26 to 54). Although HLA class I genes are highly polymorphic, it is possible to cluster HLA class I A and B locus alleles into supertypes that are defined by similarities in peptide sequence binding motifs [38,39]. No between group differences were observed in the distribution of subjects expressing alleles belonging to the A1, A3, A24, A26, B7, B44, B27, B38 or B62 MHC class I supertypes.

Screening for HIV-Specific CD8+ T Cell Activity

PBMC from each test subject were screened for HIV-specific IFN-γ secretion with a panel of HIV peptides. The HIV peptides included in each screening panel were selected because they contained sequence motifs able to bind one of the MHC class I alleles expressed by the subject being tested, based on the information available at the Los Alamos HIV Molecular Immunology Database. The peptide panels used to test each individual were restricted to a median of 4 (range 3 to 5) MHC class I alleles. The number of

peptides included in the screening panels was comparable between groups (Table 2).

A similar proportion of Group I and II EU exhibited HIV-specific effector activity [7 of 20 (35.0%) mucosally versus 6 of the 27 (22.2%) intravenously exposed subjects; p=0.51, Fisher's Exact test] as defined by responding to a minimum of 1 HIV peptide from the panel (Table 2). Responders from both groups of EU recognized a median of 1 HIV peptide (range 1-2 for Group I; 1-7 for Group II). Fig 1A shows the magnitude of individual HIV-peptide specific responses for all stimulatory peptides tested in Group I and II individuals as well as in 14 low risk healthy control heterosexual adults and 5 low risk control IDUs. None of the 19 low-risk, negative controls had positive IFN-γ ELISPOT responses (p=0.0083 for comparison versus Group I; p=0.035 for comparison vs. Group II, Fisher's Exact test).

Similar Magnitude and Breadth of the HIV-Specific Response in Group I and Group II EU

The magnitude of the HIV specific response for each individual was calculated by adding the number of SFC/10^6 PBMC generated from each HIV peptide stimulus that induced a positive response. The mean magnitude of the HIV-specific immune response to the peptide panel used to screen each subject in Group I versus Group II was 130.0 ± 67.5 and 182.9 ± 184.2 SFC/10^6 PBMC, respectively (p=0.49, unpaired t-test) (Table 2 and Figure 1B). The mean magnitude per stimulatory peptide was 82.7 ± 36.5 versus 78.4 ± 34.6 SFC/10^6

PBMC for Group I and Group II subjects, respectively (p=0.76, unpaired t-test, Table 2).

The mucosally (Group I) and parenterally (Group II) exposed populations responded to a similar proportion of HIV peptides used for screening: 11 of 230 (4.78%) and 14 of 269 (5.20%), respectively (Table 3). These proportions were significantly higher than that seen for the 19 low risk controls (0/154 peptides tested; p=0.0040 for comparison with Group I EU and p=0.0029 for Group II EU) (Fig 1A). Comparing the distribution of stimulatory peptides by HIV gene product did not reveal any statistically significant between-group differences, although in Group I EU the greatest number of stimulatory peptides were Nef derived (5) followed by RT (1), Env (1), and Gag (1), whereas in Group II the greatest number of stimulatory peptides were RT derived (8) followed by Gag (5) and Nef (1) (Table 3).

Figure 2 illustrates the breadth of the response for each of the Group I (panel A) and Group II (panel B) responders. Only 1 HIV peptide induced IFN-γ secretion from both sets of EU: RT 153-167. Group I subjects recognized 1 peptide, Nef 81-100, on more than one occasion. Group II subjects recognized RT 397-405 more than once with an intensity of 185 SFC/10^6 PBMC (OM-FED) and 92.5 SFC/10^6 PBMC (OM-SRS). The breadth of the HIV-specific CD8+ T cell response by the Group II EU HSLX-PRT is particularly noteworthy. This subject responded to 7 different peptides restricted to 3 different alleles with a cumulative magnitude of 480 SFC/million PBMC.

Responses were classified according to the supertype of their MHC class I restriction allele. Seven of the 11 (63.6%) peptides that stimulated HIV-specific IFN-γ secretion in Group I EU were restricted to alleles belonging to the A3 supertype (Figure 2, panel A). The remaining 4 responses were restricted to alleles belonging to the A1, A2, B7, and B62 [1/11, (9.1%)] supertypes. The 14 peptides that stimulated responses in Group II EU were restricted to MHC class I alleles belonging to the B7 supertype [6/14, (42.9%)], the B44 supertype [4/14, (28.6%)], the A3 supertype [3/14, (21.4%)], and the A2 supertype [1/14 (7.1%)] (Figure 2, panel B). There were no statistically significant between-group differences in the number of stimulatory peptides that were restricted to any of the supertypes.

HLA-A*0201 EU Rarely Respond to HLA-A2-Restricted Peptides Immunodominant in HIV infected subjects

The HLA allele A*0201 belonging to the A2 supertype was differentially expressed in the 2 EU study populations. Eleven of 20 (55%) Group I EU expressed A*0201, whereas only 5 of 27 (18.5%) Group II EU expressed this allele (p=0.0134, Fisher's Exact test). All were screened with 2 peptides that are "immunodominant" in chronically HIV infected HLA-A*0201 expressing individuals: Gag 77-85 (SLYNTVATL, SL9) and RT 484-476 (ILKEPVHGV, IL9). One individual from each group secreted IFN-γ in response to stimulation with SL9 (1/11 vs 1/5; p=NS, Fisher's Exact test) while none of the A*0201 positive EU in either group responded to IL9 stimulation. Using similar methods, we

screened 13 HLA-A*0201 expressing individuals in the chronic phase of HIV infection. Nine (69.2%) recognized Gag 75-85 and 4 (30.8%) recognized IL9 [40]. This frequency of responses in HLA-A*0201 positive, chronically infected HIV subjects is similar to that reported by others for these HIV peptides [41-44]. HLA-A*0201 positive EUs from Groups I and II recognized these epitopes significantly less frequently than do HIV positive subjects ($p= 0.0027$ for responses to Gag 77-85 and $p=0.0301$ for responses to IL-9; Fisher's Exact test).

DISCUSSION

This report compares two groups of EUs exposed to HIV by different routes for the presence of IFN-γ secreting, HIV-specific T cells. Seven of 20 (35.0%) mucosally (Group I) versus 6 of 27 (22.2%) intravenously exposed (Group II) subjects exhibited HIV-specific effector activity to at least one of the HIV peptides in the MHC class I restricted screening panel used in the ELISPOT assay (p=0.51, Fisher's Exact test). The proportion of responding individuals in both these groups is significantly greater than that seen in 19 low-risk, negative control individuals, none of whom responded to similar MHC class I restricted HIV peptide panels.

The average magnitude per stimulatory peptide was similar between both EU groups [82.7 ± 36.5 versus 78.4 ± 34.6 SFC /10^6 PBMC for Group I and Group II subjects, respectively (p=0.76, unpaired t-test)]. This was over 3-fold lower than the average per peptide response of 275 ± 162 SFC/10^6 PBMC generated by 20 therapy naïve HIV-infected subjects in the chronic phase of infection [40]. The overall magnitude per responder was also comparable between Group I and II EU [130.0 ± 67.5 vs. 182.9 ± 184.2 SFC /million PBMC (p=0.49, unpaired t-test)]. The magnitude of the response to HIV peptides in EUs was approximately 10-fold lower than the mean of 1409 ± 1459 SFC/10^6 PBMC generated by 20 HIV infected subjects in the chronic phase of infection [median 6.1 yrs infected (range 1 to 15 yrs)] who were naïve to anti-retroviral drug therapy at the time they were tested using a similar testing strategy [40].

There were no statistically significant differences in the breadth of the HIV-specific T cell responses between mucosally exposed (Group I) and parenterally exposed (Group II) individuals. Group I EU recognized 11 of 230 (4.78%) peptides tested while Group II EU were stimulated to secrete IFN-γ by 14 of 269 (5.20%) peptides tested. Although the hierarchy of HIV peptide recognition in Group I individuals was Nef>RT>Env>Gag whereas that in Group II subjects was RT>Gag>Nef, comparisons did not achieve statistical significance (Table 3). It is possible that this observation may be related to the low number of stimulatory peptides seen in these EU populations (11 peptides for Group I vs. 14 peptides for Group II).

In selecting subjects for inclusion into Groups I and II, care was taken to exclude individuals reporting HIV exposure by more than one route. Only IDU reporting no sexual exposure (with or without a condom) to commercial sex workers, other IDUs, HIV seropositive partners, or individuals with unknown HIV serostatus at any time were included in the parenterally exposed group (Group II). So too, only EUs reporting no injection drug use at any time were included in the mucosally exposed group (Group I).

Due to a limited number of PBMC available for testing, it was not possible to screen the cells for HIV-specific IFN-γ secretion with peptides spanning the entire HIV genome. As such, peptide panels for each study subject were designed to include epitopes likely to be restricted by the MHC class I alleles expressed by the test subject and therefore having a likelihood of stimulating a positive ELISPOT response. The Los Alamos HIV Molecular

Immunology database, from which the panel of HIV peptides was drawn, lists epitopes that are frequently recognized by HIV-infected individuals in the chronic phase of infection expressing particular MHC class I alleles. It has been shown that EU differ from chronically HIV infected subjects in terms of the immunodominance pattern of HIV epitope recognition [45, 46]. Our results regarding reactivity to Gag 77-85 and RT 476-484 support this observation. This phenomenon has also been described for HIV infected subjects in acute primary HIV infection versus the chronic phase of infection [47]. Any bias resulting from the design of the peptide panels, however, is unlikely to be a confounding factor in this study since both groups under investigation are EU. The approach used to design the peptide screening panels was the same for both study groups and the control group, and would not be expected to alter the conclusion that mucosal and intravenous exposure to HIV can induce virus specific responses of similar quality. The greatest limitation of this approach is that it likely underestimates the frequency, breadth, and magnitude of the HIV-specific T cell response in this population of EU individuals.

Although the peptide screening panels were designed to detect CD8+ T cell responses, the possibility exists that CD4+ T cells contributed to the observed HIV-specific responses. Most of the peptides used were either 15 or 20 amino acids in length, thereby conceivably containing epitopes capable of stimulating CD4+ T cells. Indeed, recent studies have indicated a growing role for CD4+ T cells as a potential correlate of immunity in EU [27,46, 48, 49]. The anti-HIV response in EU is likely multifactorial, employing several arms of the

immune system. The results presented in this report suggest that parenteral and mucosal stimulation of these processes may produce HIV-specific immune responses of similar quality.

The observation made by Kaul et al. that a similar frequency and specificity of CD3+/CD8+ T cells able to secrete IFN-γ in response to HIV-stimuli in the genital tract and blood of HIV-1 infected women supports a linkage between the mucosal and peripheral compartments [50]. The implication of this observation is that screening for HIV-specific responses in PBMC from mucosally exposed EU is a valid correlate of the presence and quality of the HIV-specific response present at the site of exposure.

We have shown previously that IDU EUs having HIV-specific effector activity are at a 40-fold reduced risk of seroconverting compared to similarly exposed IDU subjects with undetectable HIV-specific effector activity [8]. In this report we show that both mucosal and intravenous exposure to HIV can induce HIV-specific T cell responses of similar magnitude and breadth in a similar proportion of subjects. Based on these observations, it is reasonable to hypothesize that individuals who develop HIV-specific T cell responses detectable in the periphery will be at a reduced risk for seroconversion. Such responses may result equally well from either parenteral or mucosal exposure.

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LEGEND

Figure 1. Scatter plot of HIV specific responses in seronegative subjects exposed to HIV mucosally (Group I) and parenterally (Group II). Peripheral blood mononuclear cells (PBMC) from each HIV exposed uninfected (EU) subject were stimulated with a panel of HIV peptides restricted to the MHC class I alleles expressed by the individual being tested. Peptide specific stimulatory capacity was measured using an IFN-γ ELISPOT assay. Panel A shows the magnitude of individual HIV peptide specific responses (spot forming cells per million peripheral blood mononuclear cells [SFC/10^6 PBMC]) separated by group. Group I EU are mucosally exposed uninfected (EU) individuals, Group II EU are parenterally exposed EU. Low risk controls are separated into 2 groups: Group A, healthy heterosexual adults (n=14); Group B, IDU controls (n=5). The horizontal line is drawn at 47 SFC/10^6 PBMC, which is 3-fold over the average negative response. For a response to be considered positive it must exceed this value and be 3-fold greater than the autologous negative control (see methods section). Panel B, displays for each individual exhibiting a positive response the total positive response calculated by adding the number of SFC/10^6 PBMC generated from each HIV peptide stimulus that induced a positive response. Shown are the magnitudes of the 7 Group I and 6 Group II responders; the line through each scatter plot represents the mean magnitude of the HIV-specific immune for each group.

