Correlates of protective immunity among HIV-exposed seronegative female sex workers in Abidjan, Côte d'Ivoire

Correlaten van protectieve immuniteit in seronegatieve prostituées met frequente blootstelling aan HIV in Abidjan, Ivoorkust

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen aan de Universiteit Antwerpen te verdedigen door

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Promotoren:
Prof. L. KESTENS
Prof. R. COLEBUNDERS

Antwerpen, 2003
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Cover: “Coiffure Moderne” (detail), Kouassi Aka, Abidjan, Côte d’Ivoire, 2001
Preface

Infection with the human immunodeficiency virus (HIV) leads to the acquired immunodeficiency syndrome (AIDS). Twenty years since its discovery, HIV has infected over 65 million people, and has killed more than 20 million of them. Among adults, HIV seroprevalence has increased to 30% and more in some African countries, and fast upcoming epidemics in Eastern Europe and South-East Asia do not predict a slowing down of the infection rate. If left to run its natural course, this pandemic, the HIV epidemic circling the globe, will cause social and economical devastation on an unprecedented scale.

Promotion of condom use and wide availability of antiretroviral therapy for HIV patients should get the highest priority in countries that are most affected by HIV. Unfortunately, in many countries, condom use is confronted with social and religious restrictions and as a consequence HIV continues to spread. Antiretroviral therapy may also reduce the risk of HIV transmission from infected to non-infected persons, but is both costly and dependent on suitable health care infrastructure that is often lacking in developing countries. Certainly, the best hope for controlling the HIV pandemic is a protective HIV vaccine.

A vaccine is a suspension of weakened viruses or parts of viruses that stimulate the immune system to make antibodies and cells that prevent infection when exposed to this virus. To date, it remains unclear how such a vaccine should be designed for HIV. In order to make a protective vaccine, one needs to know what parts of the human immune system are necessary for the protection. This information is still lacking. Candidate HIV vaccines that are currently being tested have been designed based on the principle of trial and error. Unlike previous lucky hit vaccines against other viruses, this methodology has so far not been successful for HIV.

In this doctoral thesis, the mechanisms by which the human immune system can protect against HIV infection are investigated. We have identified female sex workers in Côte d’Ivoire who are not infected with HIV despite extensive and frequent exposure to the virus through unprotected sexual contacts. We hypothesise that these women are protected against HIV infection by their immune system. By comparing these women to frequently exposed women that became infected with the virus, or to women who are not exposed to HIV at all, we hope to identify the parts of the human immune system that go together or correlate with protection against infection. These correlates of protective immunity are the Holy Grail in HIV vaccine development.
Chapter 1 introduces HIV and how HIV infection leads to AIDS. The role of the immune system in HIV infection and progression to AIDS is reviewed. Already proposed correlates of protection against HIV infection are summarised. Chapter 2 addresses general immune factors that may be directly or indirectly associated with protection against HIV infection among the female sex workers in Côte d'Ivoire. These factors include immune activation, HIV-suppressive factors and transplantation rejection. In Chapter 3, the role of the HIV-suppressive β-chemokines in protection against HIV disease progression is studied. Chapter 4 focuses on the detection of HIV-specific memory T cell responses in the population of female sex workers in Côte d'Ivoire. Chapter 5 discusses the findings presented in this thesis, their relevance in protection against infection with HIV, and their significance for the development of a protective HIV vaccine.
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# Chapter 1

## Introduction

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1.1. HIV/AIDS: FROM DISCOVERY TO PANDEMIC

The acquired immunodeficiency syndrome (AIDS) was first observed in 1981 among young homosexual men in New York and California who suffered from an unusual clustering of rare diseases, notably Kaposi’s sarcoma and opportunistic infections such as *Pneumocystis carinii*, and cases of persistent lymphadenopathy [1]. It soon became evident that these individuals had a common immunological deficit in cell-mediated immunity, resulting predominantly from a significant reduction in the number of circulating CD4+ T cells [2, 3]. Clustering of AIDS cases in diverse risk groups, including homosexual men, intravenous drug users, haemophiliacs, and sex-partners of risk-group members, could be explained only if AIDS were caused by an infectious microorganism [4]. The search for such a microorganism led in 1983 to the isolation of a new retrovirus named lymphadenopathy-associated virus (LAV) [5], or also human T-lymphotropic virus type III (HTLV-III) [6], and was later renamed human immunodeficiency virus (HIV) [7]. Further epidemiological and virological data established that HIV was the causative agent of AIDS [8-10], and of “slim disease” in Africa [11].
Twenty years after its discovery, the HIV has acquired the status of number one killer among infectious disease-causing agents. By the beginning of 2003, HIV had infected a cumulative total of more than 65 million people, over 20 million of whom subsequently died. Of 42 million people who are currently incubating the virus, 95% live in the developing world; 70% of the cases are in sub-Saharan Africa where in some regions the HIV seroprevalence among adults exceeds 30% (figure 1A) [12]. In these countries, HIV is responsible for a dangerous weakening of various societal institutions, including schools, civil services, armed forces and health care [13]. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), this evolution provides positive proof that HIV, if left to run its natural course, will cause devastation on an unprecedented scale.

1.2. HIV CLASSIFICATION, STRUCTURE AND REPLICATION CYCLE

1.2.1. HIV classification

HIV is a member of the Retroviridae family, based on its ability to reverse transcribe RNA into DNA (Latin retro: backward), and belongs to the genus of lentivirinae that cause persistent infections with slow disease progression (Latin lenti: slow) [14]. Apart from a number of animal lentiviruses, two human lentiviruses are distinguished: HIV type 1 (HIV-1), the predominant HIV type throughout the world, and HIV-2, that is found primarily in West-Africa [15]. Phylogenetic analyses indicate that HIV-1 originated from a simian immunodeficiency virus (SIV) that infects chimpanzees [16, 17], while HIV-2 arose from SIV in sooty mangabeys and rhesus macaques [18]. As the result of high genetic variability, HIV-1 isolates have been classified into a number of subtypes designated by alphabetical letters, together with a number of circulating recombinant forms (CRF) of these subtypes, each having a specific geographic location to the world. The regions in which HIV-1 has been present the longest have the most complex array of subtypes (figure 1B) [19-21].

1.2.2. Structure of HIV

HIV virions are spherical particles of about 100 nm in diameter. Infectious virions contain two copies of positive single-stranded RNA surrounded by p24 capsid proteins and p17 matrix proteins, which, in turn, are surrounded by an envelope derived from the host cell membrane that contains the viral proteins gp41 and gp120, and host proteins (figure 2A). The viral genome contains three essential
structural genes: gag (group specific antigen), env (envelope), and pol (polymerase); in addition there are six accessory genes (figure 2B) [14].

1.2.3. Replication cycle of HIV

HIV virions infect CD4-bearing cells including CD4⁺ T cells, monocytes, macrophages and dendritic cells. Binding of HIV to CD4 molecules and chemokine receptor molecules on the cell surface leads to fusion of viral and cellular membranes [22]. Following entry, viral RNA is reverse transcribed into double stranded DNA, and integrated into the host cell genome. Activation of the infected cell initiates the transcription of viral mRNA and translation of structural and enzymatic proteins that assemble with two copies of single-stranded viral RNA. Enveloped infectious particles are formed by budding from the host cell during which part of the host cell membrane is acquired [14].

Figure 2. Structure of HIV-1. A, Virion structure. B, Genome structure. Virion structural genes are heavily shaded and accessory genes lightly shaded. Precursor proteins Gag-Pol, Gag, and Env are enzymatically processed to yield mature virion proteins: Gag-Pol and Gag undergo several cleavage steps mediated by the viral protease, Env is cleaved once by a cellular protease. p17, matrix protein; p24, capsid protein; p9, nucleocapsid protein; p11, protease; p66/p51, reverse transcriptase; p32, integrase; gp120, surface protein; gp41, transmembrane protein. Adapted from ref. 22.
1.3. HIV PATHOGENESIS

Following transmission of HIV, HIV-infected target cells home to the lymphoid organs where systemic infection is established. During the acute phase of HIV infection, virus replication accelerates and massive viraemia leads to the wide dissemination of virus throughout the body, resulting in a decrease of the number of CD4+ T cells. By three to four months after HIV infection, HIV-specific humoral and cellular immune responses have developed that limit viral replication and establish persistent chronic infection. Because of only partial immunological control, HIV production and infection continues, associated with a rapid turnover and a slow but steady decline of CD4+ T cells. CD4+ T cells are central to immune responses to pathogens in general and their depletion will ultimately result in the development of the symptomatic phase of the disease, occurrence of opportunistic infections, and AIDS [23, 24].

Although HIV infection eventually leads to death in all infected individuals, the clinical course of the disease may vary widely. The period between the time of HIV infection and the onset of AIDS varies from 8-10 years in typical progressors, to < 3 years in rapid progressors, and > 15 years in long-term non-progressors [25]. Multiple factors may affect disease progression in HIV-infected individuals, including genetic characteristics of the infecting virus and the host, as well as qualitative and quantitative aspects of HIV-specific immune responses [26, 27]. During the last decade, highly active antiretroviral therapy (HAART) has been particularly successful in suppressing viral replication to undetectable levels, slowing down the disease progression and prolonging the life of many HIV-infected patients [28]. However, HAART has only marginally become available for the developing countries that are most affected by the epidemic.

1.4. IMMUNE RESPONSES AGAINST HIV

1.4.1. Innate immune responses

The innate immune system acts in a broadly specific manner against invading pathogens, and may be important in controlling infection before adaptive immune responses have developed. Therefore, innate immune responses are generally more rapid, but at the same time less complete, compared with adaptive immune responses. Soluble innate factors with anti-HIV activity include complement, β-chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α and MIP-1β, noncytotoxic CD8+ T cell activity, defensins, and cytokines such as tumor necrosis
factor (TNF)-α, interferon (IFN)-α and IFN-γ. Cellular innate responses that may potentially protect against HIV include, among others, neutrophils, natural killer (NK) cells, IFN-producing cells, dendritic cells, and γδ T cells [29, 30].

1.4.2. HIV-specific immune responses

**Cytotoxic CD8+ T lymphocytes (CTL).** CTL are known as killer T cells because they recognise, bind and kill infected cells that display foreign antigens on their membrane, and their role in protective immunity against viral infections in general has been well-documented [31]. CTL may exert considerable pressure on HIV replication during the primary and chronic phases of the infection: the initial control of HIV infection in humans is dominated by an acute CTL response [32-34], and in experiments with chronic SIV-infected rhesus macaques, a marked increase in viral load was seen when CD8+ T cells were depleted in these animals [35, 36]. However, CTL are not able to clear HIV from infected hosts: evasion of HIV from CTL responses has been observed as the result of HIV mutations [37-39], HIV persists in latently infected cells and in viral reservoirs [40], and CTL responses become impaired with progressing HIV disease and lack of CD4+ T cell help [41, 42].

**CD4+ T helper cells.** CD4+ T cell help is central to the induction and the maintenance of both humoral and CTL responses to pathogens in general. Several reports have demonstrated the importance of CD4+ T helper cells in HIV-infected subjects, and decreasing numbers of HIV-specific CD4+ T cells were associated with loss of HIV-specific CTL function and progression to AIDS [43-46]. Numbers of HIV-specific CD4+ T cells are generally low in HIV-infected subjects, possibly because of early elimination during acute HIV infection. HIV has been found to preferentially infect HIV-specific CD4+ T cells: these cells are activated at the site of viral replication and become therefore more susceptible to HIV [47, 48].

**Humoral immune responses.** CTL only become activated once a productive infection is established, and can therefore only suppress or eliminate infections that are already established. In contrast, circulating neutralising antibodies may provide sterilising immune protection by binding to the pathogen and preventing adhesion to the host target cells, a strategy proven successful in many previous vaccines [49]. In HIV infection, the initial control of viral replication results, at least in part, from the activity of neutralising antibodies. However, the high frequency of genetic mutations in HIV leads to neutralisation escape, and failure of neutralising antibodies to prevent disease progression [50, 51]. Anti-HIV antibodies are generally targeted against the highly variable gp120 region of HIV. Effectively
neutralising antibodies to conserved regions of gp120 are rare in HIV-infected subjects [52], and have been difficult to achieve by immunisation [53, 54].