Figure 1A:

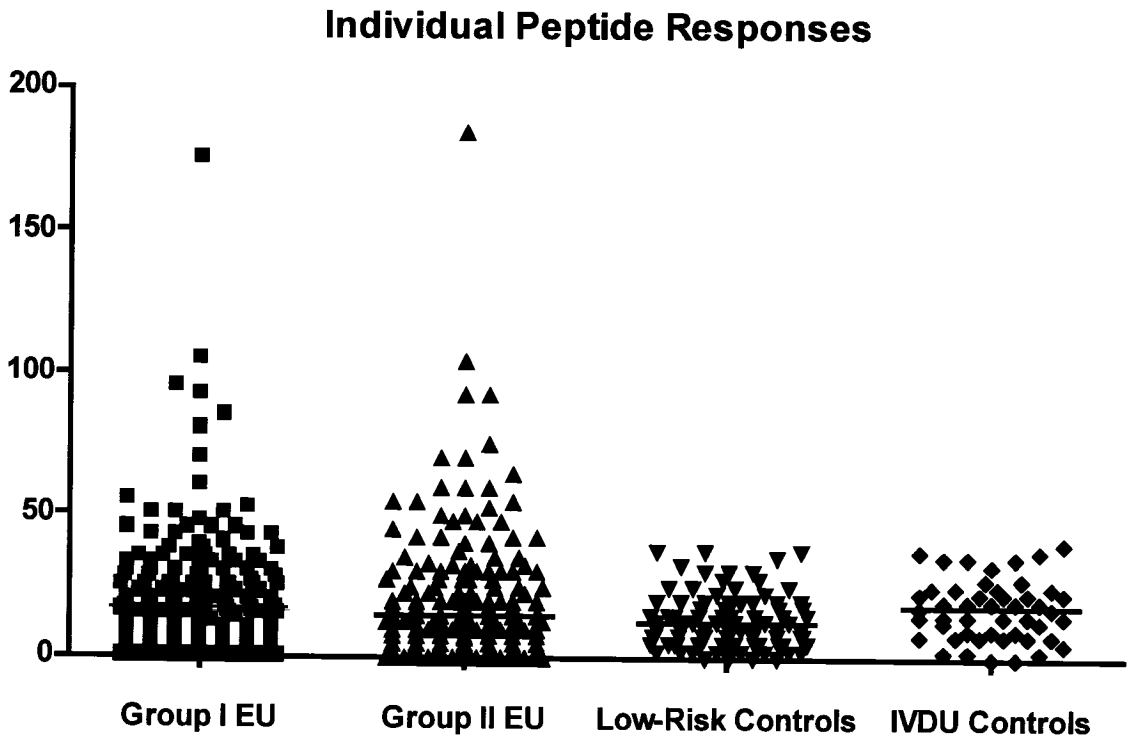
Individual Peptide Responses
200
150
100
50
0
Group I EU
Group II EU
Low-Risk Controls
IVDU Controls

Figure 1B:

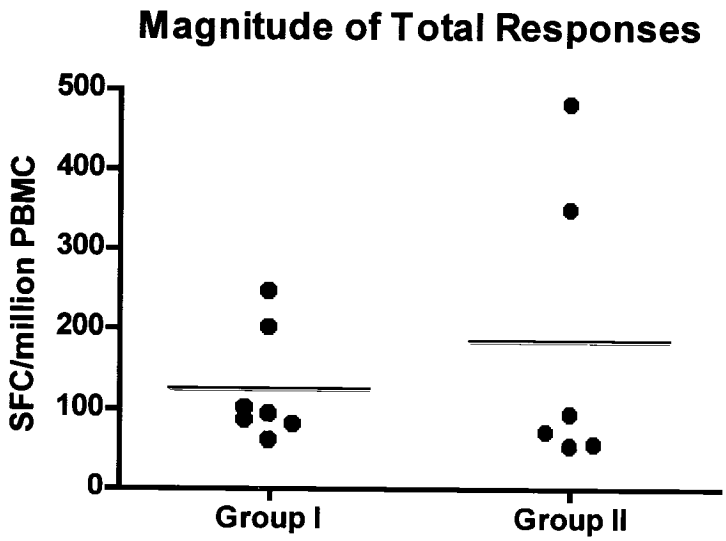
Magnitude of Total Responses
SFC/million PBMC
500
400
300
200
100
0
Group I
Group II

Figure 2. Breadth of HIV specific T cell responses. Each stacked bar represents an individual EU having a positive HIV specific Interferon-γ ELISPOT response. Panel A shows responses for Group I EU and Panel B shows responses for Group II EU. Each stack in the bar corresponds to an individual HIV peptide response. The identity of the stimulatory peptide is provided in shorthand form in the legend for each panel, with gene product from which the peptide was derived, its location in the sequence of that gene product and the supertype to which the peptide is restricted in parentheses. The table in panel C lists the sequence of each HIV peptide able to stimulate a positive response in these study populations. RT=reverse transcriptase, p24=Gag p24, p17= Gag p17, gp160= Env. SFC/10^6 PBMC= spot forming cells per million peripheral blood mononuclear cells.

Figure 2A:

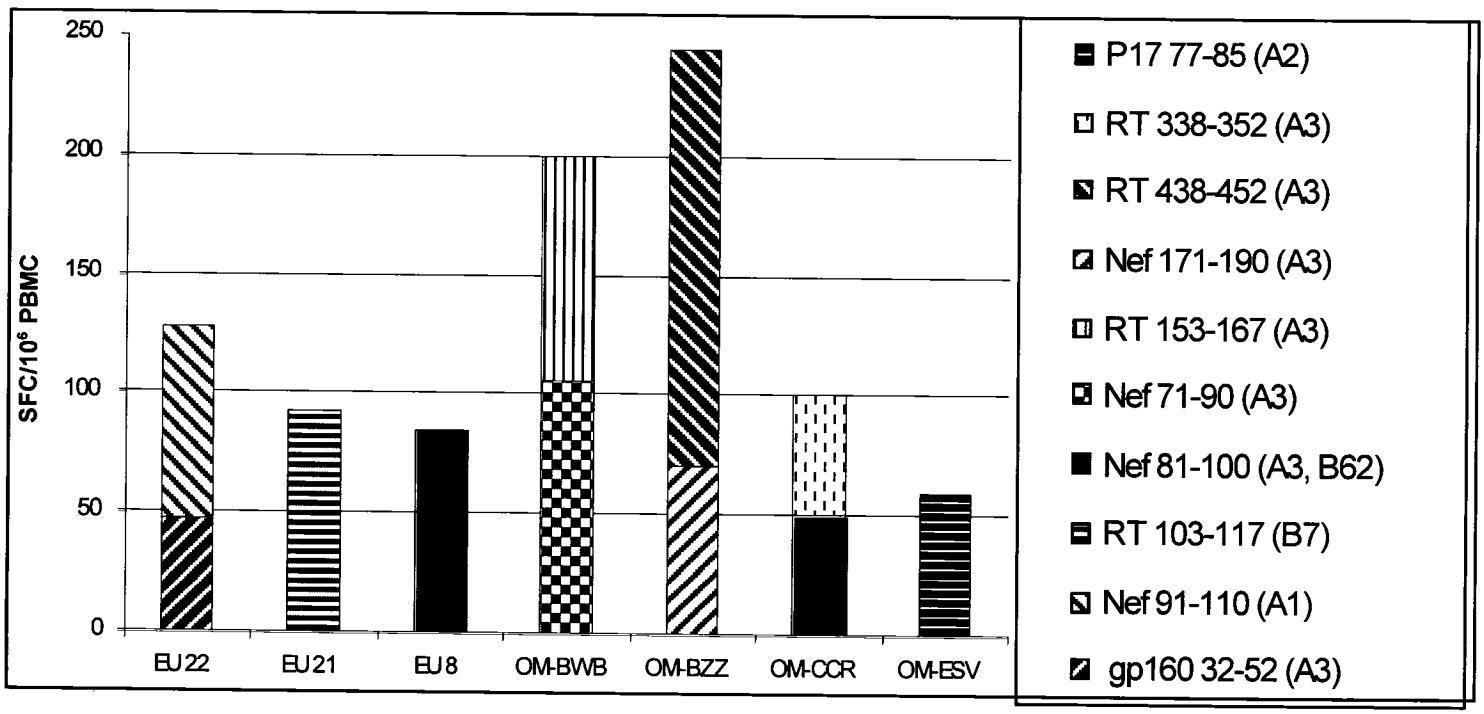
250
200
150
100
50
0
SFC/10⁶ PBMC
EU22
EU21
EU8
OM-BWB
OM-BZZ
OM-CCR
OM-ESV
P17 77-85 (A2)
RT 338-352 (A3)
RT 438-452 (A3)
Nef 171-190 (A3)
RT 153-167 (A3)
Nef 71-90 (A3)
Nef 81-100 (A3, B62)
RT 103-117 (B7)
Nef 91-110 (A1)
gp160 32-52 (A3)

Figure 2B:

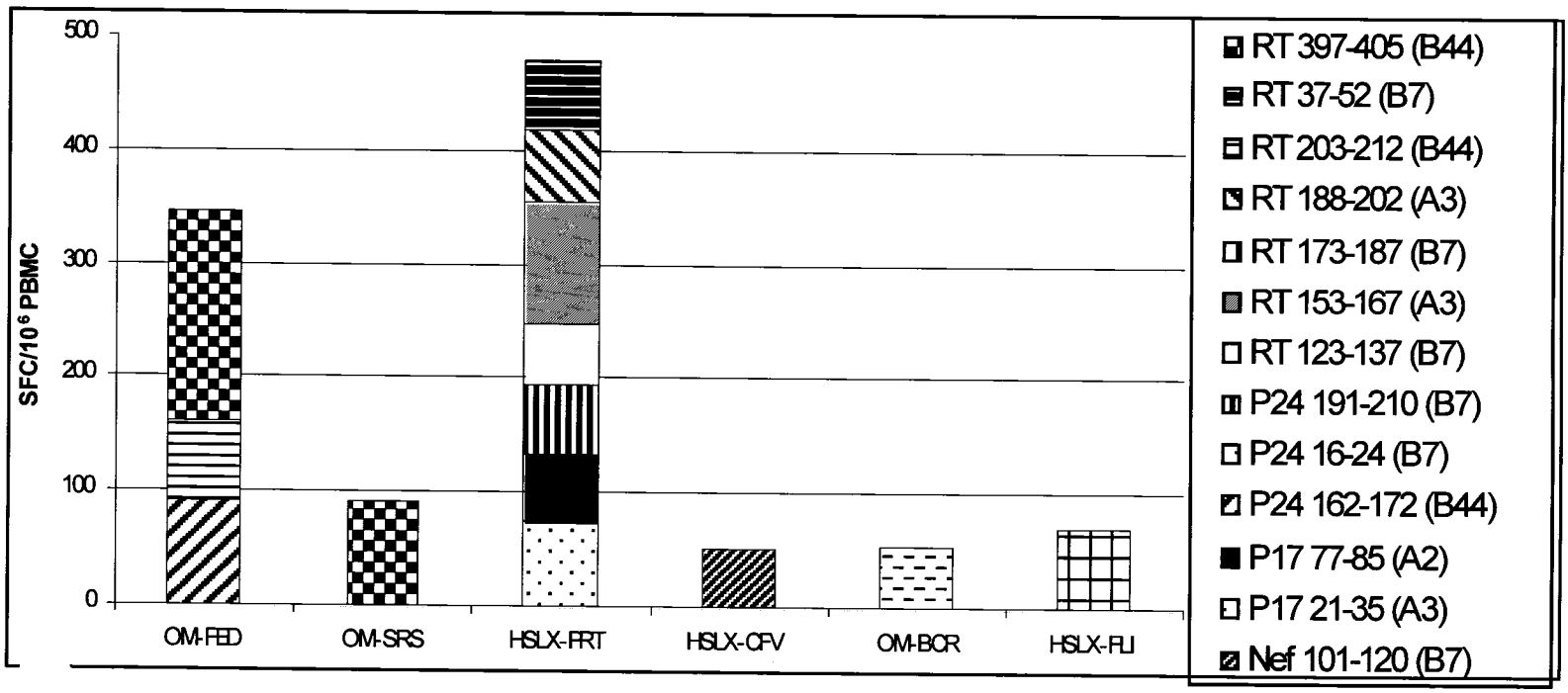
SFC/10^6 PBMC
500
400
300
200
100
0
OM-FED
OM-SRS
HSLX-FRT
HSLX-CFV
OM-BCR
HSLX-FLJ
RT 397-405 (B44)
RT 37-52 (B7)
RT 203-212 (B44)
RT 188-202 (A3)
RT 173-187 (B7)
RT 153-167 (A3)
RT 123-137 (B7)
P24 191-210 (B7)
P24 16-24 (B7)
P24 162-172 (B44)
P17 77-85 (A2)
P17 21-35 (A3)
Nef 101-120 (B7)

Figure 2C:

Peptides Recognized by Group 1 EU

Gene Location	Sequence
gp160 aa 32-52	EKLWVTVYYGVPVWKEATTT
Gag p17 aa 77-85	SLYNTVATL
RT aa 103-117	KKSVTVLDVGDAYGS
RT aa 153-167	WKGSPAIFQSSMTKI
RT aa 338-352	TYQIYQEPFKNLKTG
RT aa 438-452	ETFYVDGAANRETKL
Nef aa 71-90	PQVPLRPMTYKAAVDLSHFL
Nef aa 81-100	KAAVDLSHFLKEKGGLEGLI
Nef aa 91-110	KEKGGLEGLIHSQRRQDILD
Nef aa 171-190	HGMDDPEREVLEWRFDSRLA

Peptides Recognized by Group 2 EU

Gene Location	Sequence	HLA Supertype
Gag p17 aa 77-85	SLYNTVATL	A2
Gag p17 aa 21-35	LRPGGKKKYK LKHIV	A3
Gag p24 aa 16-24	SPRTLNAWV	B7

Gag p24 aa 162-172	RDYVDRFYKTL	B44
Gag p24 aa 191-210	VQNANPDCKTILKALGPAAT	B7
RT aa 37-52	ICTEMEKEGKISKIGP	B7
RT aa 123-137	DFRKYTAFTIPSINN	B7
RT aa 153-167	WKGSPAIFQSSMTKI	A3
RT aa 173-187	KQNPDIVIYQYMDDL	B7
RT aa 188-202	YVGSDLEIGQHRTKI	A3
RT aa 203-212	EELRQHLLRW	B44
RT aa 397-405	TWETWWTEY	B44
Nef aa 101-120	IHSQRRQDILDLWIYHTQGY	B7

Table 1: Population Characteristics

Group 1- Mucosally Exposed Individuals

Subject	Sex	Age	HLA Supertypes	Months of Exposure Prior to Sample Date
OM-AGZ	M	40	A2, A24, B7, B62	6
OM-BWB	M	42	A2, A3, B62	6
OM-BZZ	M	27	A2, A3, B44	29
OM-CCR	M	33	A1, A3, B7, B44	10
OM-CFN	M	23	A2, B7, B44	20
OM-DDZ	M	51	A2, A3, B7, B44	21
OM-MPS	M	37	A1, A2, B27, B44	38
OM-RWS	M	40	A3, A26, B7, B44	29
OM-WCR	M	47	A2, B39, B62	6
OM-ESV	M	24	A2, A3, B27	6
OM-NCB	M	32	A3, B8 (outlier), B44	6
OM-SHZ	M	44	A24, A29 (outlier), B7, B44	6
EU 8	F	54	A2, B27, B62	23
EU 11	M	38	A3, B60	43
EU 14	M	55	A1, A2, B8 (outlier), B62	192
EU 21	M	50	A3, B7	150
EU 22	M	27	A1, A3, B44, B58	120
EU 23	F	37	A3, A24, B7	24

EU 24	F	41	A2, A3, B7, B62	10
EU 25	M	55	A3, A26, B7	72

Group II- Parenterally Exposed Individuals

Subject	Sex	Age	HLA Supertypes	Months of Exposure Prior to Sample Date
HSLM-ABD	M	54	A24, A26, B7, B62	13
HSLM-HCE	M	42	A3, B27	6
HSLM-ITF	M	45	A3, B7, B27	6
HSLM-JEF	M	36	A24, A26, B7	6
HSLM-NMP	M	49	A1, A3, BB44	18
HSLM-NVE	M	43	A3, B27, B62	60
HSLX-ADX	M	38	A1, A26, B8 (outlier)	6
HSLX-BWC	M	37	A3, B7, B27	36
HSLX-CFV	M	30	A29 (outlier), B7	12
HSLX-EUPT	M	30	A1, B7, B8 (outlier)	6
HSLX-GBX	F	26	A2, B44, B62	6
HSLX-MIWE	M	49	A2, A24, B44	24
HSLX-RGW	M	34	A3, B7, B44	6
HSLX-RCK	M	39	A3, B27	6
HSLM-BGW	M	43	A1, A3, B7, B8 (outlier)	60

OM-BCR	M	38	A3, B7	14
HSLX-PRT	M	36	A2, A3, B7	6
HSLX-IBL	M	39	A2, B7	36
HSLX-JVK	M	27	A1, B7, B8 (outlier)	42
HSLX-FLI	M	38	A3, B7, B62	12
OM-FED	M	48	A2, B39, B44	10
OM-PFX	M	26	A3, B7, B44	6
OM-SRS	M	35	A24, B7, B44	22
HSLX-DKFZ	M	33	A1, A3, B7, B8 (outlier)	6
HSLM-JZC	M	44	A1, A3, B7, B44	6
HSLX-DTZM	M	38	A1, B7, B44	6
HSLX-DTL	M	52	A3, A24, B44	12

Table 2: Summary of HIV-Specific T Cell Responses in Individuals Exposed to HIV Mucosally or Parenterally

	Group I- Mucosal Exposure (n=20)	Group II- Parenteral Exposure (n=27)	p-value
Mean number of peptides tested per panel ± SD	11.5 ± 3.89	9.96 ± 3.04	0.14[a]
Subjects with anti-HIV specific effector activity, n (%)	7 (35.0%)	6 (22.2%)	0.51[b]
Mean Magnitude* of positive responses ± SD	130.0 ± 67.5 SFC	182.9 ± 184.2 SFC	0.49[a]
Mean Magnitude* per stimulatory HIV peptide ± SD	82.7 ± 36.5 SFC	78.4 ± 34.6 SFC	0.76[a]

[a] Unpaired t-test

[b] Fisher's Exact test

SFC: Spot forming cells/million peripheral blood mononuclear cells

Table 3: Breadth of HIV-Specific ELISPOT Responses

HIV Peptides	Group 1 (Mucosal EU)	Group 2 (Parenteral EU)	P-Value (Fisher's Exact test)
Env	1/15[a]	0/16	0.4839
Gag	1/88	5/106	0.2237
RT	4/77	8/93	0.5500
Nef	5/50	1/54	0.1029
Total	11/230	14/269	1.000

[a] Peptides that stimulated a positive response/ Peptides tested

Chapter 3:

The Functional Breadth of the HIV-Specific T Cell Response in EU Subjects

Rationale:

In order to characterize the breadth of the HIV-specific T cell response among EUs, T cell functions other than IFN-γ secretion were studied. The salient properties of memory T cells include immediate IL-2 production and rapid proliferation upon antigenic restimulation[275]. Since EUs do not have any persisting virus in their system, any HIV-specific T cell response would be, by definition, a memory response. It follows that if EUs truly possess memory T cell responses directed against HIV, then it should be possible to detect HIV-specific IL-2 release and T cell proliferation.

Interleukin-2 secretion as a marker of T cell function

IL-2 is a protein growth factor that is secreted by T cells to promote the proliferation and differentiation of antigen-specific T cells that will be able to respond to pathogenic infections swiftly and efficiently. Antigenic stimulation of the TCR triggers the transcription factor NF-AT to bind to the promoter of the IL-2 gene, resulting in enhanced transcription of the gene[401]. Costimulatory signals arising from the interaction between B7 on the APC and CD28 on the T cell activate the transcription factors AP-1 and NF-κB[402]. These proteins stabilize the IL-2 mRNA, thereby increasing the yield of IL-2 100-fold[403].

TCR stimulation, as well as costimulation, also induce the expression of CD25, the α chain of the IL-2 receptor, on the cell surface. CD25 associates with the constitutively expressed β and γ chains of the IL-2 receptor to form a high-affinity receptor[404]. The γ chain of the IL-2 receptor is a subunit that is shared by

many cytokine receptors (the common "γc" subunit). The binding of IL-2 to its receptor triggers a cascade of signalling events mediated by Jak-3[405], which has been shown to be associated not only with the common γc but also with the TCR/CD3 complex[406]. IL-2- induced activation of Jak 3 leads to the phosphorylation of Stat 3[407] and Stat 5a/b[408,409], which allows T cells to progress through the cell replicative cycle[410]. A single T cell can give rise to thousands of daughter cells that all bear TCR's of identical specificity. IL-2 signalling also directs the differentiation of these cells into effector cells.

IL-2 is a central factor for lymphocyte proliferation and survival. The selective impairment of IL-2 secretion following TCR stimulation is a critical determinant of immune dysfunction during HIV infection, resulting in the inability of HIV-specific memory T cells to mediate protective immunity upon restimulation[291,411,411]. Since EUs preserve their CD4+ T cell compartment, HIV-specific IL-2 secretion should serve as a reliable marker of functional HIV-specific memory T cells.

The CFSE Proliferation assay

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) is a membrane permeable dye that becomes highly fluorescent and impermeable to the cell membrane once endogenous esterases in the cytoplasm remove the two acetate groups. The succinimidyl ester reacts with a free amine group on an intracellular protein, for example a component of the cytoskeleton, to form a long-lived dye-protein adduct. During cellular proliferation, CFSE is evenly distributed between

daughter cells. As a result, an analysis of the serial halving of the fluorescence intensity of the dye by flow cytometry allows a quantitative measurement of lymphocyte proliferation. Up to ten discrete cycles of cell division can be detected with this assay. [412]

A major advantage of this technique is that appropriately conjugated fluorescent probes can be simultaneously used to visualize cell surface markers and/or internal cytokines. As a result, CFSE can track the proliferation of minor subsets within a heterogeneous population of T cells, as well as monitor the kinetics of expression of differentiation markers and cytokines linked to cell division. Other commonly used assays of cellular proliferation offer only qualitative information, since they usually measure division at a population level. By using the CFSE dilution assay, low-level antigen-specific T cell responses that may have gone undetected by conventional methods will be amplified and readily observed.

Hypothesis:

EUs develop functional memory T cells that can mediate protective immunity against HIV. If these responses are associated with protection from HIV infection, it is reasonable to expect a greater frequency of HIV-specific memory T cell functions in EUs who remain seronegative than in EUs who seroconvert at a subsequent time point.

Objectives:

1) To detect HIV-specific T cell proliferation in EUs.

2) To identify the phenotype of the responding T cell populations.

3) To compare the frequency, magnitude, and breadth of the T cell proliferative responses between EUs who remain seronegative, EUs who seroconvert, and low-risk negative controls.

4) To detect HIV-specific IL-2 secretion in EUs.

5) To correlate HIV-specific T cell proliferation with IL-2 secretion.

HIV-specific T cell proliferation in exposed, uninfected individuals is associated with protection from HIV infection

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Running head: EU proliferation to HIV peptide pools

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Word Count: 3890

ABSTRACT

Objective(s): To determine whether HIV exposed uninfected subjects (EUs) possess HIV-specific immune responses characteristic of memory T cells.

Design: A cross sectional study of HIV-specific proliferation was performed on peripheral blood mononuclear cell samples from EUs versus EUs who seroconverted a median of 6 months (range 1.5- 28.5) after screening in order to assess whether the presence of such responses was associated with persistent seronegativity.

Methods: EUs were screened for HIV-specific T cell proliferation by the CFSE dilution assay. HIV peptide pools corresponding to all expressed viral gene products were used as stimuli. A subset of EUs having proliferative responses was assayed for HIV-specific IL-2 secretion by ELISPOT.

Results: Persistently seronegative EUs were more likely to proliferate to at least one HIV stimulus than low-risk controls (8/17 EUs versus 0/20 controls; $p<0.001$ Fisher's Exact test). All EU responders had HIV-specific $CD4^+$ T cells and 6 also had HIV-specific $CD8^+$ T cells with proliferative capacity. A statistically significant correlation was observed between HIV-specific T cell proliferation and IL-2 production detected by ELISPOT [Spearman $r = 0.4325$; $p = 0.03$]. None of 7 EU subjects who later seroconverted (EU-SC) had detectable HIV-specific proliferative responses (EU versus EU-SC, $p=0.034$, Fisher's test).

Conclusion: EUs possess HIV-specific memory T cells able to expand upon antigen restimulation, and this function is associated with a reduced risk of HIV infection.

Key Words: cellular immunity, CD4, CD8, IL-2 secretion, T cell proliferation, drug users, homosexual men

INTRODUCTION

Several groups of persistently seronegative individuals repeatedly exposed to HIV have been studied with the goal of identifying immune responses that play a role in protecting them from HIV infection [1-14]. The identification of HIV-specific $CD8^+$ T cell responses in resistant Kenyan sex workers provided support for the concept that candidate HIV vaccines should be designed to induce this activity [15-18]. Recent studies in other cohorts of HIV exposed, uninfected subjects (EUs) have shown that HIV-specific $CD4^+$ T cells are also present in EU individuals and may play a role in preventing the establishment of a productive HIV infection [19-23].

HIV-specific immune responses detected in HIV exposed individuals with no evidence of persistent infection are likely memory responses. While much of the recent research on EUs has focused on measuring HIV-specific interferon (IFN)-γ secretion as a correlate of HIV-specific T cell activity, memory T cells also characteristically secrete interleukin (IL)-2 and tumor necrosis factor-α (TNF-α) [24,25]. A hallmark of a memory T cell response is rapid proliferation and expansion upon re-encounter with antigen [24]. Several studies have demonstrated HIV-specific IL-2 secretion by EUs [7,9,22,26-29]. In contrast, the presence of low level HIV-specific T cell proliferation in EUs has been inconsistently demonstrated [7,9,26,30,31]. In two studies, the presence of IL-2 secretion was observed in the absence of HIV-specific T cell proliferation [32]. Thus, no study of EUs to date has established a link between these two memory T cell functions.

Proliferation detected by ^{3}H thymidine incorporation in bulk antigen stimulated T cell cultures is highly variable and the downstream result of several processes. Newer methods to evaluate proliferation such as the flow cytometry based 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay are more sensitive than ^{3}H thymidine incorporation, and when combined with cell surface staining permit the identification of the proliferating cell subset [32]

In this report we show that a significantly higher proportion of EUs than low-risk controls have HIV-specific T cell proliferative activity detected by the CFSE dilution assay. We also establish a correlation between HIV-specific T cell proliferation and HIV-specific IL-2 secretion. EUs having T cells able to expand in response to HIV stimuli are less likely to seroconvert than similarly exposed EUs in whom these responses are not detectable.

METHODS

Study Population

Individuals at highest risk for HIV infection were identified from the Omega Cohort of men having sex with men (MSM) [33], and the Saint Luc Cohort of injection drug users (IDU) for inclusion as EUs [34,35]. High-risk exposure was defined as having used syringes already used by a self reported HIV-positive IDU, or having had unprotected sex with a self-reported HIV-positive sex partner. All but 1 EUs included in this cross sectional study were seen at 3 to 6 month intervals for a minimum of 12 months in order to monitor their HIV serostatus and to document their exposure history. Subject MGBR (an IDU) was followed for 8 months. At the time point selected for immune response screening in this study, all EU participants reported at least one exposure to HIV in the preceding year, and were seronegative for HIV antibodies using a standard HIV-1 antibody enzyme immunoassay (HIV-1 EIA). HIV incidence was 3 per 100 person-years in the St Luc cohort overall and 0.56 per 100 person-years in the Omega cohort [33,34] Risk of HIV acquisition was 4.5 times higher for EUs having parenteral exposure as defined by the criteria for inclusion in the highest exposure IDU subgroup [34]. Only 25 MSM subjects from the Omega cohort were classified as EU-MSM according to the criteria for inclusion in the highest exposure MSM subpopulation and provided serial blood samples. None of these EU-MSM seroconverted during the observation period. Therefore, increased risk of seroconversion in this subgroup compared to the Omega cohort overall could not be evaluated.

Seven individuals from the Saint Luc cohort who had subsequently seroconverted were included in this study as a comparison group (EU-SC). These individuals were screened for HIV-specific T cell proliferation a median of 6 months (range 1.5- 28.5) before seroconversion. The possibility that some of the study subjects were in the acute or early phase of infection prior to seroconversion was ruled out using a nested PCR designed to detect proviral HIV-1 Nef, Vif, and Pol sequences [36].

Twenty subjects at low-risk for HIV infection were also assayed for HIV-specific responses as controls.

Sample Processing

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected in ethylenediamine tetra-acetic acid (EDTA) anticoagulant by density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsala, Sweden) and frozen in 90% fetal calf serum (GIBCO BRL Life Technologies, Burlington, Ontario) containing 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis MO). PBMC from a single time point from 2 persistently seronegative EUs (MGBR and XAKP) were collected by apheresis and processed for freezing in a similar fashion.

HIV Peptide Stimuli

Sets of synthetic peptides corresponding to all expressed HIV genes were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD). Two sets of peptides were used to screen EUs and low risk controls. All were 15 amino acids (aa) in length (15-mers) with 11 aa overlaps, except for the Pol peptide set used to screen the first batch of samples, which was composed of 20-mers with 10 aa overlaps. Ten EUs, 7 EU-SC and 8 low risk controls were screened for proliferative responses using peptide pool set 1. The first peptide set included 122 peptides corresponding to HIV-1 Gag HXB2 separated into two superpools, G1.1 and G1.2, each containing 61 peptides. The peptide set spanning HIV-1 Pol HXB2 was divided into two superpools of 50 peptides, P1.1 and P1.2. A single pool, N, was made with the 49 peptides corresponding to HIV-1 Nef Consensus B. Two superpools, A1.1 and A1.2, were designed for the consensus sequence of the

accessory proteins of HIV-1; A1.1 included the synthetic peptide sets spanning Tat (23 peptides), Vpu (19 peptides), and Rev (27 peptides); A1.2 included Vif (46 peptides) and Vpr (22 peptides). Two hundred and twelve (212) peptides spanning the entire sequence of HIV-1 Env MN were split into pools E1.1, E1.2, and E1.3. Because these Env pools induced high levels of background proliferation in both the low risk controls and test subjects, responses to these pools were not considered in the analyses described in this report.

Ten EUs and 14 low risk controls were screened for proliferative responses using peptide pool set 2 based entirely on consensus clade B sequences. The second set included 123 Gag peptides split onto 3 pools of 41 peptides each (G2.1, G2.2 and G2.3), 249 15-mer Pol peptides split into 5 pools or 49 to 50 peptide each (P2.1, P2.2, P2.2, P2.4 and P2.5), 1 Nef pool of 49 peptides identical to the pool in peptide set 1, 3 accessory gene product pools in which the A2.1 pool contained the 23 Tat and 19 Vpu peptides the A2.2 pool contained 27 Rev and 22 Vpr peptides and the A2.3 pools contained 49 Vif peptides. The second set of Env pools contained 211 peptides separated into 5 pools of 42 or 43 peptides (E2.1, E2.2, E2.3, E2.4 and E2.5). Peptide superpools were diluted in RPMI 1640 medium (Sigma, Burlington, Ontario, Canada).