1.5. CORRELATES OF PROTECTIVE IMMUNITY AGAINST HIV

A protective HIV vaccine may prove the best hope for controlling the HIV epidemic, especially in developing countries where HIV prevalence is high, health care infrastructure is poor, and antiretroviral therapy is unlikely to become fully available in the near future. A safe, effective, broad-spectrum HIV vaccine is needed that generates long-term immune memory and protection at the site of infection. Of ample importance for the design of such a vaccine is the understanding of the immunological mechanisms that protect against HIV infection. However, despite numerous research efforts, a clear ‘correlate of protective immunity’ against HIV infection has yet to be identified. Development of an HIV vaccine without the lead of clear correlates of protection against infection is possible, but will prove much more difficult, as recently exemplified by the failure of the VaxGen gp120 subunit vaccine [54]. The majority of the current candidate AIDS vaccines being tested in phase I and II clinical trials are also based on assumptions rather than evidence of protective immunity.

Throughout the development of previous vaccines for other viruses like smallpox, measles or polio, it was clear that people who recovered from infection were immune against a subsequent attack by the same virus. Similarly, the recent epidemic of severe acute respiratory syndrome (SARS) showed a high mortality rate, but cases of disease recovery and viral clearance were evident [55, 56]. Such cases first of all indicate that immunological protection against viral infection exists, and that a successful vaccine may be possible. In addition, analysing the nature of the immune responses that correlate with protection may provide the main lead for the design of such a vaccine. However, no one has been reported to recover from, or to completely clear, HIV infection. This raises the fundamental question whether protective immune responses exist for HIV, and whether a protective AIDS vaccine will be possible at all [57].

Although clearance of HIV infection is not evident, small proportions of subjects exist that do not progress to disease despite long-term chronic infection in the absence of antiretroviral therapy, characterised by normal and stable CD4+ T cell counts and low HIV RNA levels (long-term non-progressors) [58-61]. SIV-infected chimpanzees, sooty mangabeys and African green monkeys do not develop SIV-associated disease despite extensive viraemia in these animals [62-65]. Defining the immunological factors that cause these particular conditions may provide clues
for prophylactic or therapeutic vaccine development. Unlike HIV, other persisting virus infections in humans such as Epstein-Barr virus and cytomegalovirus appear to be efficiently controlled by the human immune system [66], and the understanding of the protective immune responses to these viruses may also contribute. In addition, correlates of protection may be deduced from future HIV vaccine efficacy trials in humans, and from experimentally vaccinated macaques. Even with largely ineffective vaccines, analysing the immune responses that may correlate with protection against HIV or SIV infection in some of the vaccinated humans or animals, may provide the key information for the improvement of these vaccines [67, 68]. Recent studies have described cases of HIV superinfection, which is the establishment of a secondary HIV infection in already HIV-infected subjects [69, 70]. While HIV superinfection is believed to be rare, studying the immunological characteristics that protect against HIV superinfection in at-risk populations may also help in our understanding of HIV-protective immunity.

An historical scientific approach led to the development of a vaccine for smallpox. Edward Jenner noticed that milkmaids who had previously contracted cowpox, were resistant to smallpox infection. This observation was the critical event leading to the finding that the cowpox virus (vaccinia) cross-reacted immunologically with the smallpox virus (variola) and could therefore be used to protect against smallpox [71]. Jenner’s milkmaids were the key population needed to develop the vaccine.

In analogy with Jenner’s experiment, and in an effort to identify the correlates of protection against infection with HIV, several populations are being identified that are exposed to HIV without becoming infected (HIV-exposed seronegative or ESN). These populations include HIV-seronegative partners of HIV-seropositive subjects (HIV-discordant couples) reporting unprotected sexual intercourse [72-77], men who have sex with men [78, 79], commercial sex workers in high HIV prevalence areas [80-85], intravenous drug users exposed to HIV by needle-sharing [86-89], haemophiliacs that have been repeatedly infused with HIV-contaminated blood products [90-93], HIV-uninfected children born to HIV-infected mothers [94-97], and health care workers exposed to contaminated blood [98-100]. Since the main focus of this thesis is the analysis of potential correlates of HIV protection in a population of African ESN female sex workers, previously proposed correlates of protection in other ESN populations will be reviewed.

1.5.1. Host genetic variability

*Homozygous CCR5 deletion.* In addition to CD4, the β-chemokine receptor CCR5 is used as a coreceptor by primary macrophage-tropic HIV-1 strains that are
sexually transmitted [101-104]. A 32 base pair deletion in the gene encoding for CCR5 results in a frame shift and premature stop codon in the fifth transmembrane domain, which translates in a truncated protein that is not expressed on the cell surface. Several ESN individuals were found to be homozygous for the mutant allele, and displayed nearly complete protection against in vitro infection with macrophage-tropic HIV-1 [105-107]. Frequencies of the homozygous 32 base pair deletion range from 15% in Northern Europeans, to about 1% in Caucasians in general, and complete absence in Africans [108-110]. A point mutation in an alternate HIV-1 coreceptor CCR2 has also been reported (CCR2-64I), but this mutation was found to be associated with slower HIV disease progression, and did not correlate with increased protection against infection with HIV [111, 112].

Specific HLA polymorphisms. ESN female sex workers in Nairobi were found to display rare HLA alleles, thus being capable of manifesting alloimmune responses against the largest proportion of the population [113]. Similarly, HLA class I discordance was associated with a decreased risk of HIV-1 transmission from mother to child [114, 115], and in HIV-discordant couples [116]. In addition, particular HLA alleles have been identified that were associated with a reduced risk of HIV transmission: HLA-A2 and A*6802 among female sex workers and children born to HIV-infected mothers in Nairobi [117, 118], HLA-B18 among female sex workers in Northern Thailand [82], and HLA-DR5 among seronegative partners in HIV discordant couples in Scotland [116].

1.5.2. Production of HIV-suppressive factors

β-chemokines. The β-chemokines RANTES, MIP-1α, and MIP-1β are the natural ligands for the CCR5 coreceptor and their HIV-suppressive capacity has been shown in vitro [119]. The antiviral effect of β-chemokines is mediated by inhibition of HIV binding to the cell surface by blocking and down-regulation of CCR5 [120]. Increased β-chemokine production in ESN individuals was associated with resistance of CD4+ T cells to in vitro HIV-1 infection [121, 122]. Elevated β-chemokine levels were also found among HIV-negative hemophiliacs repeatedly inoculated with HIV-contaminated blood products [91], and in Chinese seronegative partners in HIV-discordant couples [123].

Noncytotoxic CD8+ T cell activity. CD8+ T lymphocytes can inhibit HIV replication in CD4+ T cells by a noncytotoxic factor. Although a number of soluble factors including β-chemokines, interleukin-16, interferons, antithrombin III, and α-defensins have been proposed, the exact identity of this activity remains unclear [124-127]. Nontotoxic CD8+ T cells were shown to play a role in preventing
HIV disease progression in long-term non-progressors [128]. Noncytotoxic CD8+ T cell activity has been observed in ESN subjects in HIV-discordant couples [75, 129, 130], among intravenous drug users [88, 129], and in children born to HIV-infected mothers [131].

1.5.3. HIV-specific immune responses

HIV-specific immunoglobulin (Ig) A antibodies. HIV-specific IgA antibodies were detected in serum and vaginal secretions of ESN partners of HIV-infected subjects and in ESN female commercial sex workers [82, 83, 132, 133]. IgA purified from ESN subjects neutralised an HIV primary isolate [134], prevented in vitro transcytosis of HIV through an epithelial membrane [135, 136], and targeted a region within the alpha-helix of gp41 [137]. The presence of IgA antibodies was found to be dependent on recent HIV exposure [76]. HIV-specific antibodies may function by neutralising HIV in the mucosa before productive infection can be established. Antibodies to cellular proteins such as HLA class I, CD4 and CCR5 have also been found in ESN subjects [138-140].

HIV-specific CTL responses. HIV-specific CTL have been detected in children born to HIV-infected mothers [94], in regular partners of HIV-infected subjects [73, 74, 76, 77, 95], in intravenous drug users [89], and female commercial sex workers [80, 81, 85, 141]. HIV-specific CTL in ESN female sex workers from Nairobi were capable of lysing HIV-infected target cells in vitro [81], were found enriched at the mucosal sites [141], and specifically recognised different HIV epitopes compared with HIV-infected subjects [142]. These epitopes were found to be presented by the HIV-protective HLA alleles that were previously identified in that population [117]. The long-term protective role of HIV-specific CTL remains unclear since waning of HIV-specific CTL and subsequent seroconversion was reported in association with a decrease in the sexual exposure to HIV [143].

HIV-specific T helper responses. HIV-specific CD4+ T helper cells have been detected in occupationally-exposed health care workers [98], in children born to HIV-infected mothers [97], in regular partners of HIV-infected subjects [72, 73, 76, 130], and in commercial sex workers in developing countries [144]. Reduced HIV-stimulated T helper cell activity was found in cord blood after short-course antiretroviral treatment for prevention of maternal-infant transmission, suggesting a direct relationship between HIV-specific T helper cells and the extent of antigenic exposure [145]. The majority of the studies used methods based on lymphocyte proliferation or bulk cytokine secretion that lack specificity compared with the more recent assays like ELISPOT and cytokine flow cytometry. Although less well
characterised than HIV-specific CTL, it is plausible that HIV-specific T helper cells make an as yet undefined contribution to the quality of the responding HIV-specific CTL, as is the case for HIV-infected subjects [146].

1.6. AIM, OBJECTIVES AND OUTLINE OF THIS THESIS

Defining the correlates of HIV protection in subjects that remain HIV-seronegative despite frequent unprotected HIV exposure is of great importance for the design of a protective HIV vaccine. Factors that are found to protect highly exposed individuals against HIV infection may be induced in HIV-seronegative populations by vaccination, thereby halting the HIV epidemic from further spreading.

The overall aim of this thesis was to study potential correlates of protective immunity against HIV. Innate and adaptive immune responses with potential anti-HIV activity were analysed in African ESN FSWs at high risk for HIV infection in comparison with HIV-seronegative low-risk female blood donors in Abidjan, Côte d’Ivoire. The specific objectives of this thesis were:

**Objective 1.** Analysis of cellular activation and Th1 and Th2 cytokine expression in CD4+ and CD8+ T cells of ESN FSWs and female blood donors.

**Objective 2.** Comparison of the expression levels of the HIV-1 coreceptors CCR5 and CXCR4 on CD4+ T cells of ESN FSWs and female blood donors.

**Objective 3.** Study of the role of the β-chemokines RANTES, MIP-1α, and MIP-1β in protection against infection with HIV in ESN FSWs, and in protection against HIV disease progression in HIV-infected subjects.

**Objective 4.** Analysis of alloimmune responses in ESN FSWs and blood donors.

**Objective 5.** Detection of HIV-specific CD4+ T helper cells and CD8+ CTL in ESN FSWs in association with sexual behaviour characteristics.

The objectives 1 to 5 are addressed in four different chapters outlined as follows.

**Chapter 2** addresses non-HIV-specific immunological factors that may be directly or indirectly associated with decreased susceptibility to HIV infection among ESN FSWs. These factors include both innate and adaptive immune responses such as in vivo CD4+ and CD8+ T cell activation (objective 1), cellular expression levels of the HIV-1 coreceptors CCR5 and CXCR4 (objective 2), in vitro production of
CCR5-binding β-chemokines (objective 3), Th1 and Th2 cytokine expression by T cell subsets (objective 1), and humoral and cellular alloimmune responses (objective 4).

In Chapter 3, the role of β-chemokines in protection against HIV disease progression is studied further in HIV-infected subjects from Abidjan and Antwerp (objective 3), by making use of both novel and established methods of detection.

Chapter 4 focuses on the detection of HIV-specific T cell responses (T helper responses and CTL responses) in the population of ESN FSWs in Abidjan (objective 5). For the detection, both sensitive and specific laboratory methods are used, and a newly modified and highly sensitive method is described.

Chapter 5 discusses the findings presented in this thesis, the relevance of the observed correlates in protection against infection with HIV, and their significance for the development of a protective HIV vaccine.