CFSE Dilution Assay

Thawed PBMC were resuspended in complete RPMI medium (cRPMI) containing 2 mM L-glutamine (ICN Biomedical, Canada), 50 IU/ml penicillin (ICN Biomedical, Canada), 50 mg/ml streptomycin (ICN Biomedical Canada), and 50

μM 2-mercaptoethanol (Sigma-Aldrich) supplemented with 2% human AB serum (Wisent Inc, St-Bruno, Quebec, Canada), and stained with 5 μM CFSE (Molecular Probes, Eugene, Oregon, USA) in the dark for 8 minutes according to the manufacturer's directions. CFSE labelled PBMC were stimulated with HIV superpools in cRPMI supplemented with 10% human AB serum so that the final concentration of each peptide in the mixture was 2 μg/ml. Stimulation with cRPMI with 10% human AB serum served as a negative control, whereas stimulation with 2 μg/ml of Staphylococcus Enterotoxin B (SEB) (Sigma) was used as a positive control for all experiments. Following a six-day incubation period at 37°C, 5% CO_2, cells were stained for surface markers using anti-CD3- peridinin chlorophyll protein (PerCP), anti-CD8-allophycocyanin (APC), and anti-CD4-phycoerythrin (PE) monoclonal antibodies (mAb) (Becton Dickinson [BD] Biosciences, Mississauga, Ontario, Canada). Data acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences). Between 50,000 and 100,000 lymphocyte gated events were acquired per sample and analyzed by Cell Quest software. Additional analysis was performed on FlowJo 6.0 (TreeStar, San Carlos, CA, USA).

Data from the experiments conducted on the 8 low-risk seronegative controls tested with peptide pool set 1 and 14 tested with peptide pool set 2 were used to establish the criteria for a positive response (2 low risk control subjects were screened with both peptide sets). Positive responses were defined as those greater than the mean plus 3 standard deviations of the percent of $CFSE^{lo}$ cells generated following stimulation of PBMC from low risk controls with the same

peptide superpool used to stimulate samples from EU subjects and responses greater than 3-fold over the autologous (no stimulation) background control.

The Interleukin-2 ELISPOT Assay

Frozen PBMC samples were thawed and resuspended in cRPMI supplemented with 10% fetal bovine serum (FBS, Wisent Inc, St-Bruno, Quebec, Canada). Cells were plated in duplicate at 2 x 10^5 cells per well into 96-well polyvinylidene difluoride-backed plate(s) (MAIPS 45; Millipore, Bedford MA) pre-coated with 3 μg/ml of human IL-2 capture mAb (BD Biosciences). HIV superpools were added to the test wells so that the final concentration of each peptide in the pool was 2 μg/ml. Positive and negative control stimuli for these experiments were anti-CD3 mAb (Research Diagnostics Inc, Flanders N.J.) and R10 medium, respectively. After an incubation of 18 to 20 hr, PBMC were washed away and IL-2 secreting cells were detected as spots by the sequential addition of human IL-2 detection mAb (BD Biosciences), diluted 1:1000 in PBS, streptavidin-alkaline phosphatase (Mabtech, Stockholm, Sweden), diluted 1:2000 in PBS, and p-nitro blue tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine substrate (Bio-Rad Laboratories, Richmond, CA). The resulting spots were counted under a stereomicroscope (Carl Zeiss, London, Ontario, Canada). Results were expressed as spot forming cells (SFC) per 10^6 PBMC.

The cut off for a positive response was determined as described for the CFSE dilution analysis, using 13 low risk controls. A response was deemed

positive if, after subtraction of the background, the magnitude was greater than 43 SFC/10^6 PBMCs and at least 2 times the average of the autologous negative control wells.

Statistical Analyses

All statistical analyses were performed on GraphPad InStat Version 3.0 software, San Diego, California, USA. The Fisher's exact test was used to evaluate proportional differences between persistently seronegative EUs, EU subjects that subsequently seroconverted (EU-SC), and low-risk negative controls. A Spearman nonparametric correlation analysis was performed to determine the relationship between IL-2 secretion and proliferation. All p-values are two-tailed with an alpha-error of 0.05.

RESULTS

Association between HIV-specific T cell proliferation and exposure to HIV

Table 1 shows the characteristics of the EU study population screened for HIV-specific responses by either HIV peptide pool set 1 or 2 or both peptide sets. Twenty four separate EU subjects (17 EU and 7 EU-SC) and 20 low risk controls included in this study were screened; 10 EUs, 7 EU-SC and 8 low risk controls were tested using peptide pool set 1. Of these all were male, and had a median age of 42 yrs (range 27-51). Six of these EUs were IDU, while 4 were MSM. The seven EU-SC study subjects tested with the same peptide set seroconverted a median of 6 months (range 1.5- 28.5) after the sampling date. The EU-SC were all male IDU, aged a median of 42 years (range 39-47). In addition to IDU exposure to HIV, the EU-SC with the identification code MEEG also reported unprotected sex with an HIV infected partner. There was no statistically significant difference in the duration of consistent exposure prior to sampling between the EU and EU-SC groups (p=0.67, t-test).

We addressed the reproducibility of the CFSE dilution assay by testing cells from the same time point from 2 EU subjects on 3 occasions. PBMC from subject XAKP proliferated on all three occasions to HIV pool P2.3 with an average ± standard deviation of 1.3 ± 0.2 CD4+CD3+ $CFSE^{lo}$ cells, to pool E2.4 with 1.9 ± 0.6 CD4+CD3+ $CFSE^{lo}$ cells and to no stimulus with 0.3 ± 0.2 CD4+CD3+ $CFSE^{lo}$ cells. Subject MGBR proliferated on 3 occasions to the P2.3 peptide pool with 1.3 ± 0.3 and to no stimulus with 0.2 ± 0.2 CD4+CD3+ $CFSE^{lo}$

cells. The average inter-assay coefficient of variation for positive response stimuli was 23.4%.

To address whether HIV-specific proliferation could also be detected in other EUs using peptide sets based on consensus clade B sequence we screened additional subjects. Ten persistently seronegative EUs (9 IDU and 1 MSM) with a median age of 44 yrs (range 33 to 50) and 14 low risk controls were screened using peptide pool set 2, which included 5 pools of Env peptides. Two different time points from EU subjects XAKP, OM-BCR and MCGX separated by 29 mos, 7 mos and 32 mos, respectively, were screened, one time point with peptide pools et 1 and the other with peptide pool set 2. The same time point from 2 low risk controls were tested with both stimulatory peptide platforms.

A summary of the CFSE dilution assay results is presented in Table 2. Five of the 10 (50%) persistently seronegative EUs screened with peptide set 1 proliferated to an HIV stimulus (Table 2) versus none the 8 low-risk control individuals (p=0.036, Fisher's exact test). One peptide superpool stimulated proliferation of $CD4^+$ T cells in each of the 5 responders. The N peptide pool stimulated 3 of the 5 $CD4^+$ T cell responses; P1.1 and P1.2 superpools each stimulated $CD4^+$ T cells to proliferate in one subject. The same peptide pool induced responses in 3 of the 4 EU responders with proliferative potential in their $CD3^+CD8^+$ compartment. Figure 1 illustrates representative results from a CFSE dilution experiment. OM-BWB (Fig 1 A) and HSLM-CGX (Fig 1 B) exhibited N pool-specific and P1.1-specific proliferation, respectively, in both their CD4+ and CD8+ T cell compartments.

In a set of experiments using HIV peptide pools set 2 as stimuli, we again observed that EUs were more likely that low risk controls to proliferate to HIV peptides (4 of 10 (40%) versus none of 14 controls exhibited HIV-specific proliferation; p=0.02, Fisher's Exact test). All 4 EU responders displayed above background proliferation in the CD4+CD3+ compartment. Responses observed were to A2.3 in subject XHXE, P2.1 in subject XRHG, to P2.3 and E2.4 in subject XAKP and to P2.3 in subject MGBR. CD8+CD3+ cells also proliferated same peptide pool that induced proliferation of CD4+ cells in XRHG and XHXE (Table 2).

If results for EUs and low risk controls screened with either peptide platform are pooled, 8 of 17 EUs versus none of 20 controls proliferated to HIV stimuli ($p<0.001$, Fisher Exact test).

HIV-specific proliferation is associated with a lower incidence of HIV infection

Because we had access to a pre-seroconversion time point PBMC sample on 7 individuals who eventually became infected, we were able to assess whether the presence of HIV-specific proliferative responses was associated with remaining seronegative. For these analyses we only considered the 17 EU individuals who were screened using the peptide set 1. A significantly greater proportion of persistently seronegative EU than EU-SC exhibited proliferation in response to stimulation with the same panel of HIV peptide superpools. (Table 2) (5/10 EU vs. 0/7 EU-SC; p=0.04 Fisher's exact test).

HIV-specific T cell proliferation is correlated with HIV-specific Interleukin-2 secret

XAKP was tested for HIV-specific IL-2 secretion and T cell proliferation on the same time point. Peptide pool P1.2 stimulated responses in both assays. (Table 2 and Figure 2). Cells from 2 EUs with exposure between time points (XAKP [29 months apart] and OM-MPS [6 months apart]) were tested for HIV-specific proliferative responses and exhibited responses to the same gene product on both occasions. We therefore tested additional EUs for both HIV-specific IL-2 secretion and T cell proliferation using PBMC from time points a median of 6 months (range 0 – 13.5 months) apart. Three of the 5 (60%) EUs exhibited HIV-specific IL-2 secretion to the same stimulus that induced CFSE proliferation, with magnitudes of 57.5, 57.5, and 87.5 SFC/10^6 PBMC after subtraction of background (not shown). Figure 2 illustrates the relationship between the CFSE proliferation and the IL-2 ELISPOT data. A statistically significant correlation was observed between HIV-specific T cell proliferation and IL-2 secretion in EU individuals (Spearman r = 0.4325, p = 0.03).

DISCUSSION:

Our results show that persistently seronegative EUs are more likely than low risk controls to exhibit HIV-specific proliferative responses (8 of 17 [47%] EUs versus none of 20 controls, 0<0.001, Fisher's Exact test). Proliferation occurred in the CD4+ T cell compartment in all 8 responders and in the $CD8^+$ T cell compartment as well in 6 responders. In contrast, no HIV-specific proliferative response was observed in seven similarly exposed EU individuals who seroconverted a median of 6 (range 1.5 to 28.5) months after the time point tested [EU 5/10 vs. EU-SC 0/7; p=0.04 Fisher's Exact test].

Three of the 5 subjects able to proliferate in response to HIV peptides also secreted IL-2 to these stimuli. The fact that not all of the stimulatory superpools in the CFSE dilution assay induced a positive response in the IL-2 ELISPOT assay suggests that the latter technique may underestimate the presence of HIV reactivity. The likely explanation for this is that proliferation of HIV-specific cells amplifies low frequency responses. Alternatively, the specificity of the T cell response against HIV stimuli may have changed during the interval between the time points used for the screening of HIV-specific proliferation and IL-2 secretion.

All of the EUs recruited into this study reported HIV exposure, either by sharing syringes with a self-reported HIV-infected individual or by engaging in unprotected sex with an HIV-infected partner. These modes of exposure represent behaviour at the highest risk for HIV acquisition [37]. The exact frequency of exposure to HIV was available for some, but not all of the study subjects. For this reason, an analysis relating the observed T cell responses to

the frequency of exposure was not possible. In our earlier work, however, there was no correlation between the frequency of exposure via needle-sharing and the frequency of HIV-specific T cell responses in our cohort of EU IDU [38]. Thus, the frequency of exposure to HIV in this cohort of EUs is unlikely to alter our findings.

In infected individuals, $CD8^+$ T cells are thought to be responsible for controlling HIV replication, but effective virological suppression requires maintenance of the ability of both HIV-specific $CD4^+$ and $CD8^+$ T cells to proliferate [39-42]. IL-2 production from HIV-specific $CD4^+$ T cells has been shown to be an important prerequisite for HIV-specific T cell proliferation to occur [42,43]. Since EU subjects are not infected, these $CD4^+$ T cell responses would be expected to be fully functional in providing help to HIV-specific $CD8^+$ T cells. Indeed, in our population of HIV exposed uninfected persons, we confirm the correlation between HIV-specific T cell proliferation and IL-2 production (Spearman $r = 0.4325$, $p = 0.03$).

A major advantage of the CFSE dilution assay over the commonly used ^{3}H-thymidine incorporation assay is that it affords a quantitative identification of the cell subset responsible for the observed proliferation amidst a heterogeneous population of cells. In combination with cell surface phenotyping, the CFSE technique allows an analysis to be performed at the single-cell level [32]

Based on the observation of HIV-specific immune responses in the absence of infection in EUs, we hypothesize that these individuals encountered HIV in a manner that induced an immune response that cleared the infection.

This situation may be analogous to that seen in murine models of acute lymphocytic choriomeningitis virus (LCMV) infection, where virus is also cleared. Effector T cells that control and clear LCMV differentiate into effector memory T cells, and eventually into resting central memory T cells which are able to secrete IFN-γ, TNF-α and IL-2, to proliferate, and to rapidly develop lytic activity upon re-exposure to antigen [24]. The use of the CFSE dilution assay has permitted the detection of proliferative responses to HIV antigens by both $CD4^+$ and $CD8^+$ T cells, suggesting EUs have developed functional memory that can be triggered upon antigen re-encounter. The fact that HIV-specific IL-2 secretion and proliferation in EU subjects appear to be correlated supports the notion that EU individuals develop protective functional memory responses that are characteristic of central memory T cells. Schenal et al. have recently reported that, compared to low-risk controls and HIV infected subjects, EUs possess a greater proportion of Gag-specific T cells of the CD45RA-/CCR7+ phenotype able to secrete IL-2, a phenotype and function consistent with central memory cells [44].

The preferential detection of HIV-specific T cell proliferation in EUs who remain seronegative compared to those who eventually seroconvert suggests that this response may be an immune correlate of protection against HIV infection and should be targeted by candidate vaccines.

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Figure Legends

Figure 1: HIV-specific T cell proliferation in EUs. PBMC from OM-BWB (A) and MCGX (B), representing mucosal and parenteral exposure to HIV, respectively, were stained with CFSE and then stimulated with a panel of HIV peptide pools. **A)** HIV-specific T cell proliferation ($CFSE^{lo}$) in OM-BWB directed against the Nef pool by both $CD4^+$ T cells (left panel) and $CD8^+$ T cells (right panel). **B)** HIV-specific T cell proliferation ($CFSE^{lo}$) by MCGX stimulated by Pol 1 in both the $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell compartments. For both subjects, negative control (medium) and positive control (SEB) stimulations are shown for comparison. The percentage of T cells that proliferated is indicated in the upper left quadrant of each dot plot.

Figure 2: Correlation between HIV-specific T cell proliferation and IL-2 secretion. Five EU study subjects were assayed for both HIV-specific T cell proliferation and IL-2 secretion. A total of 24-paired responses ($SFC/10^6$ PBMC; x-axis and $\%CD3^+CD4/8^+$ CFSElo; y-axis) were plotted on the graph. A Spearman nonparametric correlation analysis was performed. Indicated p-value is two-tailed, $\alpha=0.05$.

Table 1: Population Characteristics

	Study Subject	Age / Gender	Exposure to HIV[a]	Duration of Exposure (months)[b]	Time to Seroconversion (months)[c]	Peptide pool set
EU	XAKP	42/M	IDU	25		1
	XAKP	44/M	IDU	31		2
	MBMF	42/M	IDU	12		1
	MCGX	47/M	IDU	12		1
	MCGX	50/M	IDU	18		2
	MNMP	50/M	IDU	23		1
	OM-BCR	39/M	IDU	33		1
	OM-BCR	39/M	IDU	39		2
	OM-PFX	27/M	IVDU	19		1
	OM-BWB	51/M	MSM	12		1
	OM-MPS	38/M	MSM	24		1
	OM-NCB	33/M	MSM	12		1
	OM-WCR	48/M	MSM	27		1
	XHXE	33/M	IDU	33		2
	XEHF	52/M	IDU	24		2
	XLDN	47/M	IDU	18		2
	XIBL	43/M	IDU	34		2
	SJST	44/m	IDU	22		2
	XRHG	37/M	IDU	9		2
	HAAS	33/M	IDU	6		2
	MGBR	52/M	IDU	6		2
EU-SC	MHKV	45/M	IDU	12	6	1
	XRHX	39/M	IDU	12	1.5	1
	XMFM	39/M	IDU	42	6	1

	XNVC	39/M	IDU	12	3	1
	MLFW	42/M	IDU	12	6	1
	MEEG	47/M	IDU + MSM	25	5	1
	MITF	45/M	IDU	18	28.5	1

a: IDU= injection drug user who shared a syringe with an HIV seropositive partner(s); MSM= man who had unprotected sex with HIV seropositive man.

b: Time from entry into EU cohort until sample date for EU; until date midway between seroconversion and last negative sample date for EU-SC.

c: Time between sample date and date of seroconversion.

Table 2: Summary of HIV-specific proliferative responses in Exposed uninfected subjects and low risk controls

		% CFSE^{lo} CD4+CD3+ cells		% CFSE^{lo} CD8+CD3+ cells	
		Neg control stimulus	Stimuli inducing positive responses[a]	Neg control stimulus	Stimuli inducing positive responses[a]
	Peptide set 1				
EU	XAKP	0.14	1.35 (P1.2)	0.04	None
	MBMF	0.46	none	0.04	None
	MCGX	0.24	1.28 (P1.1)	0.09	0.51 (P1.1)
	MNMP	0.13	None	0.05	None
	OM-BCR	0.59	None	0.15	None
	OM-PFX	0.55	2.26 (N)	0.02	0.37 (G1.2)
	OM-BWB	0.80	3.28 (N)	0.10	0.39 (N)
	OM-MPS	1.56	6.23 (N)	0.31	1.58 (N)
	OM-NCB	0.47	None	0.21	None
	OM-WCR	0.58	None	0.17	None
EU-SC	MHKV	0.01	None	0.01	None
	XRHX	0.05	None	0	None
	XMFM	0.14	None	0	None
	XNVC	0.09	None	0.02	None
	MLFW	0.67	None	0.05	None
	MEEG	0.01	None	0.03	None
	MITF	0.05	None	0.07	None
Low risk controls N=8		0.41 (0.17 to 0.79)[b]	0.23 (0.04 to 1.23)[c]	0.09 (0.06 to 0.12)	0.03 (0.01 to 0.19)
	Peptide set 2				
EU	XAKP	0.17	1.18 (P2.3), 1.26 (E2.4)	0.04	None
	MCGX	0.06	None	0.02	None

	OM-BCR	0.56	None	0.31	None
	XHXE	0.19	0.98 (A2.3)	0.06	0.43 (A2.3)
	XEHF	0.15	None	0.19	None
	XLDN	0.21	None	0.05	None
	XIBL	0.18	None	0.03	None
	SJST	0.36	None	0.09	None
	XRHG	0.46	14.87 (P2.1)	0.27	5.48 (P2.1)
	MGBR	0.11	1.21 (P2.3)	0	None
Low risk controls N=14		0.26 (0.12 to 0.63)	0.34 (0.02 to 1.23)	0.04 (0.02 to 0.2)	0.05 (0 to 0.36)

a: Criteria defining a positive response: Average response by low risk controls to same peptide stimulus + 3 standard deviations and at least 3-fold over autologous background (stimulation with no peptide).

b median (range) of responses by low risk controls to no peptide stimulus.

e median (range) of responses by low risk controls to stimulatory HIV peptide pools.

Figure 1A:

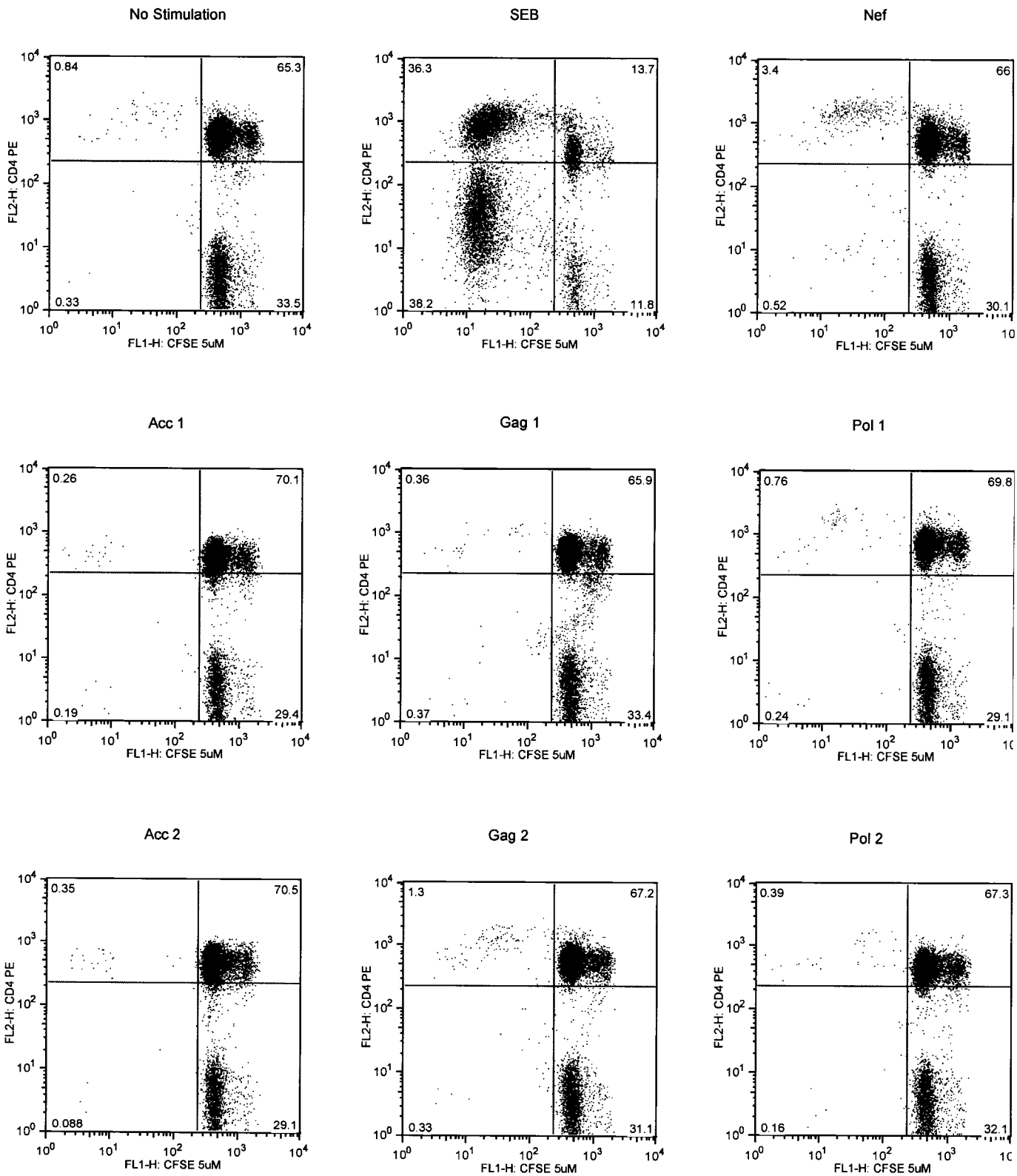

No Stimulation
SEB
Nef
Acc 1
Gag 1
Pol 1
Acc 2
Gag 2
Pol 2
FL2-H: CD4 PE
FL1-H: CFSE 5uM
0.84
65.3
0.33
33.5
36.3
13.7
38.2
11.8
3.4
66
0.52
30.1
0.26
70.1
0.19
29.4
0.36
65.9
0.37
33.4
0.76
69.8
0.24
29.1
0.35
70.5
0.088
29.1
1.3
67.2
0.33
31.1
0.39
67.3
0.16
32.1

Figure 1A:

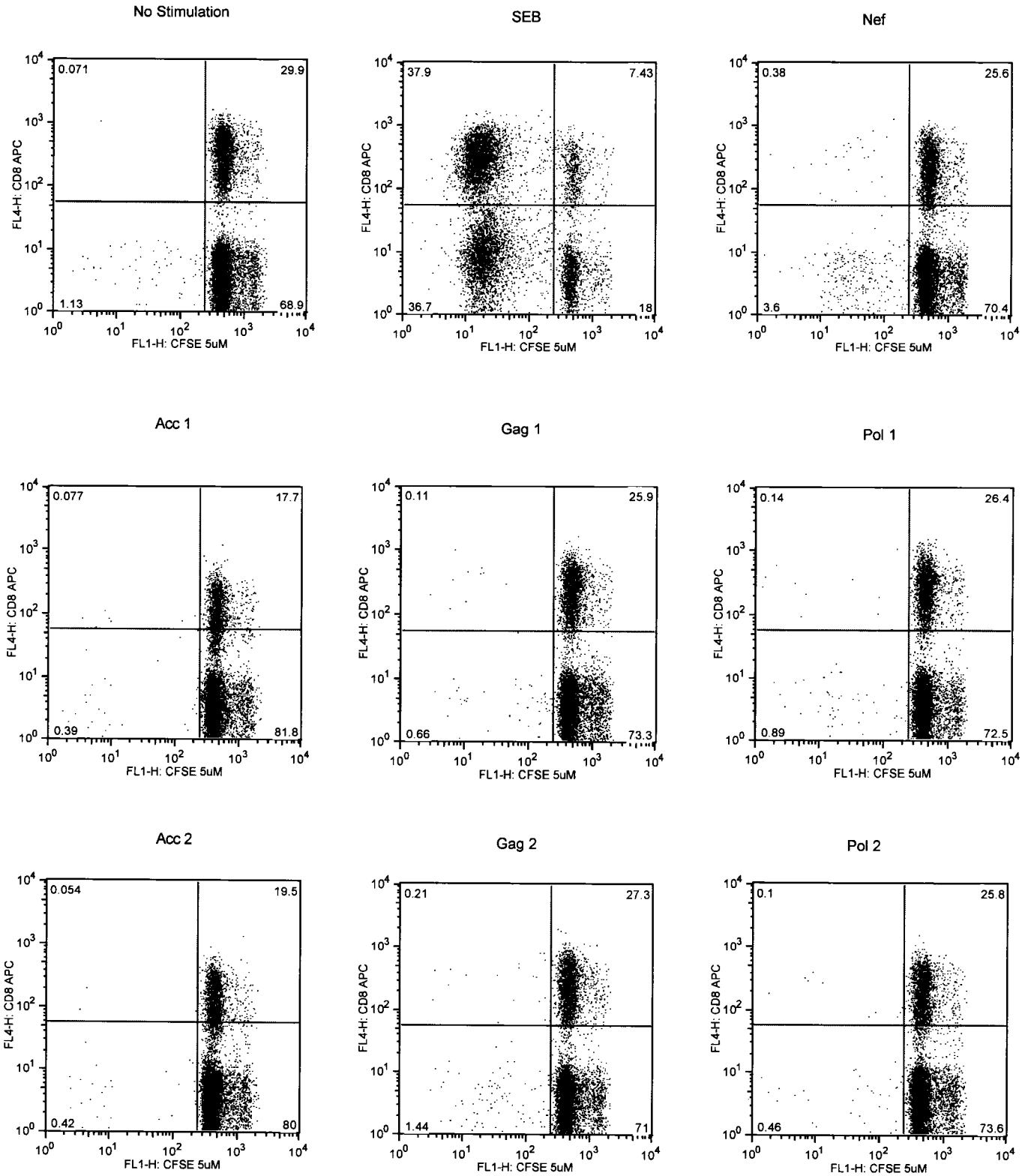
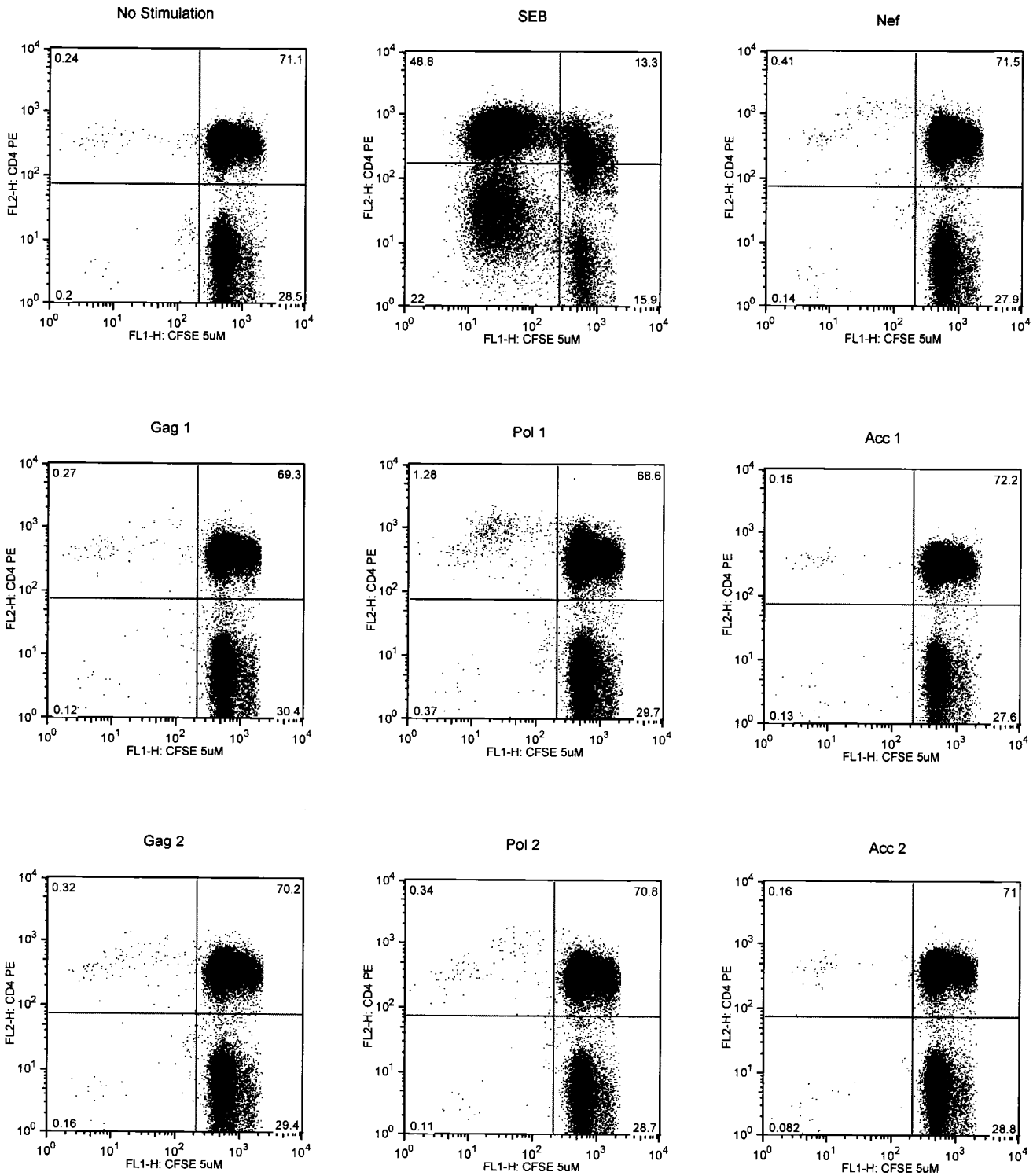