1.7. REFERENCES

22 / CHAPTER 1

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Chapter 2

Non-HIV-specific immunological factors with protective potential against HIV infection

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   Tolerance towards alloantigens in HIV-exposed seronegative female sex workers in Abidjan, Côte d'Ivoire. Submitted.
Cellular HIV-protective factors: a comparison of HIV-exposed seronegative female sex workers and female blood donors in Abidjan, Côte d'Ivoire

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Journal of Infectious Diseases 2003, 187:206-214

ABSTRACT

Cellular factors that may protect against human immunodeficiency virus (HIV) infection were investigated in 27 HIV-exposed seronegative (ESN) female sex workers (FSWs) and 27 HIV-seronegative female blood donors. Compared with blood donors, ESN FSWs had significantly decreased expression levels of C-X-C chemokine receptor 4 (CXCR4), but not of C-C chemokine receptor 5 (CCR5), on both memory (P < 0.001) and naive (P = 0.041) CD4+ T cells. CXCR4 down-regulation was associated with prolonged duration of commercial sex work by ESN FSWs. CD38 expression on CD8+ T cells was significantly increased among ESN FSWs, compared with that among blood donors (P = 0.017). There were no differences in HLA-DR and CD62L expression between blood donors and ESN FSWs. Proportions of T cells producing the β-chemokines RANTES (regulated on activation, normally T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α, and MIP-1β or the cytokines interleukin (IL)-2, IL-4, interferon-γ, and tumor necrosis factor-α, were similar in the 2 groups. These data indicate that ESN FSWs differ from HIV-seronegative female blood donors with respect to immunological factors that have no clear protective potential against HIV transmission.
INTRODUCTION

In several populations, small proportions of individuals remain human immunodeficiency virus (HIV)-seronegative, despite frequent sexual exposure to HIV, which suggests that host factors may contribute to protection against infection [1-5]. Proposed correlates of protection against HIV infection in HIV-exposed seronegative (ESN) subjects that are well documented include inherited and acquired host factors, such as a homozygous 32 base pair deletion in the gene encoding the HIV-1 coreceptor CCR5 [6-11], particular genetic HLA polymorphisms [12, 13], mucosal and systemic anti-HIV IgA [14-18], and HIV-specific helper and cytotoxic T cells [19-25].

In addition to CD4, the β-chemokine receptor CCR5 is used as a coreceptor by primary macrophage (M)-tropic HIV-1 strains that are sexually transmitted [26-29], whereas the α-chemokine receptor CXCR4 is used by T cell (T)-tropic variants of HIV-1 that emerge at later disease stages [30-32]. Decreased expression levels of CCR5 on CD4+ T cells, in the absence of the 32 base pair deletion, have been correlated to decreased susceptibility to M-tropic HIV-1 in vitro [33, 34], to decreased HIV-1 load levels in infected subjects [35], and to increased protection against HIV-1 infection in ESN subjects [36]. However, studies exist in which the latter could not be confirmed [37, 38]. Although susceptibility to T-tropic HIV-1 strains in vitro has been correlated directly to CXCR4 expression levels [39-41], no associations have been found between CXCR4 levels and the transmission of HIV-1 in vivo.

β-chemokines are the natural ligands for the CCR5 coreceptor, and, although their HIV-suppressive capacity has been shown in vitro [42], their role in protection against HIV infection in vivo remains controversial. Some studies have found increased β-chemokine levels in ESN subjects, compared with those in unexposed control subjects [25, 36, 43-45]. However, other studies have not found any difference [37, 38, 46]. Similarly, Th1 cytokines could play a role in protection against infection with HIV in association with cell-mediated immunity [47]. Indeed, in ESN populations, increased levels of Th1 cytokines have been shown in the presence of mucosal and systemic cellular immune activation [48] or together with a decreased Th2 cytokine profile [49, 50]. However, in other studies, decreased Th1 cytokine production was found among ESN subjects [46, 51].

In the present study, we determined whether African ESN female sex workers (FSWs) differed from African HIV-seronegative female blood donors in one or several immunological aspects with potential HIV-protective capacity. Freshly obtained whole-blood samples were used to measure ex vivo cell surface
expression levels of immune activation markers and HIV-1 coreceptors and to detect intracellular β-chemokine and cytokine production, all at the level of a single cell, by flow cytometry. In addition, we verified whether these potential correlates of HIV protection were associated with indicative markers of HIV exposure among FSWs, such as the duration of commercial sex work, the number of clients per day, the consistency in using condoms, and the occurrence of sexually transmitted infections (STIs).

SUBJECTS AND METHODS

Study population. HIV-seronegative FSWs were enrolled consecutively as part of an ongoing surveillance study at a confidential clinic in Abidjan [52], and blood samples and standard questionnaires with information on sociodemographics and sexual behavior were collected. Voluntary HIV-seronegative female blood donors were enrolled at the national blood transfusion center in Abidjan; only blood samples were collected. The study lasted 2 months and took place in 2 distinct 1-month time periods. In November 1999, 14 FSWs and 15 blood donors were enrolled; in May 2000, 13 FSWs and 12 blood donors were enrolled. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention (Atlanta, GA, USA), and the Ethical Committees of the Côte d’Ivoire Ministry of Health (Abidjan, Côte d’Ivoire) and the Institute of Tropical Medicine (Antwerp, Belgium). Informed consent was given by all study subjects before enrollment.

Laboratory method for HIV and STI diagnosis. Whole blood was drawn from FSWs and control subjects into EDTA tubes (Becton Dickinson, San Jose, CA, USA). Within 4 h of blood collection, plasma was separated from cells by centrifugation at 500 g, aliquoted and stored at -70 °C.

The HIV-seronegative status of all subjects was determined in plasma using the current HIV testing algorithm of Projet RETRO-CI, defined by a combination of ELISAs and Western Blots [53, 54]. The HIV-negative status of seronegative FSWs was confirmed by reverse HIV-1 reverse transcriptase (RT) polymerase chain reaction (PCR) in plasma samples [55].

Bacterial and protozoan STIs were tested in the 27 FSWs: antitreponemal antibodies were detected using Treponema pallidum hemagglutination assay (TPHA, Fujirebio, Malvern, PA, USA) and the rapid plasma reagin (RPR) test (Macro-Vue, Becton Dickinson, San Jose, CA, USA). A diagnosis of syphilis was made if results for both the TPHA and RPR tests were positive [56]. Neisseria
gonorrhoeae infection was diagnosed by culture on modified Thayer-Martin medium or by PCR [56]. Chlamydia trachomatis infection was diagnosed by Microtrak EIA (Syva, Palo Alto, CA, USA) or by PCR. Trichomonas vaginalis infection was diagnosed by use of wet mount techniques.

**T cell activation markers.** Phenotypic analyses of peripheral blood lymphocytes were performed on fresh whole blood within 4 h of collection in EDTA. To quantify the levels of CD4+ and CD8+ T cell activation, whole blood was incubated in the presence of the following monoclonal antibody (MAb) combinations: anti-CD8, anti-CD38, and anti-HLA-DR; anti-CD3, anti-CD8, and anti-HLA-DR; anti-CD4, anti-CD45RO, and anti-CD62L MAbs. All MAbs were fluorochrome-labelled and purchased from Becton Dickinson, San Jose, CA, USA. The whole-blood staining procedure was done as described elsewhere [57]. Samples were analyzed using a FACScan flow cytometer and CellQuest software (both from Becton Dickinson, San Jose, CA, USA). Calibration beads (Dako, Copenhagen, Denmark) were run on a weekly basis, to ensure the stability of the flow cytometer. Percentages of HLA-DR+ cells were analyzed within the CD4+ and CD8+ T cell subsets. Percentages of CD38+ and CD38+ HLA-DR+ double-positive cells were analyzed within the bright CD8+ lymphocyte subset. Percentages of CD62L+ cells were analyzed within CD45RO+ (memory) and CD45RO− (naive) CD4+ lymphocyte subsets. For all analyses, quadrant markers were set using negative isotype controls.

**HIV-1 coreceptor expression.** Levels of HIV-1 coreceptor expression were analyzed on fresh whole blood within 4 h of collection in EDTA. Cells were stained with a combination of peridinin-chlorophyll-protein-labeled anti-CD4 and fluorescein isothiocyanate-labeled anti-CD45RO (both from Becton Dickinson, San Jose, CA, USA), and phycoerythrin (PE)-labeled anti-CCR5 and anti-CXCR4 MAbs (both from Pharmingen, San Diego, CA, USA), to assess the membrane expression of CCR5 and CXCR4 on CD45RO+ (memory) and CD45RO− (naive) CD4+ lymphocytes. Whole-blood staining was done as described above for T cell activation markers. HIV-1 coreceptor expression was calculated as the percentage of positive cells or as the median logarithmic fluorescence intensity (MFI).

**Intracellular staining of β-chemokines and cytokines.** Peripheral blood mononuclear cells (PBMC) were separated from fresh whole blood within 4 h of collection and stained intracellularly for β-chemokines and cytokines, as described elsewhere [57]. In brief, PBMC were incubated overnight in the presence of monensin (1.5 µg/ml; Sigma, St. Louis, MO, USA) for the constitutive intracellular accumulation of the β-chemokine RANTES (regulated on activation, normal T cell
expressed and secreted) and for 5 h with phorbol myristate acetate (0.02 µg/ml) and ionomycin (1 µg/ml) in the presence of brefeldin A (10 µg/ml) (all from Sigma, St. Louis, MO, USA) for the stimulation-induced intracellular accumulation of the β-chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β and the cytokines interleukin (IL)-2, IL-4, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. PBMC were subsequently surface stained for CD3 and CD8, fixed, permeabilized, and stained intracellularly with anti-RANTES, anti-MIP-1α (both from Pharmingen, San Diego, CA, USA); anti-IL-2, anti-IL-4, anti-IFN-γ, or anti-TNF-α (all from Becton Dickinson, San Jose, CA, USA) PE-labeled MAbs or anti-MIP-1β carboxyfluorescein-labeled MAb (R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature. Samples were analyzed using a FACSscan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). Percentages of β-chemokine-positive and cytokine-positive cells were analyzed within the CD4+ and CD8+ T cell subsets. For all analyses, quadrant markers were set using negative isotype controls.

Statistical analysis. Because there were 2 different study periods and because we wanted to minimize bias as a result of changing laboratory conditions, ESN FSWs and blood donors with approximately the same date of laboratory analysis were used for comparisons (n = 20 for both groups). Median values were calculated for normal and non-normal distributions. Differences between groups were tested for statistical significance by use of the non-parametric Mann-Whitney U test. Correlation analyses were performed for all 27 subjects in both study groups by use of the non-parametric Spearman rank correlation test. The level of significance for all statistical tests was P < 0.05.

RESULTS

Characteristics of the study population. The median age of the 27 ESN FSWs was 23 years (range, 17 – 37 years), compared to 23 years (range, 18 – 52 years) for the 27 female blood donors (P = 0.326). The median duration of commercial sex work reported by 27 ESN FSWs was 6 months (range, 1 – 120 months). Three FSWs (11 %) reported commercial sex work duration of ≥ 3 years, 11 (41 %) reported doing commercial sex work for 6 months to 3 years, and 13 (48 %) reported doing commercial sex work for < 6 months. The reported number of clients on the last working day ranged from 1 to 14 (median, 3 clients). Ten FSWs (37%) reported “always” using condoms, 14 (52%) reported “often” using condoms, and 3 (11%) reported “rarely” or “never” using condoms. The reported consistency in condom use decreased with prolonged duration of commercial sex work (r = -0.517, P = 0.006) and increased with higher reported numbers of clients per day (r = 0.554, P
The number of clients per day tended to decrease with prolonged duration of commercial sex work \((r = -0.364, P = 0.062)\). Of the 27 ESN FSWs, 5 (19\%) had 1 STI: 2 tested positive for \(T.\ vaginalis\), 2 tested positive for \(C.\ trachomatis\), 1 tested positive for \(N.\ gonorrhoea\), and none tested positive for \(T.\ pallidum\).

**Table 1.** Expression of T cell activation markers on CD4\(^+\) and CD8\(^-\) T cell subsets from HIV-seronegative blood donors and ESN FSWs.

<table>
<thead>
<tr>
<th>T cell activation marker, T cell subset</th>
<th>Blood donors ((n = 20))</th>
<th>ESN FSWs ((n = 20))</th>
<th>(P^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR(^+), CD4(^+) T cells</td>
<td>12.9 (9.4 – 18.2)</td>
<td>12.7 (9.7 – 16.4)</td>
<td>0.766</td>
</tr>
<tr>
<td>HLA-DR(^+), CD8(^+) T cells</td>
<td>22.0 (14.5 – 32.0)</td>
<td>23.0 (18.6 – 33.3)</td>
<td>0.589</td>
</tr>
<tr>
<td>CD38(^+), CD8(^-) T cells</td>
<td>70.1 (62.1 – 78.1)</td>
<td>76.6 (72.3 – 84.6)</td>
<td>0.017</td>
</tr>
<tr>
<td>HLA-DR(^+)/CD38(^-), CD8(^+) T cells</td>
<td>56.6 (48.7 – 67.2)</td>
<td>63.3 (52.7 – 71.8)</td>
<td>0.144</td>
</tr>
<tr>
<td>CD62L(^+), CD45RO(^+)/CD4(^+) T cells</td>
<td>56.4 (51.4 – 66.4)</td>
<td>60.1 (48.4 – 67.4)</td>
<td>0.607</td>
</tr>
<tr>
<td>CD62L(^+), CD45RO(^-)/CD4(^+) T cells</td>
<td>96.1 (93.7 – 97.9)</td>
<td>94.0 (86.8 – 96.3)</td>
<td>0.062</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) ex vivo percentage of T cell activation marker expression. \(^2\)Two-tailed non-parametric Mann-Whitney U test. \(P\) values < 0.05 are in bold.

**ESN FSWs had increased T cell activation, compared with blood donors.**

ESN FSWs had significantly higher percentages of CD38\(^+\) cells in CD8\(^+\) lymphocytes than did blood donors (table 1). Moreover, ESN FSWs tended to have lower percentages of true naive (CD62L\(^+\) CD45RO\(^+\)) CD4\(^+\) T lymphocytes, compared with blood donors. No differences were found in percentages of HLA-DR\(^+\) cells in CD4\(^+\) and CD8\(^+\) T cell subsets. Since multiple statistical comparisons were performed (table 1), there is an increased probability that the statistically significant result for CD38 expression on CD8\(^+\) T cells has occurred by chance. Among ESN FSWs, no associations were found between CD38, CD62L, or HLA-DR expression and the duration of commercial sex work, number of clients per day, condom use, or the occurrence of an STI.

**ESN FSWs had similar expression levels of the HIV-1 coreceptor CCR5 but significantly decreased levels of CXCR4, compared with blood donors.** Figure 1 shows representative histograms for the analysis of percentages of positive cells and MFI levels of CCR5 and CXCR4 in one blood donor and one ESN FSW for CD45RO\(^+\) CD4\(^+\) and CD45RO\(^-\) CD4\(^+\) lymphocytes. For CCR5 (figure 1A), the
markers M1 and M2 clearly discriminate CCR5<sup>+</sup> and CCR5<sup>+</sup> cell distributions in the CD45RO<sup>-</sup>CD4<sup>+</sup> and CD45RO<sup>+</sup>CD4<sup>+</sup> lymphocyte subsets. Therefore, relative cell numbers of CCR5<sup>+</sup> cells (marker M2) were recalculated in percentages of CCR5<sup>+</sup> cells and in median logarithmic fluorescence intensity (MFI) levels of the CCR5<sup>+</sup> distributions within the CD45RO<sup>-</sup>CD4<sup>+</sup> and CD45RO<sup>+</sup>CD4<sup>+</sup> lymphocyte subsets. MFI levels of the CCR5<sup>+</sup> clusters were calculated as a measurement of CCR5 expression density on CCR5<sup>+</sup> cells. CXCR4 expression on lymphocytes was not characterized by discrete distributions of positive and negative cells (figure 1B). Therefore, MFI levels of the whole CXCR4 distributions in the CD45RO<sup>-</sup>CD4<sup>+</sup> and CD45RO<sup>+</sup>CD4<sup>+</sup> lymphocyte subsets were calculated. In both ESN FSWs and blood donors, CCR5 was almost exclusively expressed on CD45RO<sup>-</sup>CD4<sup>+</sup> T cells, whereas the expression of CXCR4 was higher on CD45RO<sup>-</sup>CD4<sup>+</sup> cells than on CD45RO<sup>-</sup>CD4<sup>+</sup> T cells (table 2), which confirms previous observations [58, 59].
Table 2. Expression of HIV-1 coreceptors CCR5 and CXCR4 in CD4⁺ lymphocyte subsets from HIV-seronegative female blood donors and ESN FSWs.

<table>
<thead>
<tr>
<th>HIV-1 coreceptor, Lymphocyte subset</th>
<th>Blood donors (n = 20)</th>
<th>ESN FSWs (n = 20)</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All CD4⁺ cells (%)</td>
<td>19.6 (15.2 – 24.1)</td>
<td>17.8 (15 – 21.2)</td>
<td>0.534</td>
</tr>
<tr>
<td>All CD4⁺ cells (MFI)</td>
<td>20.6 (17.9 – 25.2)</td>
<td>24.6 (20.2 - 30.3)</td>
<td>0.144</td>
</tr>
<tr>
<td>CD45RO⁻/CD4⁺ cells (%)</td>
<td>1.5 (0.6 - 3)</td>
<td>1.4 (0.7 - 3.5)</td>
<td>0.946</td>
</tr>
<tr>
<td>CD45RO⁻/CD4⁺ cells (MFI)</td>
<td>14.6 (12.1 – 17.6)</td>
<td>13.6 (12.9 – 19.3)</td>
<td>0.808</td>
</tr>
<tr>
<td>CD45RO⁺/CD4⁺ cells (%)</td>
<td>29.4 (25.6 – 32)</td>
<td>27.7 (23.5 - 36.4)</td>
<td>0.978</td>
</tr>
<tr>
<td>CD45RO⁺/CD4⁺ cells (MFI)</td>
<td>20.6 (18.4 – 25)</td>
<td>24.7 (20.2 - 31.2)</td>
<td>0.168</td>
</tr>
<tr>
<td>CXCR4&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All CD4⁺ cells (MFI)</td>
<td>55.3 (45.3 – 75.3)</td>
<td>47.4 (37.3 – 56.5)</td>
<td>0.045</td>
</tr>
<tr>
<td>CD45RO⁻/CD4⁺ cells (MFI)</td>
<td>93.5 (79.5 – 109.2)</td>
<td>81.7 (65.1 - 87.6)</td>
<td>0.041</td>
</tr>
<tr>
<td>CD45RO⁺/CD4⁺ cells (MFI)</td>
<td>37.1 (29.6 – 49.9)</td>
<td>25.7 (22.2 – 29.6)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) ex vivo percentages or median logarithmic fluorescence intensity (MFI). <sup>a</sup>Two-tailed non-parametric Mann-Whitney U test. <sup>b</sup>P values < 0.05 are in bold. <sup>c</sup>CCR5 MFI levels were calculated as MFI levels of CCR5⁺ cells. <sup>c</sup>CXCR4 MFI levels were calculated as MFI levels of all cells.

Percentages and MFI levels of CCR5⁺ memory, naive and total CD4⁺ T cells were similar between ESN FSWs and blood donors (table 2). Percentages of CCR5⁺ memory CD4⁺ cells among ESN FSWs correlated significantly with percentages of HLA-DR⁺ CD4⁺ T cells (r = 0.621, P = 0.003), HLA-DR⁺ CD8⁺ T cells (r = 0.502, P = 0.024), and HLA-DR⁺ CD38⁺ CD8⁺ T cells (r = 0.582, P = 0.007). None of these correlations was found among blood donors. Among ESN FSWs, no associations were found between CCR5 expression levels and the duration of commercial sex work, number of clients per day, condom use, or the occurrence of an STI.

MFI of CXCR4 expression on memory, naive, and total CD4⁺ T cells was significantly lower in ESN FSWs than in blood donors (table 2). No associations were found between CXCR4 expression and T cell activation among either ESN FSWs or blood donors. CXCR4 expression levels tended to correlate inversely with the duration of commercial sex work for ESN FSWs, within both naive (r = -0.279, P = 0.176) and memory (r = -0.276, P = 0.182) CD4⁺ T cells. To explore further the relationship between CXCR4 expression and the duration of commercial sex
work, 10 ESN FSWs who had done sex work for < 6 months were compared with 10 ESN FSWs who had done sex work ≥ 6 months, with approximately the same date of laboratory analysis, to eliminate bias as a result of changing laboratory conditions over time (figure 2). ESN FSWs who had done sex work for ≥ 6 months had lower CXCR4 MFI levels than did ESN FSWs who had done sex work for < 6 months, reaching significant levels in the naive (CD45RO−) CD4+ T cell subset but not in the memory (CD45RO+CD45RO−) CD4+ T cell subset. Although CXCR4 MFI levels on naive CD4+ cells tended to correlate inversely with age among ESN FSWs (r = -0.331, P = 0.106), this was not found to be the case among blood donors. In addition, duration of sex work did not correlate with age (r = 0.183, P = 0.360) among ESN FSWs, and median age in the groups of ESN FSWs who had done sex work for ≥ 6 months or < 6 months was not different (P = 0.239). Therefore, age could be excluded as a significant confounding factor in the analysis. Among ESN FSWs, no associations were found between CXCR4 expression and the number of clients per day, consistency of condom use, or the occurrence of an STI.

Figure 2. CXCR4 median logarithmic fluorescence intensity (MFI) levels of CD45RO− naive and CD45RO+ memory CD4+ cell subsets for 20 female blood donors (white boxes), 10 ESN FSWs who had done sex work for < 6 months (gray boxes), and 10 ESN FSWs who had done sex work for ≥ 6 months (black boxes). Box plots represent the lowest value, the 25th percentile, the 50th percentile (median), the 75th percentile, and the highest value, respectively, indicated by horizontal lines in ascending order. Differences between groups were tested for statistical significance by use of the non-parametric Mann-Whitney U test. P1, female blood donors vs. all ESN FSWs; P2, ESN FSWs who had done sex work for < 6 months vs. ESN FSWs who had done sex work for ≥ 6 months.
Table 3. Percentages of β-chemokine-positive cells and Th1 and Th2 cytokine-positive cells in CD4⁺ and CD8⁺ T cell subsets from HIV-seronegative female blood donors and ESN FSWs.

<table>
<thead>
<tr>
<th>Chemokine or cytokine, T cell subset</th>
<th>Blood donors (n = 20)</th>
<th>ESN FSWs (n = 20)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-chemokines</strong>(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% RANTES⁺ of CD4⁺ T cells</td>
<td>15.5 (12.0 – 19.4)</td>
<td>13.1 (8.9 – 21.8)</td>
<td>0.387</td>
</tr>
<tr>
<td>% RANTES⁺ of CD8⁺ T cells</td>
<td>59.8 (40.2 – 67.3)</td>
<td>56.9 (42.3 – 68.1)</td>
<td>0.829</td>
</tr>
<tr>
<td>% MIP-1α⁺ of CD4⁺ T cells</td>
<td>4.1 (2.9 – 7.5)</td>
<td>5.0 (2.9 – 7.1)</td>
<td>0.626</td>
</tr>
<tr>
<td>% MIP-1α⁺ of CD8⁺ T cells</td>
<td>9.4 (5.5 – 18.3)</td>
<td>11.1 (5.2 – 22.2)</td>
<td>0.626</td>
</tr>
<tr>
<td>% MIP-1β⁺ of CD4⁺ T cells</td>
<td>4.9 (3.2 – 8.3)</td>
<td>5.1 (2.4 – 7.6)</td>
<td>0.892</td>
</tr>
<tr>
<td>% MIP-1β⁺ of CD8⁺ T cells</td>
<td>25.5 (13.5 – 33.7)</td>
<td>20.4 (14.7 – 33.8)</td>
<td>0.808</td>
</tr>
<tr>
<td><strong>Cytokines</strong>(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL-2⁺ of CD4⁺ T cells</td>
<td>22.2 (18.4 – 30.0)</td>
<td>25.2 (19.2 – 33.4)</td>
<td>0.402</td>
</tr>
<tr>
<td>% IL-2⁺ of CD8⁺ T cells</td>
<td>6.0 (4.3 – 8.9)</td>
<td>6.6 (5.2 – 11.4)</td>
<td>0.245</td>
</tr>
<tr>
<td>% IFN-γ⁺ of CD4⁺ T cells</td>
<td>17.6 (11.8 – 21.9)</td>
<td>17.9 (12.4 – 23.3)</td>
<td>0.665</td>
</tr>
<tr>
<td>% IFN-γ⁺ of CD8⁺ T cells</td>
<td>18.7 (13.2 – 26.1)</td>
<td>21.8 (14.3 – 32.1)</td>
<td>0.499</td>
</tr>
<tr>
<td>% TNF-α⁺ of CD4⁺ T cells</td>
<td>27.2 (16.2 – 40.3)</td>
<td>37.7 (22.9 – 46.6)</td>
<td>0.245</td>
</tr>
<tr>
<td>% TNF-α⁺ of CD8⁺ T cells</td>
<td>9.6 (5.9 – 17.4)</td>
<td>12.2 (6.4 – 21.4)</td>
<td>0.499</td>
</tr>
<tr>
<td>% IL-4⁺ of CD4⁺ T cells</td>
<td>5.2 (3.9 – 6.1)</td>
<td>6.4 (4.5 – 7.7)</td>
<td>0.105</td>
</tr>
<tr>
<td>% IL-4⁺ of CD8⁺ T cells</td>
<td>3.5 (2.7 – 4.6)</td>
<td>3.6 (2.9 – 5.8)</td>
<td>0.516</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) in vitro percentages of β-chemokine-positive cells and Th1 and Th2 cytokine-positive cells. IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor. \(^b\)Two-tailed non-parametric Mann-Whitney U test. \(^c\)Intracellular accumulation was stimulation induced, except for RANTES. IL-2, IFN-γ, and TNF-α are Th1 cytokines; IL-4 is a Th2 cytokine.