No Stimulation
0.071
29.9
1.13
68.9
SEB
37.9
7.43
36.7
18
Nef
0.38
25.6
3.6
70.4
Acc 1
0.077
17.7
0.39
81.8
Gag 1
0.11
25.9
0.66
73.3
Pol 1
0.14
26.4
0.89
72.5
Acc 2
0.054
19.5
0.42
80
Gag 2
0.21
27.3
1.44
71
Pol 2
0.1
25.8
0.46
73.6
FL4-H: CD8 APC
FL1-H: CFSE 5uM

Figure 1B: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0.24
71.1
0.2
28.5
SEB
48.8
13.3
22
15.9
Nef
0.41
71.5
0.14
27.9
Gag 1
0.27
69.3
0.12
30.4
Pol 1
1.28
68.6
0.37
29.7
Acc 1
0.15
72.2
0.13
27.6
Gag 2
0.32
70.2
0.16
29.4
Pol 2
0.34
70.8
0.11
28.7
Acc 2
0.16
71
0.082
28.8
FL2-H: CD4 PE
FL1-H: CFSE 5uM

Figure 1B:

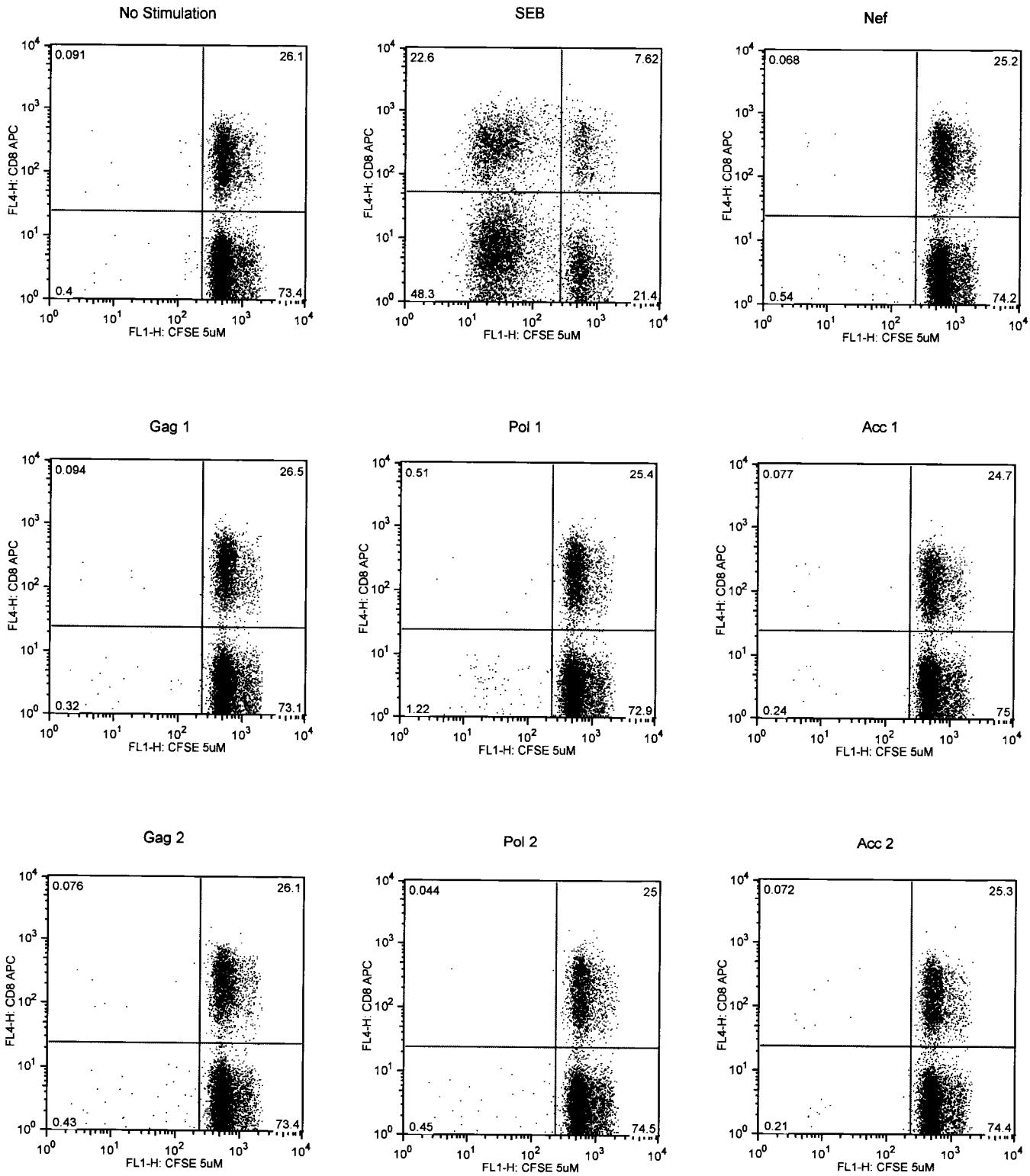
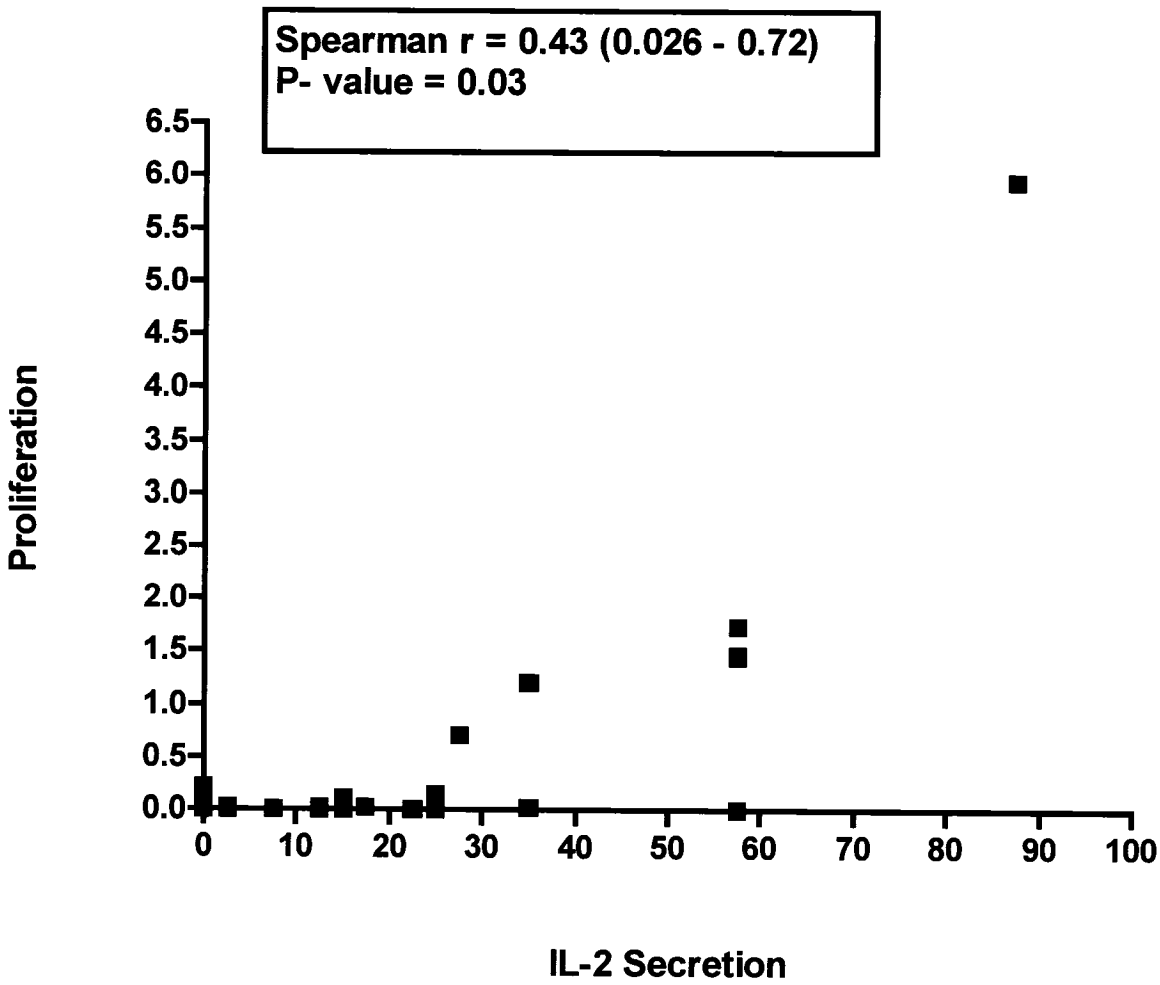

No Stimulation
0.091
26.1
0.4
73.4
SEB
22.6
7.62
48.3
21.4
Nef
0.068
25.2
0.54
74.2
Gag 1
0.094
26.5
0.32
73.1
Pol 1
0.51
25.4
1.22
72.9
Acc 1
0.077
24.7
0.24
75
Gag 2
0.076
26.1
0.43
73.4
Pol 2
0.044
25
0.45
74.5
Acc 2
0.072
25.3
0.21
74.4
FL4-H: CD8 APC
FL1-H: CFSE 5uM

Figure 2:


Spearman r = 0.43 (0.026 - 0.72)
P- value = 0.03
Proliferation
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
90
100
IL-2 Secretion

Chapter 4:

Original Scholarship and Advancement of Knowledge in the Domain of HIV Exposed Uninfected Individuals

1. HIV-specific IFN-γ secretion has been demonstrated in a population of EU IVDU. At the time, most of the information on HIV-specific immunity in EUs had been derived from sexually exposed subjects. While HIV-specific IFN-γ production had been identified in the Nairobi cohort of CSW, the only immunological responses that had been reported heretofore in IVDU EUs was T cell proliferation and IL-2 secretion. Thus, this was the first study reporting a quantitative determination of HIV-specific T cell responses in EU IVDU.

2. HIV-specific IFN-γ secretion has been associated with the maintenance of HIV seronegativity. HIV-specific IFN-γ secretion, in response to peptide stimulation, was detected in the majority of EU IVDU tested (66.7%), but was completely absent in a group of EU that seroconverted for HIV at a subsequent time point. Statistical analysis revealed that EUs who develop an HIV-specific T cell response, as determined by the IFN-γ ELISPOT, are 40 times less likely to seroconvert than those who cannot. In the absence of direct challenge experiments, these results provide compelling evidence that EUs develop T cell responses that help mediate protection from HIV infection.

3. HIV-specific immunity is comparable in EUs who have been exposed to HIV mucosally and parenterally. This was the first study to analyze HIV-specific immunity in EUs as a function of route of exposure. The determination that the frequency, magnitude, and breadth of the HIV-specific IFN-γ response in EUs is similar regardless of the route of exposure implies that both mucosal and

parenteral administration of HIV vaccines will be equally efficient at inducing HIV-specific immunity in the recipients. These results provide vaccinologists with a premise with which to design vaccine efficacy experiments.

4. HIV-specific IL-2 secretion and T cell proliferation are correlated. Even though these effector functions have been independently shown previously in EU subjects, no study has reported a direct relationship between these two correlates of antiviral immunity. This association strongly suggests that EUs develop HIV-specific T cell memory.

Section 3: Discussion

The development of a vaccine that will induce sterilizing immunity to HIV is contingent upon the identification of the relevant immune correlates that will protect a host from HIV infection. Two primate models have been used to study mechanisms of resistance to HIV: SIV infection of rhesus macaques and HIV exposed uninfected individuals (EUs).

The SIV/Macaque Paradigm

By virtue of its genetic similarity to HIV-1, SIV has been used as an important tool to further our understanding of HIV pathogenesis. Molecular manipulation of SIV has produced several strains of the virus with varying pathogenic potential. Infection of rhesus macaques with these laboratory strains of SIV has afforded researchers a relevant primate model with which to assess the significance of both viral and host factors on the rate of disease progression.

The other main attribute of the SIV/macaque model is the ability to assess the efficacy of vaccine strategies. Several candidate vaccine constructs have been tested for their ability to stimulate SIV-specific cellular and humoral immunity in rhesus macaques. Given the difficulty in inducing broadly-reactive neutralizing antibodies, the focus of vaccinologists in the last few years has been to generate a potent T cell response able to mediate, if not sterilizing immunity, at least slower disease progression.

The most promising regimen involves priming the host with a DNA plasmid expressing one or more viral proteins, followed by a boost with recombinant, replication defective viral vectors expressing homologous or heterologous viral

proteins[413,414]. In theory, the viral protein- expressing DNA plasmid primes the T cells against desirable viral epitopes. Upon subsequent immunization with a recombinant virus that expresses a multitude of antigenic proteins, the same response is boosted. Without the priming step, T cell immunodominance may skew the host T cell response towards irrelevant viral antigens. For example, an SIV Gag plasmid DNA prime, recombinant modified vaccinia Ankara (rMVA) expressing SIV Gag boost efficiently primed 20% of T cells to respond to a dominant SIV Gag epitope during an ex vivo stimulation[413,415,416]. DNA-based vaccines administered with adjuvants like IL-2 have been shown to induce even stronger antigen-specific T cell responses[417]. Other viral vectors that are being developed as antigen delivery vehicles in vaccine protocols include fowl pox[418], canary pox[419,420], replication-deficient adenovirus[414], Semliki Forest virus[421], and Venezuelan equine encephalitis virus[422].

Vaccine efficacy trials conducted in rhesus macaques have yet to demonstrate sterilizing immunity as a result of a T cell-based vaccine. The vaccinated macaques did, indeed, become infected, but their viral loads were 1000-fold lower than that in unvaccinated controls[413,414,417,423]. Thus, a strong cellular immune response that increases the threshold for new infections, retards disease progression, and lowers the probability of transmitting the virus is now deemed an adequate objective for candidate HIV vaccines.

There are two important caveats to the results obtained from vaccine studies conducted in macaques. First, the dose of SIV used to challenge vaccinated macaques is typically 100 times higher than the dose of HIV

encountered during a single episode of sexual contact in humans[424,425]. This is done to ensure that all animals in the positive control population become infected, which in turn reduces the number of control and vaccinated monkeys needed in the comparison groups to observe statistically significant effects. Second, SHIV 89.6P is normally used to challenge vaccinated macaques. SHIV 89.6P is an extremely pathogenic hybrid virus that is composed of genes from both HIV and SIV[426]. Macaques infected with SHIV 89.6P have plasma viral loads that are typically 10-100 fold greater than those of HIV-1 in chronically infected patients[413,414,417]. SHIV 89.6P depletes CD4+ T cells in its host within a few weeks of infection, and causes end-stage disease in most infected monkeys within six months of infection[413,414,417].

It is, thus, not entirely surprising that sterilizing immunity has not been achieved in vaccine studies of macaques. Furthermore, results from vaccine trials conducted in animals do not necessarily translate well when applied to humans. In addition to differences in pathogenicity between the heterogeneous population of HIV and the array of SIV and SHIV strains used in vaccine studies in macaques, the human immune system may respond adversely to vaccine formulations that were optimized in non-human primates.

HIV Exposed Uninfected Individuals as a Paradigm

Despite the limited potential for experimental manipulation, EUs offer a realistic depiction of HIV-specific immunity. Cohorts of commercial sex workers (CSW), men having sex with men (MSM), intravenous drug users (IVDU), health

care workers (HCW), HIV serodiscordant heterosexual couples, haemophilia patients, and infants born to HIV infected mothers, representing parenteral, mucosal, and vertical exposure to HIV, have all revealed several possible mechanisms of natural resistance to HIV infection. Compelling data from the study of EUs suggests that acquired T cell immunity may mediate sterilizing protection from HIV infection.

A common criticism of studies involving EUs is the question of whether or not these individuals have actually been exposed to HIV. Validity of self-reporting has been intensely investigated in several domains of epidemiological research, especially with regards to illicit intravenous drug use. By assaying for the presence and concentration of drug metabolites in the plasma, urine, and hair of IVDU, researchers have been able to assess the veracity of the reported frequency of drug use within a statistically acceptable range of error. In almost all settings, IVDU are more likely to underreport than to overstate their drug habits[427-430]. This is thought to be a function of their desire for social acceptance and/or fear of legal liability[431]. As such, the EU IVDU in our cohort may have actually experienced more exposure to HIV than what is reported. In the St-Luc cohort of IVDU, individuals are interviewed by trained professionals in a one-on-one manner in a private room[427]. Studies have also shown that privacy does not influence the data, as EU subjects are just as likely to report their behaviour alone as in the presence of an interviewer[427,428]. Thus, false-reporting is likely not a limitation of the presented results.

Naturally acquired resistance to HIV is a controversial phenomenon. There is yet to be a documented case in which a person successfully controlled and cleared a primary HIV infection. Although CD8+ T lymphocytes are associated with control of viral replication in HIV infected patients, HIV-specific T cell immunity has not been explicitly shown to prevent HIV infection of seronegative individuals. Furthermore, the frequencies of HIV-specific CTL are approximately ten fold lower in EUs than in HIV-infected individuals[317,342]. As such, the literature on HIV-specific immunity among EUs has been met with a healthy dose of scepticism.

It is possible that these repeatedly exposed individuals have serendipitously eluded HIV, but chance alone cannot reconcile the findings presented in this thesis. In our cohorts of IVDU, MSM, and serodiscordant couples, we have identified HIV-specific IFN-γ secretion, IL-2 production, and T cell proliferation that was completely absent in accompanying low-risk control populations. IVDU who did not report any needle-sharing with HIV infected peers were included in the negative control groups, thereby strengthening the validity of the results.

HIV-Specific T cell Responses in EUs are Associated with Protection

Due to ethical considerations, it is not possible to directly demonstrate a causal relationship between HIV-specific T cell responses and resistance to HIV infection in humans. As an indirect approach, EUs who remain seronegative were compared to EUs who later seroconverted for the presence of HIV-specific T cell

activity. We have shown that HIV-specific IFN-γ secretion and T cell proliferation are all present in a high proportion of EUs who remain seronegative, whereas these functional responses are missing in a population of equally exposed individuals who seroconverted soon after the sample date. If the observed state of immune activation was simply a reflection of high risk behaviour, and not correlated to HIV resistance, then it is expected that these HIV-specific T cell responses would have also been detected in the EUs that later seroconverted. Despite the modest size of the sample populations, our data suggests a statistically significant relationship between the presence of HIV-specific T cell activity and the persistent seronegativity of individuals with repeated exposure to HIV.

The demonstration of an HIV-specific T cell response in the seroconverter population that wanes immediately prior to seroconversion would have been ideal, but we did not have access to PBMC samples from multiple pre-seroconversion time points in order to conduct such a longitudinal analysis. In the study of female CSW from Nairobi, Kaul showed that pre-existing CTL responses were absent in those who later seroconverted[350]. The CTL responses were detected prior to a vacation from sex work, almost two years before seroconversion. Although these responses were not shown to wane immediately before seroconversion in those individuals who became infected, they were shown to be lost following interruption of sexwork[350]. Our results, demonstrating that functional T cell responses are present in EUs who remain seronegative and absent in EUs who seroconvert immediately after testing, together with Kaul's

findings, support the notion that HIV-specific T cell responses are important immune correlates of protection from HIV infection.

Experiments attempting to prove causality between HIV-specific CTL and protection from HIV infection have been carried out in a mouse model. SCID/beige mice, which are deficient in T, B, and NK cells, were reconstituted with PBMC from an EU individual and then challenged with M and T-tropic strains of HIV-1. The mice were resistant to infection, whereas mice that had received PBMC from a low-risk HIV negative control were not. When the resistant mice were depleted of their CD8+ T lymphocytes, they became susceptible to HIV infection upon subsequent challenge with virus. [432]

How Are HIV-Specific T cells stimulated in the Absence of Infection?

The presence of systemic virus-specific T cell responses in HIV seronegative individuals suggests that the host has encountered the virus, but that a productive infection was not achieved. The generation of HIV-specific T cell responses necessitates the processing and presentation of viral antigens. The exogenous pathway of antigen presentation represents a viable mechanism by which CD4+ T cell responses are engineered, but the stimulation of HIV-specific CD8+ T cells requires the intracellular production of viral proteins i.e. infection. There are three conceivable mechanisms by which HIV-specific T cell responses may be stimulated in the absence of overt infection:

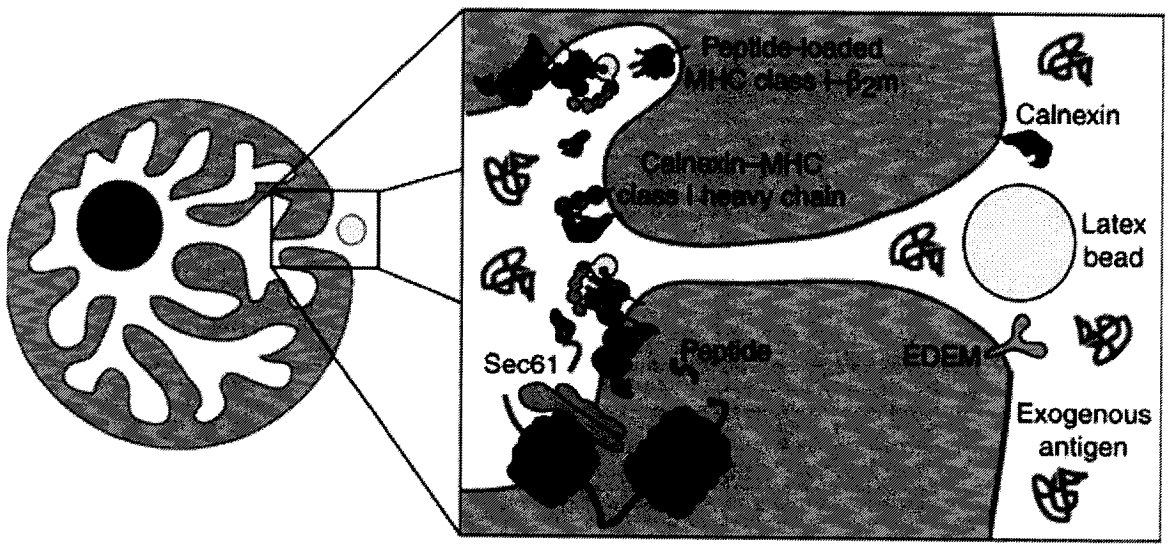
1. The infecting strain of HIV lacked sufficient fitness to establish a productive infection. A weakly pathogenic virion may be competent enough to gain entry into macrophages or dendritic cells, but may lack the genetic initiative to integrate into the host DNA, thereby allowing the antigen presenting cell to process the viral proteins and present them to CD8+ T cells in the absence of an infection.

2. Viral uncoating immediately after cellular entry may trigger the endogenous pathway of antigen presentation, stimulating an effective CTL response that kills the infected cell before the virus can transcribe its RNA genome into DNA, translocate into the nucleus, and integrate itself into the host chromosomal DNA. The T cell responses among our EUs were mainly directed against peptides from Nef and Gag, and to a lesser extent Pol, all of which are contained in the infecting virion. Kebba recently showed that EUs from serodiscordant couples preferentially recognized Vif peptides[358]. Vif is packaged into mature virions and is a critical mediator of HIV infection. These findings support the plausibility of such a mechanism.

3. Cross presentation of exogenous antigens by MHC class I molecules is a property of dendritic cells that is considered crucial for the priming of CTL responses directed against many infectious pathogens[433-435]. During phagocytosis of exogenous antigens, the endoplasmic reticulum (ER) membrane fuses with the plasma membrane to form the initial phagosome[436] [Figure 22]. Early phagosomes formed in this way harbor the translocon Sec61, a pore

complex found on ER membranes that is known to retrotransport ER proteins to the cytoplasm for degradation by the proteasome[437,438]. Cytoplasmic proteolysis is responsible for epitope generation in the cross-presentation pathway, since the process is highly sensitive to specific inhibitors of the proteasome[439]. It was later discovered that early phagosomes also possess all the functional elements necessary for TAP-dependent loading of peptides onto cognate MHC class I molecules[440-442]. TAP is present in association with a properly assembled MHC I loading complex that contains calreticulin, a disulfide-linked ER p57-tapasin conjugate, and endoglycosidase H-sensitive MHC class I heavy chains[440-442]. ER aminopeptidase, which trims antigenic peptides, is also present in the early phagosome[440,441]. The proteasomes are bound to the cytoplasmic face of phagosomes, implying that these organelles could be completely self-sufficient for mediating cross-presentation.

Figure 22: The Mechanism of Cross Presentation



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Thus:

- The Sec61 pore translocates large protein domains derived from exogenous antigens into the cytoplasm.
- Proteasomes attached to the cytoplasmic face of the phagosome degrade these proteins into peptides.
- The peptides are delivered back into the phagosomal lumen via TAP.
- The MHC class I loading complex would then facilitate peptide binding to the MHC I- β2 microglobulin dimer, producing mature MHC class I peptide complexes derived from internalized exogenous antigens. [443]

The first mechanism, though possible, represents a passive immunological mechanism and implies the host is at the mercy of the virus. The second mechanism, despite the support from the patterns of T cell reactivity in EUs, is unlikely to account for the observed anti-HIV T cell responses in EUs because HIV pathogenesis dictates a rapid spread of HIV throughout the lymphoid compartment and the rapid seeding of viral reservoirs. It is unlikely that a CTL response generated in this manner would suffice to clear all of the HIV infected cells, especially those with latent proviruses.

Cross presentation is a recently described mechanism that has garnered much support throughout the immunology community. By being able to prime CTL responses to proteins internalized from the environment, dendritic cells render the immune system reactive to universal danger signals. In the context of HIV, cross presentation may resolve the dispute over whether or not dendritic

cells are infected by HIV. Though it has been reported that HIV is internalized by dendritic cells, it appears that HIV replication does not occur within dendritic cells in vivo[444]. The current understanding is that dendritic cells internalize HIV virions, thereby preserving their pathogenicity, and later mediate infection of CD4+ T cells in trans[445]. It is possible that internalized HIV virions are processed by the exogenous pathway of cross presentation in EUs, resulting in the stimulation of CTL responses that clear the virus before HIV can infect CD4+ T cells in the lymphoid tissues. Cross presentation would also efficiently process a low dose of immunogenic, but non-replication competent HIV in EUs.

There are, however, some caveats to this mechanism. HIV exhibits a tropism for macrophages, but macrophages have a poor cross-presentation capacity because of their aggressive endocytic machinery that destroys internalized pathogens[446]. Efficient cross presentation requires that the early endocytic phagosome exhibits weak proteolytic activity[446]. Also, how the nascent MHC class I molecules loaded with peptides derived from exogenous antigens get expressed on the cell-surface is not yet understood.

Nonetheless, cross presentation provides a viable mechanism by which HIV-specific CD8+ T cell responses may be generated in the absence of infection in repeatedly HIV exposed individuals.