ESN FSWs and blood donors had similar percentages of β-chemokine-positive T cells. RANTES was the most abundant β-chemokine detected in both CD4⁺ and CD8⁺ T cells, being constitutively expressed at higher percentages than mitogen-stimulated MIP-1α or MIP-1β (table 3). Percentages of RANTES⁺, MIP-1α⁺ and MIP-1β⁺-positive cells in CD4⁺ and CD8⁺ T cell subsets were comparable between ESN FSWs and blood donors (table 3). Among ESN FSWs, percentages of HLA-DR-positive cells correlated with percentages of RANTES-positive cells in the CD8⁺ T cell subset (\( r = 0.459, P = 0.032 \)) and with percentages of MIP-1β⁺-positive
cells in both CD4+ ($r = 0.478, P = 0.033$) and CD8+ ($r = 0.549, P = 0.012$) T cell subsets. Among blood donors, percentages of HLA-DR-positive cells correlated with MIP-1β-positive cells in the CD8+ T cell subset ($r = 0.522, P = 0.018$). Among ESN FSWs, no correlations were found between proportions of β-chemokine-positive T cells and duration of commercial sex work, number of clients per day, the occurrence of and STI, or condom use.

*ESN FSWs and blood donors had similar percentages of cytokine-positive cells.* Percentages of CD4+ and CD8+ T cells that were positive for the Th1 cytokines IL-2, IFN-γ, and TNF-α and the Th2 cytokine IL-4 were comparable among ESN FSWs and blood donors (table 3). Percentages of CD38+ HLA-DR+ CD8+ T cells correlated with percentages of IL-2-positive ($r = 0.538, P = 0.01$), IFN-γ-positive ($r = 0.621, P = 0.002$), and TNF-α-positive ($r = 0.525, P = 0.012$) CD8+ T cells among ESN FSWs and with IFN-γ-positive CD8+ T cells ($r = 0.407, P = 0.06$) among blood donors. Among ESN FSWs, no correlations were found between proportions of cytokine-positive T cells and the duration of commercial sex work or use of condoms, but the number of clients per day correlated positively with proportions of IL-2-positive CD4+ T cells ($r = 0.512, P = 0.015$) and IL-4-positive CD4+ T cells ($r = 0.479, P = 0.024$). No statistically significant associations were found between proportions of cytokine-positive T cells and the occurrence of an STI among ESN FSWs.

**DISCUSSION**

In Abidjan, Côte d’Ivoire, HIV prevalence among FSWs has decreased extensively, from 89% in 1992 to 32% in 1998 [52], and 28% in 2000 (authors’ unpublished data), thanks to intensive prevention efforts and increasing condom use. However, although reported condom use is high with clients (79% consistent condom use with clients during the last working day in 1998 [52, 60]), only 20% of the FSWs use condoms consistently with their stable partners (authors’ unpublished data). The HIV incidence among FSWs participating in a trial on microbicides from 1998-2000 was 4% [61], indicating that seronegative FSWs in Abidjan are still frequently exposed to HIV and at risk of acquiring infection. In 2000, HIV seroprevalence among first-time blood donors (men and women) in Abidjan was estimated at 6% [62]. Ten percent of pregnant women attending an antenatal clinic in Abidjan from 1997-2000 tested HIV-seropositive [63]. These data indicate that, despite targeting a population that is at lower risk for HIV infection than HIV-seronegative FSWs, blood donors, especially young female blood donors, cannot be considered to be HIV-unexposed.
In this study, a group of ESN FSWs was compared with a group of HIV-seronegative female blood donors for the presence of immunological factors that could contribute to decreased susceptibility to HIV infection. Our findings indicate that ESN FSWs and HIV-seronegative female blood donors have comparable expression levels of the HIV-1 coreceptor CCR5, β-chemokines, and Th1 or Th2 cytokines, but that ESN FSWs have significantly decreased levels of CXCR4 expression and significantly increased levels of CD8+ T cell activation.

Increased CD8+ T cell activation could be a sign of anti-HIV activity by HIV-specific CD8+ cytotoxic T cells [48]. Indeed, several studies have described HIV-specific CTL responses in HIV-seronegative subjects in association with frequent sexual exposure to HIV [20, 21, 24, 25]. However, in the present study, CD8+ T cell activation among ESN FSWs did not correlate with any marker of sexual HIV exposure. It is possible that CD8+ T cell activation is associated with other infections and STIs in ESN FSWs, rather than being related to HIV. Similar to results of a previous study among HIV-infected FSWs in Abidjan [64], we did not observe any statistically significant association between T cell activation markers and the occurrence of protozoan or bacterial STIs. It is more likely that viral non-HIV concurrent infections and STIs (which were not analyzed in this study), rather than protozoan or bacterial STIs, play a role in T cell activation among ESN FSWs.

Previous studies in Kenya and Ethiopia reported similar expression levels of CCR5 in ESN FSWs, compared with those in unexposed control subjects, as in our study, but found increased rather than decreased levels of CXCR4 [37, 38]. In these studies, however, HIV-1 coreceptor expression was measured on fresh or cryopreserved PBMC, whereas, in the present study, fresh whole blood was used. Variation in technical procedures can highly influence the analysis of HIV-1 coreceptor expression levels. This has been shown for prolonged sample storage before analysis, which causes increased CXCR4 and decreased CCR5 levels [65] and for density gradient isolated PBMC with significantly lower levels of CCR5 [66] and CXCR4 (authors’ unpublished data), compared with whole blood. The use of different anticoagulants can also influence laboratory results [65, 66].

Decreased levels of CXCR4 among ESN FSWs in our study were found to be associated with prolonged duration of sex work. This association was found to be independent from T cell activation, since T cell activation did not correlate with either CXCR4 expression or the duration of sex work. In vitro, HIV-1 envelope gp120 has been shown to down-regulate CXCR4 [67, 68]. Similarly in vivo, chronic HIV infection results in down-regulation of CXCR4 expression [59, 69-
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...Therefore, in the same way as in HIV-infected subjects, chronic exposure to HIV among ESN FSWs could have caused the down-regulation of CXCR4. Alternatively, it is also possible that factors other than HIV are responsible for the down-regulation of CXCR4 in ESN FSWs, as has been reported for infection with human herpesvirus (HHV)-6 and HHV-7 [72, 73], or through allogeneic activation [74], both of which could have been acquired gradually by FSWs who had done sex work for a prolonged duration.

The relevance of CXCR4 down-regulation in ESN FSWs as a correlate of protection against HIV infection remains unclear and can only be addressed in a longitudinal study. Theoretically, it is unlikely that CXCR4 plays a role, since primary HIV-1 strains that are sexually-transmitted, M-tropic strains, predominantly use CCR5 as a coreceptor [27, 29, 75], an observation that has been confirmed in Abidjan (authors’ unpublished data). Moreover, the few reported cases of HIV-1 transmission via CXCR4 have all occurred in subjects homozygous for the 32 base pair deletion in the CCR5 gene [76, 77]. As shown previously for other African populations [37, 78], this 32 base pair deletion has not been detected among ESN FSWs in Abidjan [79]. Still, it would be interesting to investigate whether the down-regulated CXCR4 expression among ESN FSWs could provide a protective mechanism against T-tropic HIV-1 viruses that selectively use CXCR4 as a coreceptor. If this were proven to be the case, as a mechanism, CXCR4 down-regulation could have contributed to the selection of CCR5-using M-tropic HIV-1 strains in sexual transmission.

In most studies, β-chemokine levels in ESN subjects have been measured in plasma samples or in the supernatant of mitogen-stimulated lymphocyte cultures, without providing information on the identity of the cells that produced the β-chemokines [36-38, 43-46]. Therefore, we measured proportions of β-chemokine-producing CD4+ and CD8+ T cells in ESN FSWs by means of flow cytometry after spontaneous (for RANTES) and mitogen-stimulated (for MIP-1α and MIP-1β) intracellular accumulation in vitro. Our results show that proportions of β-chemokine-producing CD4+ and CD8+ T cells were comparable between ESN FSWs and blood donors and did not correlate with any indicative marker of HIV exposure of the FSWs. These data are in agreement with findings from other studies of ESN FSW populations [37, 38]. In a previous study, we found a positive correlation between proportions of β-chemokine-producing T cells and HIV-1 viral load in HIV-1-infected FSWs, which indicates that intracellular β-chemokine expression is a correlate of disease progression rather than of protection [57].
Proportions of T cells producing the Th1 cytokine IL-2, TNF-α and IFN-γ were similar in ESN FSWs and blood donors. Nevertheless, production of Th1 cytokines correlated more clearly with in vivo T cell activation among the ESN FSWs than among blood donors. Therefore, our data are more in line with augmented T cell activation and Th1 cytokine production in ESN subjects, as reported elsewhere [48] and do not confirm a down-regulated Th1 cytokine profile in ESN subjects in association with a postulated HIV-protective low-grade activation state of CD4⁺ T cells [51]. Our data also show comparable percentages of IL-4-producing T cells among ESN FSWs and female blood donors and, therefore, do not support the recent observation of a down-regulated Th2 cytokine profile in ESN FSWs [50].

In summary, we found that ESN FSWs in Abidjan have similar expression levels of the HIV-1 coreceptor CCR5, β-chemokines, and Th1 and Th2 cytokines, compared with HIV-seronegative female blood donors, but have significantly decreased levels of CXCR4 expression, together with significantly increased levels of T cell activation. The data indicate that ESN FSWs differ from female blood donors based on immunological factors that have no clear protective potential against HIV transmission.

ACKNOWLEDGEMENTS

We thank the community of female sex workers in Abidjan, for their cooperation; Marie-Yolande Borget, Mathieu Maran, Daniel Yavo, and N’Depo Yenon, for technical assistance; Emmanuel Abonga, for providing blood samples from female blood donors; Jef Braem and Serge Blockmans, for logistical support in the field; and Monica Nolan, for critical remarks and discussion. This work was supported by the Belgian Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen, grant G.0396.99 and by the Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention (CDC), Atlanta, GA.

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Tolerance towards alloantigens in HIV-exposed seronegative female sex workers in Abidjan, Côte d’Ivoire

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Submitted

ABSTRACT

In an effort to define correlates of protection against HIV infection, humoral and cellular alloimmune responses were analysed in a group of HIV-exposed seronegative (ESN) female sex workers (FSWs) and compared with HIV-seronegative female blood donors at lower risk for HIV infection in Abidjan, Côte d’Ivoire. Anti-HLA class I antibodies were measured ex vivo in plasma by flow cytometry, and were correlated to in vitro neutralising capacity of plasma against an HIV-1 primary isolate common in Abidjan. In vitro cellular activation and secretion of cytokines and β-chemokines was analysed in auto-, allo- and mitogen-stimulated lymphocyte cultures. Occurrence of anti-HLA class I antibodies in plasma was comparable among ESN FSWs, HIV-infected FSWs and female blood donors and was rather a function of age of the subjects. In addition, the presence of anti-HLA class I antibodies did not correlate with in vitro HIV neutralising activity. Compared with female blood donors, ESN FSWs had significantly decreased cellular alloimmune responses at the level of lymphocyte activation and at the level of cytokine and β-chemokine supernatant secretion, but showed increased mitogen-stimulated responses. These findings may indicate that frequent unprotected sexual exposure results in tolerisation rather than activation of cell-mediated immune responses towards alloantigens. The data suggest that protection against infection with HIV among ESN FSWs is not associated with increased alloimmune responses, and discourage the induction of alloreactivity as a strategy to prevent HIV transmission.
INTRODUCTION

The possibility that immune reactivity against allogeneic human leukocyte antigens (HLA) could contribute to protection against human immunodeficiency virus (HIV) infection has been proposed [1-3]. This suggestion is based on several observations. Protection against infection with simian immunodeficiency virus (SIV) was induced in macaques solely by vaccination with uninfected human cells that were used to grow the SIV [4]. The sera from these macaques contained anti-HLA class I antibodies that correlated with protection [5, 6]. Alloimmunisation with the male partner’s PBMC in women with recurrent spontaneous abortions elicited upregulation of chemokines and HIV suppressor factors, and resistance to in vitro infection with M- and T-tropic HIV-1 viruses [7, 8]. HIV acquires HLA class I and class II molecules during the budding process from the host cell that outnumber the viral gp120 envelope proteins [9, 10]. In addition, viral gp120 and HLA share a degree of homology potentially leading to cross-reactive immune responses [11, 12]. Together, these observations indicate that alloimmune responses may protect against sexual HIV transmission by two distinct mechanisms. Humoral anti-HLA antibodies may neutralise free HIV virions by binding to HLA, or possibly gp120, present on the viral envelope. Cellular alloimmune responses may directly reject the partner’s infected allogeneic cells before virus production from these cells and infection can occur [2].

Some individuals remain HIV-seronegative despite frequent exposure to the virus [13-15], and several mechanisms of protection against HIV infection have been proposed [16-19]. Genetic studies have shown particular HLA polymorphisms in these persons. HIV-exposed (ESN) female sex workers (FSWs) in Nairobi displayed rare HLA alleles, thus being capable of manifesting alloimmune responses against the largest proportion of the population [20], and possessed specific HLA supertypes that were associated with a reduced risk of HIV transmission [21]. Similarly, a decreased risk of HIV-1 transmission from mother to child and in heterosexual couples was associated with HLA class I discordance or with particular HLA alleles [22-25]. However, the alloimmune responses that are expected to result from these particular HLA polymorphisms in ESN subjects have been more difficult to detect. Anti-HLA class I alloantibodies were found in ESN FSWs and in children of HIV-infected mothers but did not correlate with protection against HIV infection [26, 27]. To date, cellular alloimmune responses and their role in protection against HIV have not been studied in ESN subjects.

In the present study, in an attempt to identify correlates of protection against HIV infection, we analysed humoral and cellular alloimmune responses in African FSWs that remained HIV-seronegative despite frequent sexual exposure for more
than three years, and compared them with African HIV-seronegative female blood donors at lower risk for HIV infection. Surprisingly, we found decreased rather than increased cellular alloimmune responses in ESN FSWs compared with controls.

MATERIAL AND METHODS

Study population. Between June 1998 and July 1999, we enrolled 21 HIV-seronegative and 20 HIV-seropositive FSWs in a confidential clinic in Abidjan, Côte d’Ivoire. These women were part of a multiple-center trial testing the efficacy of the HIV microbicide nonoxynol-9 [28]. Blood samples and standard questionnaires with information on socio-demographics and sexual behavior were collected. None of the HIV-seropositive FSWs had received antiretroviral therapy prior to enrollment. Blood samples were also collected from 20 HIV-seronegative female blood donors and from 24 HIV-seronegative male blood donors at the national blood transfusion centre in Abidjan. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA, USA, and the Ethical Committees of the Ministry of Health, Côte d’Ivoire and the Institute of Tropical Medicine, Antwerp, Belgium. Informed consent was given by all study subjects before enrollment.

Laboratory methods. Whole blood was drawn from FSWs and female blood donors in EDTA tubes (Becton Dickinson, San Jose, CA). Within 4 h of collection, plasma was separated from whole blood by centrifugation, and stored at −70 °C. The HIV status of all subjects was determined in plasma by using an HIV testing algorithm based on a combination of ELISAs [29, 30]. The HIV-negative status of seronegative FSWs was confirmed by HIV-1 reverse transcriptase-polymerase chain reaction [31]. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by gradient centrifugation using lymphocyte separation medium (ICN Biomedicals, Aurora, OH, USA), resuspended in RPMI containing 50% foetal calf serum (Life Technologies, Paisly, UK) and 10% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and stored in liquid nitrogen.

Anti-HLA class I antibodies. Antibodies directed against HLA class I molecules were detected by the HLA panel reactive antibody (PRA) screening test (One Lambda, Canoga Park, CA, USA). The method is based on polystyrene microparticle beads coated with a wide range of HLA class I antigens. Based on preliminary screening of HLA class I subtypes in 31 blood donors in Abidjan, Côte d’Ivoire (unpublished data from A. Chahroudi, CDC, Atlanta), the test covered 100% of prevalent HLA subtype A alleles, 81% of subtype B alleles, and 92% of
subtype C alleles. Testing was carried out according to the manufacturer’s instructions. In brief, beads were incubated with plasma for 30 min, washed twice in wash buffer at 9000 g, and incubated with fluorescein isothiocyanate-labeled goat anti-human IgG antibodies for 30 min. Beads were washed again twice, resuspended in PBS containing 4% paraformaldehyde (PFA), and analysed by flow cytometry. For every experiment, positive and negative control samples were run together with the test samples. Percentages of HLA class I PRA were analysed in an histogram plot with a marker M1 set at the end of the negative control. The criteria for a positive result were (1) a positive reaction with the positive control, (2) percentage PRA of at least 10%, and (3) presence of a multiple peak pattern discriminating a negative and a positive bead population. Figure 1A shows representative examples for the analysis of HLA class I PRA for one female blood donor (negative result) and one ESN FSW (positive result).

Neutralisation assays. A primary isolate, CI 20, belonging to HIV-1 subtype CRF_02 (subtype A/G) which is the predominant HIV-1 strain in Abidjan [32], was assayed for neutralisation as described elsewhere [33]. Briefly, virus was diluted in a five fold series from 1/2 to 1/6250. 10 µl plasma were added to 190 µl of each virus dilution, and pre-incubated for 24 h at 37 °C and 5% CO2. Residual virus infectivity was detected by mixing 20 µl of the virus-plasma mixture with 7.5 x 10^4 PHA-stimulated PBMC in 100 µl of RPMI (BioWhittaker, Verviers, Belgium) containing 15% FCS (BioWhittaker, Verviers, Belgium), 0.03% L-glutamine (BioWhittaker, Verviers, Belgium), 2 µg/ml hydrocortisone (Roche Diagnostics, Brussels, Belgium), 2 μg/ml hexadimetrine bromide (Sigma-Aldrich, Bornem, Belgium), 0.05 mg/ml gentamicin (BioWhittaker, Verviers, Belgium) and 20 units/ml recombinant interleukin (IL)-2 (Roche Diagnostics, Brussels, Belgium), and incubated in quadruplicate wells for 1 h at 37 °C and 5% CO2. Cells were washed three times, incubated for 7 days, refreshed, and incubated for another 7 days. Supernatants were then harvested and analysed for the presence of antigen using an in house HIV-1 specific ELISA. Neutralisation indices were calculated as the virus dilution that infects 50% of the cultures (log_{10} TCID_{50}) in the presence of negative plasma divided by log_{10} TCID_{50} in the presence of test plasma. A neutralisation index equal or higher than 0.7 (corresponding with 80% neutralisation) was considered as neutralising.

Cellular alloimmune responses. Cryopreserved PBMC were thawed, washed twice and resuspended in RPMI containing L-glutamine (Life Technologies, Paisley, UK), 5% human AB serum (Irvine Scientific, Santa Ana, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany). To prepare autologous stimulator cells, freshly
2.2. ALLOIMMUNITY AND PROTECTION AGAINST HIV INFECTION

Thawed PBMC from every subject were set at a concentration of $1 \times 10^6$ cells/ml in PBS containing 1% bovine serum albumin (BSA), labeled with 10 µM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37 °C and 5% CO₂, and washed three times in ice-cold RPMI containing 10% foetal calf serum. Cells were then fixed in PBS containing 4% PFA for 10 min at room temperature, washed again three times, and resuspended in RPMI containing 5% human AB serum. To prepare allogeneic stimulator cells, PBMC were separated from fresh whole blood in EDTA from 24 HIV-seronegative male blood donors, pooled together, and CFSE-labelled and PFA-fixed as described above. One ml cultures containing $1 \times 10^6$ responder PBMC from female blood donors or ESN FSWs together with $1 \times 10^6$ autologous stimulator PBMC (autostimulation, negative control), $1 \times 10^6$ allogeneic stimulator PBMC (allostimulation), or 0.5 µg/ml PHA (PHA-stimulation, positive control) were incubated in duplicate wells of 48-well plates at 37 °C and 5% CO₂.

To assess early responses, cellular expression of an early activation marker CD69 was measured after 40 hours of culture. Cells were washed in PBS containing 1% BSA and 0.05% NaN₃, and surface stained with fluorescein isothiocyanate (FITC)-labeled anti-CD3, phycoerythrin-labeled anti-CD69, allophycocyanin-labeled anti-CD45RO (all from Becton Dickinson, San Jose, CA, USA), and R-phycoerythrin-cyanin-5-labeled anti-CD4 (Dako, Copenhagen, Denmark) monoclonal antibodies for 15 min. Cells were then washed again, resuspended in PBS with 1% PFA, and analysed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). With a gate around the small lymphocytes, CD69 expression was analysed in lymphocyte and T lymphocyte subsets, and in total, memory (CD45RO⁺) and naive (CD45RO⁻) CD4⁺ and CD8⁺ (as CD4⁻) T cell subsets within the responder cell fraction. CFSE-stained stimulator cells showed a very bright signal in the FITC channel and were thus excluded from the responder cells in the analysis.

To assess late responses, supernatant levels of the cytokines interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-10, and the β-chemokines macrophage inflammatory protein (MIP)-1β and RANTES (regulated on activation, normal T cell expressed and secreted) were measured by ELISA after 7 days of incubation. Antibody pairs were obtained from Pharmingen, San Diego, CA, USA for IFN-γ, TNF-α, IL-10, and RANTES, and from R&D Systems, Minneapolis, MN, USA for MIP-1β. IFN-γ, TNF-α, and IL-10 recombinant standards were provided by the National Institute for Biological Standards and Controls, UK; RANTES and MIP-1β recombinant standards were obtained from Pharmingen and R&D Systems, respectively.
Statistical analysis. Non-parametric Mann-Whitney U tests were used for comparing one variable among two groups. For testing differences between two variables within the same group, the Wilcoxon signed ranks test was used. Linear or logistic regression models were used for comparing one variable among two or three groups while controlling for differences in age. The normality of the variables used in linear regression models was tested with the Kolmogorov-Smirnov test. Correlation analyses were performed by using Spearman rank correlation tests. For all analyses, the level of significance was set at \( P < 0.05 \).

RESULTS

Characteristics of the study populations. Median age of HIV-seronegative female blood donors was 22 years (interquartile range (IQR) 20 - 28), compared to 30 (IQR 24 – 38) of ESN FSWs \( (P = 0.005) \) and 29 (IQR 24 – 35) of HIV-infected FSWs \( (P = 0.009) \). All ESN FSWs had a duration of commercial sex work of \( \geq 3 \) years, with a median of 5.25 years (range 3.1 – 24.0). Ten (48%) ESN FSWs reported a sex work duration of between 3 and 5 years, 6 (28%) between 5 and 10 years, and 5 (24%) of more than 10 years.

Anti-HLA class I antibodies. Plasma antibodies directed against HLA class I molecules were analysed in twenty female blood donors, twenty ESN FSWs, and twenty HIV-infected FSWs. Female blood donors had a median anti-HLA class I antibody level of 3.1% (IQR 0.6 – 8.3) compared with 3.5% (IQR 1.2 – 19.9) among ESN FSWs \( (P = 0.400) \) and 2.4% (IQR 0.0 – 11.8) among HIV-infected FSWs \( (P = 0.486) \) (figure 1B). With an antibody level of at least 10% and the occurrence of a multiple-peak pattern in the histogram plot set as the criteria for a positive result (figure 1A), two (10%) female blood donors compared to seven (35%) ESN FSWs \( (P = 0.073) \), and three (15%) HIV-infected FSWs \( (P = 0.635) \) showed detectable anti-HLA class I antibodies (figure 1B). The trend towards a higher frequency of anti-HLA class I antibodies in ESN FSWs compared to female blood donors disappeared when the higher age of ESN FSWs was controlled for in a logistic regression model \( (P = 0.179) \). When only subjects with age \( \geq 28 \) and < 40 years were selected, 40% (2 of 5) of female blood donors showed detectable alloantibodies which was similar to 46% (6 of 13) among ESN FSWs \( (P = 0.814) \) and 25% (2 of 8) among HIV-infected FSWs \( (P = 0.571) \) (figure 1C). This analysis also indicated that for all study groups the peak prevalence of alloantibodies is found within these age limits. Among ESN FSWs, no association was found between the presence of anti-HLA class I antibodies and the duration of commercial sex work \( (P = 0.882) \).
Figure 1. Detection of HLA-class I antibodies in ESN FSWs, HIV-infected FSWs and HIV-negative female blood donors. A, Representative analysis of anti-HLA class I antibodies by flow cytometry. Dark grey, light grey, and black histograms represent HLA class I antigen-coated beads incubated with positive control, negative control and test sample, respectively. A marker M1 is set at the end of the negative control sample. One HIV-negative female blood donor (top panel, negative result) and one ESN FSW (bottom panel, positive result) are shown. B, Overview of percentages of anti-HLA class I antibodies in the three groups. A negative cut-off value is set at 10%. Star symbols indicate percentages ≥ 10% with the occurrence of a multiple-peak pattern in the histogram plot. P, level of significance for comparison of ESN FSWs and HIV-infected FSWs with female blood donors by using logistic regression analysis. C, Percentages of anti-HLA class I antibodies in the three groups in association with age. A negative cut-off value is set at 10%. Star symbols indicate percentages ≥ 10% with the occurrence of a multiple-peak pattern in the histogram plot. P, level of significance for comparison of ESN FSWs and HIV-infected FSWs with female blood donors, with age between 28 and 40 years, by using logistic regression analysis.
**HIV-1 neutralisation assays.** None of the plasma samples from 21 ESN FSWs or 20 HIV-seronegative female blood donors had neutralising activity towards CI 20, a primary isolate of HIV-1 CRF_02 (subtype A/G). In contrast, plasma from 7 out of 20 (35%) HIV-infected FSWs showed neutralising activity towards this HIV-1 strain. No association was found between the occurrence of neutralising activity and anti-HLA class I antibodies in plasma among HIV-infected FSWs ($P = 0.948$).