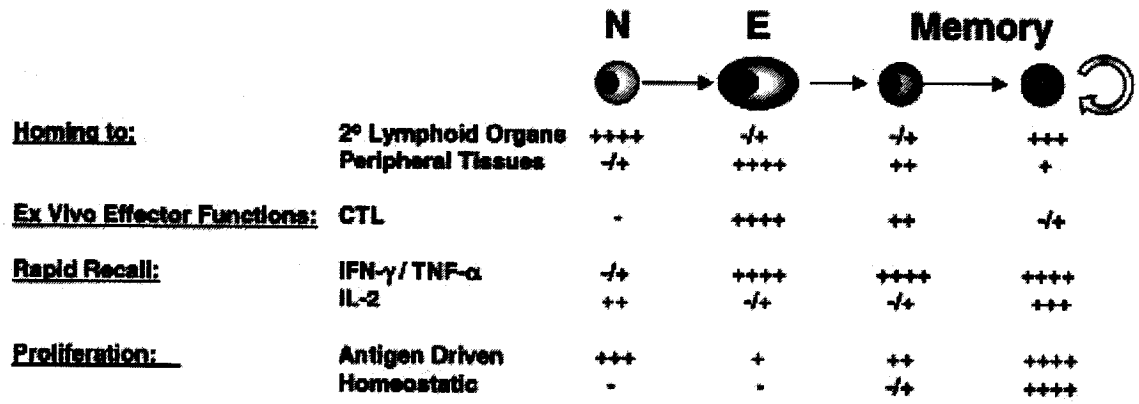
EUs Exhibit HIV-Specific T cell Effector Functions that are Hallmarks of Central Memory T cells

A statistically significant correlation between HIV-specific IL-2 secretion and T cell proliferation was established in our cohort of EUs. From the EUs that were assayed for both functional responses, those that exhibited HIV-specific IL-2 secretion also demonstrated HIV-specific T cell proliferation. All the EUs that displayed HIV-specific proliferative potential had expansions in their CD4+ T cell compartment, while two EUs showed robust HIV-specific T cell proliferation by both CD4+ and CD8+ T cells.

HIV-specific CD4+ T cells capable of rapid proliferation and IL-2 secretion are vital in containing HIV replication. HIV-specific CD4+ T cells in aviremic HIV infected patients possess a higher proliferative capacity than those from viremic patients[291]. The absence of HIV-specific proliferative responses in viremic patients was originally thought to be due to the selective deletion of HIV-specific CD4+ T cells, but this cell population was shown to be present in a functionally impaired state[411,447]. Exogenous addition of IL-2 restored the proliferative potential of HIV-specific CD4+ T cells in vitro from HIV infected patients with high viral loads[411]. Indeed, HIV-specific CD4+ T cells in aviremic HIV infected subjects persist throughout chronic infection and maintain their ability to secrete IL-2[291,411]. Thus, HIV-specific CD4+ T cell dysfunction, rather than their absolute loss, accounts for the diminished proliferative capacity of HIV-specific CD4+ T cells in viremic patients.

Rapid proliferation and IL-2 secretion upon cognate antigen restimulation are properties of central memory T cells (T_{CM})[275] [see Figure 23].

Figure 23: Functional Characteristics of Memory T cells



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Thus, in support of our working hypothesis, the HIV-specific T cell responses detected in our study of EUs are most likely memory T cell responses. Given our limited supply of PBMC with which to perform our assays, our analysis focused on the functional characterization of these HIV-specific T cell responses. It will be important to complement our results with a phenotypic characterisation of the responder T cell populations in our cohort of EUs. Staining with monoclonal antibodies for cell surface markers such as CCR7, CD62L, CD45RA/RO, CD27, and CD28 will ascertain whether or not the observed HIV-specific T cell responses in EUs are, in fact, memory T cell responses. In a recent publication, it was shown that Gag-specific CD4+ and CD8+ T cells from EUs stained for the

central memory phenotype CD45RA-CCR7+ [448]. There was also an abundance of terminally differentiated CD45RA+CCR7- CD27-CD28- effector memory T cells, compared to CD4+ and CD8+ T cells from low-risk controls and HIV-infected persons[448]. As a result, exposure to HIV that does not result in infection is sufficient to drive the maturation of memory T cell populations.

Protection from HIV Infection May Depend on the Dose of HIV to Which a Person is Exposed

The dose of HIV a host encounters is critically important in determining whether or not an HIV-specific T cell response can be generated that will eliminate the threat of a productive infection. Epidemiological studies all indicate that the concentration of HIV in the blood, semen, vaginal secretions, or breast milk is the strongest predictor of HIV transmission[141,155,156]. From an immunological perspective, this may imply that a large dose of HIV during an episode of high risk HIV exposure might overwhelm the primary immune response, resulting in successful infection by HIV. It follows, then, that exposure to a low dose of HIV may enable the stimulation of an HIV-specific CD8+ T cell response that can effectively manage the infecting bolus of virus, thus clearing the virus before a productive infection can be established.

The dose of antigen encountered by a host during a viral infection often controls the programmed production of cytokines from helper T cells that mediate protective immunity. The Th1 response is characterized by the production of IFN-γ and IL-12, whereas the Th2 response is defined by the secretion of IL-4 and IL-

10. The former phenotype favours the development of a cellular immune response, whereas the latter response promotes the production of IgG, IgE, and IgA antibodies. Infection of mice with a low dose of Leishmania major stimulates a Th1 response that leads to the containment of the parasite by cell-mediated immunity[449]. In contrast, infection with a high dose of parasite culminates in overt disease, despite the presence of a Th2, antibody immune response[449]. This dose-dependency was observed across different strains of parasite, different hosts, and different routes of infection[449]. By extension, HIV-specific cellular immunity that confers protection against HIV infection would be expected to be more efficiently primed by a low dose rather than a high dose exposure.

The current understanding of memory T cell differentiation is that the acute, memory, and terminally differentiated T cell subsets are not static in a linear model of differentiation. Instead, they represent a dynamic system, with flux occurring between these maturation subsets in response to changes in antigenic loads. There is a clear relationship between the antigenic load during viral infection and the generation of competent memory T cells. When antigen is cleared by primary T cell responses or persists at a low burden, such as during EBV and influenza infections, antigen-specific T cells are found to distribute primarily into memory subsets[284,450]. During CMV infections, when antigen loads are higher, antigen-specific T cells are observed to be primarily of the effector subset[284,450]. In HIV infected patients with persistently high viral loads, HIV-specific CD8+ T cells accumulate in a pre-terminally differentiated state[280,451]. HIV-specific T cells can mature, however, to memory given sufficient control over

the virus, for example with cART. Longitudinal phenotypic analyses for some chronically infected HIV patients showed that HIV-specific T cell responses shift toward memory as the plasma viral load decreases, and revert to an acute expansion phenotype as viremia rebounds[450].

Using these models as a premise, it can be hypothesized that seronegative individuals, exposed to a low dose of virus that is presumably cleared by the primary T cell response, develop a pool of fully differentiated memory T cells. The functional characterization of the EUs in this study, namely HIV-specific T cell proliferation and IL-2 secretion in response to HIV peptide stimulation, is in agreement with our theory.

Persistence of HIV-Specific T cell Responses in EUs

EU subject OM-MPS exhibited strong HIV-specific T cell proliferation in both the CD4+ and CD8+ T cell compartment at two consecutive time points, six months apart. The proliferation study, however, was largely cross-sectional. Data from the IL-2 and IFN-γ ELISPOT assays suggest that HIV-specific T cell responses in EUs are intermittent and appear to depend on recent high-risk activity (unpublished observations). A longitudinal analysis of persistence, however, was not possible at any point in any study because we did not identify any EUs with an HIV-specific response (IFN-γ, IL-2, or proliferation) at a time point(s) that was followed by cessation of exposure to HIV. It will be important to identify a cohort of EU responders that have discontinued their exposure to HIV in order to formally address the issue of persistence.

The development of memory T cells is critically dependent on the strength of the initial T cell stimulus, which is determined by the dose of antigen, the quality of co-stimulation, and the duration of the TCR/MHC-peptide interaction. The interplay between these parameters determines the extent to which antigen specific T cells will differentiate. In adoptive transfer experiments conducted in TCR transgenic mice, CD4+ and CD8+ T cells proliferated, differentiated, and persisted in vivo after prolonged stimulations with high doses of antigen in the presence of proper costimulation. Weaker stimulations, for example low dose and a low amount of costimulation, resulted in T cells that divided efficiently, but did not acquire fitness. [265,452]

In the setting of EUs, low dose stimulations may lead to the expansion of antigen-specific T cells that eventually die by neglect, depending on the levels of co-stimulation and the duration of the immunological synapse during the primary immune response. Such a mechanism would reconcile Kaul's finding from the study of female CSW that HIV-specific CTL responses require continuous exposure to HIV through sex work in order to be maintained[350]. Continued stimulation of unfit HIV-specific T cells in EUs by repeated exposure to viral antigens may avoid the deletion of these T cells from the circulation.

In a mouse model of memory T cell persistence, mice were immunized with vesicular stomatitis virus (VSV) and then challenged with a recombinant vaccinia virus that expressed a dominant CTL epitope. Whereas CTL precursor frequencies remained stable in vitro independently of antigen, memory T-cell

mediated resistance against re-infection of peripheral solid organs waned in proportion to diminishing levels of circulating antigen within a couple of weeks. [453]

Another mechanism by which HIV-specific memory T cells may be maintained in vivo is cross reactivity with other epitopes present in the host. It has been estimated that a single T cell receptor (TCR) is capable of recognizing more than 10^6 peptide/MHC combinations with varying affinity[454]. Antigen-specific CD8+ T cells have been shown to be cross-reactive to epitopes on the same viral protein[455,456], to epitopes on similar proteins of related viruses[457,458], and to epitopes on different proteins from heterologous viruses[459,460].

Mechanisms to Explain the Low frequency of HIV-specific T cells in EUs

HIV-specific T cells are not detected at a high frequency in EUs. This may be explained by an elevated frequency of exposure to numerous environmental immunogens. In theory, the immune system can only accommodate a finite number of antigen-specific memory T cells. In mice, sequential infection with LCMV, Pichinde virus (PV), vaccinia virus (VV), vesicular stomatitis virus (VSV), and murine cytomegalovirus (MCMV) led to the dilution of memory CD8+ T cells specific to the previously encountered viruses[461,462]. Similarly, persistent murine γ-herpes virus infection significantly reduced (by approximately 50%) the frequency of pre-existing T cells specific for influenza virus[463]. Whereas memory T cells that recognize cross-reactive epitopes survive, those specific for non-cross-reactive epitopes perish during sequential virus infections[464]. Thus, EUs that engage in high-risk sexual intercourse and needle-sharing can be predicted

to be exposed to a plethora of potential pathogens. Exposure to a variety of stimuli may induce new T cell responses that could replace pre-existing HIV-specific memory T cells.

Alternatively, T cells receiving a strong stimulation during an episode of exposure to HIV, possibly due to prolonged TCR contact with cognate peptide-MHC in the presence of enhanced dendritic cell co-stimulation, may differentiate into central memory T cells that preferentially localize to lymph tissues once the viral insult is cleared. Since most immunological assays utilize PBMC, HIV-specific CD4+ and CD8+ T_{CM} in EUs would remain largely undetected.

Relevance to Vaccine Design

1. The finding that parenteral and mucosal exposures to HIV both induce systemic HIV-specific T cell responses with similar characteristics was an unexpected finding. Intravenous inoculation of HIV presumably culminates with the bloodstream carrying the virus to the draining lymph node. During mucosal transmission, however, HIV faces both innate and cellular defenses within a mucosal and epithelial barrier. Distinct dendritic cell populations, with unique antigen presenting and costimulatory properties, reside in each locale. Given the extremely diverse nature of these immunological compartments, it was expected that the quality and frequency of HIV-specific T cell responses in the blood of sexually exposed, HIV seronegative individuals would be different than that in the blood of EU IVDUs.

Since most vaccine preparations are administered parenterally, our results suggest that intravenous priming will be just as effective as mucosal priming in generating systemic HIV-specific T cell responses in naïve individuals. Vaccine studies in rhesus macaques suggest that both systemic and mucosal HIV-specific T cell responses are absolutely necessary to prevent SIV infection[400,465]. It will be important to extend our findings and assess whether parenteral exposure to HIV antigens induces HIV-specific T cell immunity in the mucosa.

2. Based on the results presented in this thesis, the aim of any candidate HIV vaccine should be to stimulate high numbers of central memory T cells, which can rapidly proliferate and effectuate rapid antiviral immunity upon subsequent contact with antigen. IL-2 production from HIV-specific CD4+ T_{CM} cells may stimulate the expansion of HIV-specific CD8+ T_{CM} cells in the lymphoid tissues that can rapidly kill HIV-infected cells. In addition, CD8+ T_{CM} cells release cytokines, such as IFN-γ, immediately upon re-stimulation that inhibit viral spreading.

The identification of HIV-specific T cell immunity in EUs has, in part, influenced vaccine researchers to focus on developing vaccines that will induce robust T cell responses. Vaccine approaches that have been assayed in macaques effectively achieve early CD8+ T cell responses that are comparable to that seen during natural primary HIV infection[413-417]. The observed immune responses, however, wane rapidly and do not persist long-term[415]. This is likely a consequence of the vaccination protocols, in which the vaccinated macaques are challenged with virus before T_{CM} cells have had a chance to develop[466].

One of the hallmarks of T_{CM} cells is long-term persistence[275]. Since most exposures to HIV in vaccine recipients will likely occur many weeks after vaccination, candidate vaccine protocols should be evaluated largely on their ability to induce durable T_{CM} cells. Observations from EUs suggest that continuous exposure to HIV is important in preserving HIV-specific CD8+ T cell responses. It is likely, then, that a series of boosts will need to be incorporated into any vaccination protocol. Based on our results from EUs, HIV-specific T cell proliferation and IL-2 secretion may serve as excellent markers of effective, long-term immunity against HIV.

Concluding Remarks

From our study of EUs, it can be predicted that a vaccinated individual exposed to a low dose of HIV will be protected from a progressive HIV infection. Both the SIV/macaque and the EU paradigms suggest that T cell-mediated immunity is important in controlling HIV replication, however it is clear from studies of EUs that natural resistance to HIV is a reality and that it may be mediated by more than one mechanism. Acquired immunity is just one of many mechanisms of protection. Genetic factors, such as the expression of certain HLA alleles, and innate correlates, such as NK cells and mucosal antibodies, likely act in synergy with adaptive cellular immunity to protect individuals at high risk from HIV infection. Whereas the SIV/macaque paradigm predicts that a T cell based vaccine will not be able to induce sterilizing protection from HIV infection, our data from EU individuals provide hope that the presence of pre-existing HIV-

specific Tcm cells contribute to protection by raising the threshold for establishment of a progressive HIV infection.

Section 4: References

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Section 5: Appendix