**CD69 expression levels in stimulated PBMC cultures.** PBMC from 20 ESN FSWs and 18 HIV-seronegative female blood donors were incubated in the presence of inactivated autologous PBMC (autostimulation, negative control), inactivated pooled allogeneic PBMC from 24 HIV-seronegative male African blood donors (allostimulation), and PHA (positive control). To assess early responses, expression of the early activation marker CD69 was measured in various lymphocyte subsets by flow cytometry after 40 hours of culture (table 1). No statistically significant differences were observed between auto- and allostimulated CD69 expression for none of the lymphocyte subsets, while PHA stimulation induced significantly higher CD69 expression than auto- and allostimulation for both female blood donors and ESN FSWs. Compared with female blood donors, ESN FSWs showed lower allo- and autostimulated CD69 expression, reaching statistical significance for the memory subsets of CD4+ and CD8+ T cells, but higher PHA-stimulated CD69 expression, reaching statistical significance for the total lymphocyte subset. Differences between ESN FSWs and female blood donors became more pronounced for auto- and allostimulation but less pronounced for PHA-stimulation after adjusting for differences in age. Among female blood donors, PHA-stimulated CD69 expression levels correlated directly with allostimulated CD69 expression in most lymphocyte subsets, but especially within the memory CD4+ and CD8+ T cell subsets. These correlations were lost or were much less pronounced among ESN FSWs (figure 2A, data shown for memory CD8+ T lymphocytes). Among ESN FSWs, the duration of commercial sex work tended to correlate with allostimulated CD69 expression in T cell subsets. To explore this association further, 10 ESN FSWs who had done sex work for less than 5 years were compared with 11 ESN FSWs who had done sex work for more than 5 years (figure 2B, data shown for CD4+ and CD8+ memory T cells, $P$ values adjusted for differences in age). ESN FSWs who had been active in sex work for more than 5 years showed increased allostimulated CD69 expression compared with ESN FSWs who had done sex work for less than 5 years, and differences with female blood donors were most pronounced for ESN FSWs who had done sex work for more than 5 years.
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<th>ESN FSWs (n = 20)</th>
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<tr>
<td>CD8+ T cells</td>
<td>24.1 (10.8 – 36.2)</td>
<td>19.8 (15.6 – 24.5)</td>
<td>0.397</td>
<td>0.254</td>
<td><strong>0.032</strong></td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>22.2 (8.0 – 33.9)</td>
<td>18.8 (12.4 – 23.7)</td>
<td>0.520</td>
<td>0.400</td>
<td>0.062</td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>35.0 (21.3 – 42.1)</td>
<td>24.9 (18.9 – 30.3)</td>
<td><strong>0.050</strong></td>
<td><strong>0.039</strong></td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td><strong>Allogeneic stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymphocytes</td>
<td>26.9 (19.7 – 34.8)</td>
<td>22.3 (18.0 – 27.4)</td>
<td>0.179</td>
<td>0.154</td>
<td>0.061</td>
</tr>
<tr>
<td>T cells</td>
<td>27.4 (19.0 – 37.1)</td>
<td>23.6 (14.8 – 31.7)</td>
<td>0.279</td>
<td>0.209</td>
<td>0.078</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>29.8 (18.9 – 39.5)</td>
<td>23.4 (14.6 – 32.8)</td>
<td>0.219</td>
<td>0.202</td>
<td>0.095</td>
</tr>
<tr>
<td>CD4+ CD45RO+ T cells</td>
<td>27.2 (18.0 – 35.4)</td>
<td>22.3 (13.6 – 35.0)</td>
<td>0.447</td>
<td>0.443</td>
<td>0.238</td>
</tr>
<tr>
<td>CD4+ CD45RO+ T cells</td>
<td>32.2 (19.2 – 45.2)</td>
<td>25.5 (15.0 – 33.3)</td>
<td>0.136</td>
<td>0.083</td>
<td><strong>0.030</strong></td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>25.0 (17.5 – 35.8)</td>
<td>23.7 (16.4 – 29.9)</td>
<td>0.388</td>
<td>0.200</td>
<td><strong>0.050</strong></td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>24.6 (16.3 – 34.8)</td>
<td>24.0 (16.3 – 29.8)</td>
<td>0.640</td>
<td>0.358</td>
<td>0.104</td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>27.8 (23.3 – 40.5)</td>
<td>21.8 (17.0 – 27.5)</td>
<td><strong>0.035</strong></td>
<td><strong>0.024</strong></td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td><strong>PHA stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymphocytes</td>
<td>92.9 (89.8 – 94.0)</td>
<td>94.6 (93.4 – 95.7)</td>
<td><strong>0.002</strong></td>
<td><strong>0.002</strong></td>
<td><strong>0.029</strong></td>
</tr>
<tr>
<td>T cells</td>
<td>95.1 (92.8 – 96.2)</td>
<td>96.5 (95.4 – 97.7)</td>
<td><strong>0.018</strong></td>
<td><strong>0.030</strong></td>
<td>0.254</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>97.6 (96.9 – 98.4)</td>
<td>98.1 (97.1 – 98.6)</td>
<td>0.349</td>
<td>0.233</td>
<td>0.770</td>
</tr>
<tr>
<td>CD4+ CD45RO+ T cells</td>
<td>97.8 (96.2 – 98.7)</td>
<td>98.4 (97.0 – 98.8)</td>
<td>0.405</td>
<td>0.298</td>
<td>0.851</td>
</tr>
<tr>
<td>CD4+ CD45RO+ T cells</td>
<td>98.0 (96.6 – 98.4)</td>
<td>97.8 (96.8 – 98.5)</td>
<td>0.907</td>
<td>0.394</td>
<td>0.923</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>91.1 (87.4 – 93.5)</td>
<td>94.1 (93.1 – 96.4)</td>
<td><strong>0.019</strong></td>
<td><strong>0.044</strong></td>
<td>0.167</td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>90.3 (84.7 – 93.1)</td>
<td>94.0 (91.5 – 96.1)</td>
<td><strong>0.018</strong></td>
<td><strong>0.050</strong></td>
<td>0.128</td>
</tr>
<tr>
<td>CD8+ CD45RO+ T cells</td>
<td>94.2 (91.3 – 95.7)</td>
<td>95.3 (91.9 – 96.7)</td>
<td>0.373</td>
<td>0.501</td>
<td>0.916</td>
</tr>
</tbody>
</table>

Data are median (interquartile range). aMann-Whitney u test. bLinear regression, all variables are normally distributed. cLinear regression adjusting for differences in age, all variables are normally distributed. P values < 0.05 are in bold.
Cytokine and β-chemokine production in stimulated PBMC cultures. To assess late cellular responses, production levels of a set of cytokines and β-chemokines were measured by ELISA in the supernatants of auto-, allo- and PHA-stimulated PBMC cultures after 7 days of incubation. Compared with autostimulation, allostimulation revealed higher levels of MIP-1β ($P = 0.014$ for female blood donors; $P = 0.001$ for ESN FSWs) and RANTES ($P < 0.001$ for both female blood donors and ESN FSWs).
FSWs), but not of IFN-γ, TNF-α and IL-10 (table 2). PHA-stimulated production levels exceeded both auto- and allostimulation for all cytokines and β-chemokines studied. In line with CD69 expression, levels of IFN-γ, TNF-α, MIP-1β, and RANTES, but not of IL-10, were significantly lower in auto- and allostimulated PBMC cultures from ESN FSWs compared with female blood donors (table 2). PHA-stimulated responses were higher among ESN FSWs compared with female blood donors, reaching statistical significance for TNF-α and MIP-1β. Statistically significant differences between ESN FSWs and female blood donors largely remained valid after adjustments for differences in age.

Table 2. Levels of cytokine and β-chemokine production in autologous, allogeneic, and PHA-stimulated PBMC cultures from 20 ESN FSWs and 18 blood donors.

<table>
<thead>
<tr>
<th></th>
<th>Blood donors (n = 18)</th>
<th>ESN FSWs (n = 20)</th>
<th>( p_a )</th>
<th>( p_b )</th>
<th>( p_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Autostimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ(^d)</td>
<td>32.6 (12.2 – 528.9)</td>
<td>3.3 (3.3 – 16.6)</td>
<td>\textbf{0.001}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>TNF-α(^d)</td>
<td>6.3 (6.3 – 428.2)</td>
<td>6.3 (0.8 – 6.3)</td>
<td>\textbf{0.023}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>IL-10</td>
<td>28.5 (12.5 – 46.9)</td>
<td>16.5 (6.5 – 27.5)</td>
<td>\textbf{0.047}</td>
<td>\textbf{0.044}</td>
<td>0.078</td>
</tr>
<tr>
<td>MIP-1β(^d)</td>
<td>305.5 (103.1 – 1851.8)</td>
<td>66.1 (25.4 – 110.3)</td>
<td>\textbf{0.001}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>RANTES(^d)</td>
<td>341.8 (187.0 – 606.3)</td>
<td>122.3 (98.0 – 161.8)</td>
<td>\textbf{&lt;0.001}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td><strong>Allostimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ(^d)</td>
<td>39.1 (6.6 – 549.4)</td>
<td>10.4 (3.3 – 22.4)</td>
<td>\textbf{0.034}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>TNF-α(^d)</td>
<td>6.3 (0.8 – 289.4)</td>
<td>6.3 (0.8 – 6.3)</td>
<td>0.173</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>IL-10(^d)</td>
<td>19.1 (11.8 – 45.3)</td>
<td>12.8 (6.1 – 23.6)</td>
<td>0.299</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>MIP-1β(^d)</td>
<td>439.9 (195.5 – 2530.9)</td>
<td>194.0 (104.1 – 441.6)</td>
<td>\textbf{0.024}</td>
<td>N.A</td>
<td>N.A</td>
</tr>
<tr>
<td>RANTES</td>
<td>635.8 (425.4 – 1000)</td>
<td>293.4 (261.8 – 445.8)</td>
<td>\textbf{&lt;0.001}</td>
<td>\textbf{&lt;0.001}</td>
<td>\textbf{&lt;0.001}</td>
</tr>
<tr>
<td><strong>PHA-stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2210 (879.7 – 3902)</td>
<td>2505 (1793 – 3952)</td>
<td>0.447</td>
<td>0.453</td>
<td>0.706</td>
</tr>
<tr>
<td>TNF-α</td>
<td>234.0 (130.2 – 426.6)</td>
<td>406.1 (276.6 – 561.4)</td>
<td>\textbf{0.033}</td>
<td>0.095</td>
<td>0.143</td>
</tr>
<tr>
<td>IL-10</td>
<td>451.5 (274.8 – 739.3)</td>
<td>524.8 (320.1 – 707.6)</td>
<td>0.465</td>
<td>0.465</td>
<td>0.790</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>12607 (9717 – 17661)</td>
<td>22438 (16965 – 31971)</td>
<td>\textbf{0.002}</td>
<td>\textbf{0.003}</td>
<td>\textbf{0.005}</td>
</tr>
<tr>
<td>RANTES</td>
<td>3846 (2615 – 4997)</td>
<td>3837 (3001 – 6054)</td>
<td>0.569</td>
<td>0.303</td>
<td>0.372</td>
</tr>
</tbody>
</table>

Data are median value (interquartile range). \(^a\)Mann-Whitney U test. \(^b\)Linear regression. \(^c\)Linear regression adjusting for differences in age. \(^d\)Variables are not normally distributed, linear regression analyses are not performed. N.A, not available. \( P \) values < 0.05 are in bold.
DISCUSSION

In an effort to define putative correlates of protection against HIV infection, humoral and cellular alloimmune responses were analysed in a group of ESN FSWs. We found that ESN FSWs were not characterised by enhanced humoral and/or cellular alloimmune responses compared with HIV-seronegative female blood donors at lower risk for HIV infection. In contrast, cellular alloimmune responses were found to be decreased among ESN FSWs indicating that frequent unprotected sexual exposure resulted in suppression rather than activation of the immune system towards alloantigens. The data suggest that increased alloimmune responses are not associated with protection against infection with HIV in this population of ESN FSWs.

Anti-HLA class I antibodies were detected by a novel flow cytometry method applying latex beads coated with HLA class I antigens, which was found to be more accurate than the standard complement-dependent lymphocytotoxicity assay [34, 35]. ESN FSWs showed a similar prevalence of anti-HLA class I antibodies compared with HIV-infected FSWs and female blood donors. In fact, the presence of antibodies was rather a function of age than of frequent sexual allogeneic exposure, since a similar peak prevalence was found between the age of 28 and 40 among ESN FSWs, female blood donors, and HIV-infected FSWs (figure 1C). A possible common factor among these women may have been multiparity, as reported elsewhere [36]. In addition, no neutralising activity against an HIV-1 CRF_02 primary isolate was recorded in any of the ESN FSWs or blood donors, and neutralising activity detected in some of the HIV-infected FSWs did not correlate with the presence of anti-HLA class I antibodies. Together, these data confirm previous studies showing no effect of anti-HLA class I antibodies in ESN populations: anti-HLA class I antibodies could not be correlated with protection against HIV infection among ESN FSWs in Nairobi [26], nor with a reduced risk of perinatal HIV transmission among children born to HIV-infected mothers [27]. One study found higher levels of anti-HLA class I antibodies in ESN partners of HIV-1 discordant couples than in controls, but also here no correlation with HIV-neutralising activity could be demonstrated [37]. It may be possible that secretory IgA antibodies, rather than circulating IgG antibodies as measured in the present and in previous studies, protect against HIV transmission at the site of the mucosal HIV challenge. Alternatively, antibodies directed against HLA class II rather than HLA class I, or against both, may be necessary for protection [38-40].

ESN FSWs showed significantly lower allostimulated cellular responses both at the level of early lymphocyte activation and late cytokine and β-chemokine supernatant secretion compared with female blood donors. In contrast, ESN FSWs
showed significantly increased mitogen-stimulated lymphocyte activation and supernatant secretion of cytokines and β-chemokines. These opposite immunological effects seen in ESN FSWs were also supported by the fact that direct correlations between mitogen- and allostimulated lymphocyte activation among female blood donors were not present among ESN FSWs. These data indicate that frequent sexual exposure among ESN FSWs may result in a specific down-regulation of cellular alloimmune reactions, together with a general state of increased lymphocyte activation.

Our findings are in line with the notion that natural or artificial mucosal and systemic alloimmunisation results in peripheral immune-tolerance rather than activation towards allogeneic antigens. Normal pregnancy without symptoms of immune reaction towards the foetus was more likely to occur in women with prior exposure to paternal alloantigens via unprotected sexual contact or previous pregnancies [41-43]. Prior pretransplant blood transfusions were reported to prolong renal allograft survival [44, 45]. In women with recurrent spontaneous abortions, tolerance towards the foetus was achieved after alloimmunisation with the male partner’s PBMC [46, 47]. Alloimmunisation resulted in decreased expression of CD69 on T cells [48], decreased proliferation in mixed maternal versus paternal lymphocyte reactions [49], and a Th1 to Th2 cytokine shift [50]. The latter findings, including the Th1/Th2 shift, are in remarkable agreement with our results. Indeed, ESN FSWs showed decreased allostimulated production of the Th1 cytokines IFN-γ and TNF-α but not of the Th2 cytokine IL-10.

In that regards, reports from Wang et al. showing a protective effect of alloimmunisation against SIV or HIV infection may be confusing. They measured in vitro PHA-stimulated responses in macaques after xenoinmunisation with SIV grown in human CD4+ T cells or with the human CD4+ T cells alone [51], and in women with recurrent spontaneous abortions after alloimmunisation with the male partner’s PBMC [7]. In both studies, increased PHA-stimulated production of the β-chemokines MIP-1α, MIP-1β and RANTES, and of the CD8 suppressor factor was noted which is in agreement with increased PHA-stimulated TNF-α and MIP-1β production among ESN FSWs in our study. However, in vitro mitogen-stimulation of PBMC may not represent an appropriate physiological correlate of the in vivo alloimmune response. Unfortunately, in vitro alloantigen-stimulated cellular responses, like in the present study, were not analysed.

Interestingly, in our study, cellular alloimmune responses were lowest among ESN FSWs with less than 5 years of commercial sex work, and were more similar to controls among ESN FSWs who had done sex work for more than 5 years (figure
2B). In previous studies, we found a negative association between the duration of commercial sex work and the number of clients per day of ESN FSWs [52-53]. Together, this could suggest a dose-response association between the frequency of sexual exposure, which is highest among ESN FSWs with a short duration of sex work, and induction of tolerance towards alloantigens.

Whether and how decreased alloimmune responses as seen among ESN FSWs in our study can contribute to protection is not clear. Some initiating evidence for this suggestion may come from studies by Moriuchi et al., showing that in vitro allostimulation may render PBMC more susceptible to HIV infection, and may reactivate HIV in latently infected resting CD4+ T cells [54]. Also, increased alloimmune reactions following transfusion of blood products or transplantation of allogeneic bone marrow enhanced HIV replication in HIV patients [55-57]. In addition, multiparous women, expected to have acquired tolerised alloimmune responses, were found to be less prone to HIV infection [58], and were less likely to transmit HIV to their babies [59]. It is therefore possible that down-regulation of alloimmune responses in ESN FSWs is merely a mechanism to silence potential HIV enhancing effects, rather than being HIV-protective on its own. However, allostimulation has previously been shown to result in the reactivation of cytomegalovirus [60], and in the downregulation of CXCR4 expression [7, 61], both of which have been observed in previous studies in ESN FSWs in Abidjan as well [52-53]. Thus, cellular alloimmune responses may likely play an important role in ESN FSWs in Abidjan, and their role in protection against HIV infection should be the subject of further study.

In summary, we found that ESN FSWs in Abidjan have similar humoral but significantly lower cellular alloimmune responses than HIV-seronegative female blood donors at lower risk for HIV infection. These findings indicate that frequent unprotected sexual exposure may result in tolerisation rather than activation of the immune system towards alloantigens. These data do not support the induction of alloimmune responses as a potential strategy to prevent the transmission of HIV.

ACKNOWLEDGEMENTS

We thank the community of female sex workers in Abidjan for their cooperation. Mathieu Maran, Kabran N’Guessan and N’Depo Yenon for technical assistance, Emmanuel Abonga for providing blood samples from blood donors, Jef Braem and Serge Blockmans for logistical support in the field, Ward Schrooten for critical remarks and discussion. This work was supported by the Belgian Fonds voor
Wetenschappelijk Onderzoek (FWO) Vlaanderen, grant G.0396.99 and by the Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention (CDC), Atlanta, GA.

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Chapter 3

The controversial role of β-chemokines in HIV-1 infection

3.1. Association between β-chemokines and HIV-1 viral load .......................... 69

3.2. β-chemokine secretion in HIV infection .................................................. 83
Disrupted secretory capacity for β-chemokines in HIV-infected subjects. Submitted.
Positive association between β-chemokine-producing T cells and HIV type 1 viral load in HIV-infected subjects in Abidjan, Côte d'Ivoire

Wim Jennes,1 Souleymane Sawadogo,2 Stéphanie Koblavi-Dème,2 Bea Vuylsteke,1,2 Chantal Maurice,2 Thierry H. Roels,2,3 Terence Chorba,2,3 John N. Nkengasong,2,3 and Luc Kestens1

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AIDS Research and Human Retroviruses 2002, 18:171-177

ABSTRACT

The role of β-chemokines in controlling HIV replication in vivo is still controversial. Therefore, the association between HIV-1 plasma viral load and the capacity of CD4+ and CD8+ T cells to produce β-chemokines was studied in 28 antiretroviral drug-naive HIV-1-infected female sex workers in Abidjan, Côte d’Ivoire. Percentages of β-chemokine-positive T cells were measured in peripheral blood mononuclear cells by flow cytometry after intracellular staining for RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α and MIP-1β. HIV-1-infected subjects had higher percentages of MIP-1α- and MIP-1β-positive CD4+ and CD8+ T cells (P < 0.02) and of RANTES-positive CD8+ T cells (P = 0.054) than uninfected controls. Percentages of RANTES- and MIP-1β-positive CD8+ T cells correlated directly with HIV-1 plasma viral load (P < 0.02). Percentages of β-chemokine-positive CD4+ and CD8+ T cells correlated directly with percentages of HLA-DR-positive T cells (P < 0.02) and inversely (except RANTES in CD4+ T cells) with absolute numbers of CD4+ T cells (P < 0.05) in peripheral blood. These data indicate that increased percentages of β-chemokine-producing T cells in HIV-1-infected subjects correlate with disease progression and are a sign of viremia driven chronic T cell activation.
INTRODUCTION

β-chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α and MIP-1β have been identified as potent HIV suppressor factors produced by CD8+ T cells in vitro [1]. The anti-viral effect of β-chemokines is mediated by inhibition of HIV binding to the cell surface by blocking and down-regulation of the natural β-chemokine receptor CCR5 [2]. CCR5 is the major coreceptor of macrophage-tropic non-syncytium-inducing HIV-1 strains, which are preferentially sexually transmitted [3-5]. The crucial role of CCR5 in HIV transmission is supported by observations that individuals homozygous for a 32 base pair deletion in the CCR5 gene resulting in a defective receptor are highly resistant to infection [6-8].

Several studies suggest that protection against HIV infection and slow HIV disease progression correlate with enhanced production of HIV-inhibitory β-chemokines [9-15]. Increased resistance of CD4+ T cells from HIV-exposed uninfected individuals to in vitro HIV-1 infection has been associated with increased β-chemokine production [9, 10]. Overproduction of β-chemokines by mitogen-stimulated lymphocytes was found in a cohort of HIV-negative persons with hemophilia who had been repeatedly inoculated with HIV-contaminated blood products [11]. In a group of long-term survivors, HIV antigen-induced β-chemokine production by CD8+ T cells correlated inversely with HIV-1 viral load [12]. Among HIV-1-infected subjects, increased MIP-1β production has been associated with a decreased risk of HIV disease progression [13]. Thus, MIP-1α and MIP-1β production by CD8+ T cells was found to be higher among asymptomatic HIV-1-infected subjects compared with those with symptoms of AIDS [14]. Recently, preinfection production levels of RANTES have been found to correlate inversely with postinfection HIV-1 viral load and CD4+ T cell decline rates [15].

In other studies, however, mitogen-stimulated β-chemokine production levels did not differ between long-term nonprogressors and patients with progressive HIV disease [16, 17]. Increased serum β-chemokine levels were associated with HIV disease progression rather than with asymptomatic infection or long-term survival [18-21], and failed to correlate with HIV-1 viral load [21, 22]. Thus, conclusions concerning the in vivo relevance of β-chemokines in HIV infection may differ depending on the study population and the applied methodology.

In the study presented here, we compared percentages of RANTES-, MIP-1α- and MIP-1β-positive CD4+ and CD8+ T cells between antiretroviral drug-naive HIV-1-infected female sex workers (FSWs), exhibiting a broad range of HIV-1 viral load
levels, and HIV-uninfected controls. Among HIV-1-infected FSWs, we explored the association between β-chemokine-positive CD4$^+$ and CD8$^+$ T cells on the one hand and T cell activation, HIV-1 viral load levels and CD4$^+$ T cell counts on the other.

**MATERIALS AND METHODS**

*Study population.* From November through December 1999 and in May 2000, blood samples were collected from 28 HIV-1-infected FSWs consecutively enrolled at a confidential clinic in Abidjan. None of the FSWs had received HIV antiretroviral therapy at the time of specimen collection. Informed consent was obtained from all subjects prior to participation in this study. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention (Atlanta, GA, USA), and the Ethical Committees of the Côte d’Ivoire Ministry of Health (Abidjan, Côte d’Ivoire) and the Institute of Tropical Medicine (Antwerp, Belgium). As a control group, we collected blood samples from 35 voluntary HIV-seronegative female blood donors at the national blood transfusion center in Abidjan.

*Laboratory methods.* All laboratory analyses were performed in Abidjan using freshly obtained whole blood samples. Whole blood was drawn from FSWs and control subjects into EDTA tubes (Becton Dickinson, San Jose, CA, USA). Within 4 h of blood collection, plasma was separated from cells by centrifugation at 500 g, aliquoted, and stored at −70 °C. HIV antibody status was tested using an enzyme-linked immunosorbent assay (ELISA)-based parallel testing algorithm [23].

*HIV-1 plasma RNA viral load determination.* HIV-1 viral load was quantified in plasma by the Amplicor HIV-1 Monitor assay version 1.5 (Roche Diagnostic Systems, Branchburg, NJ, USA). This assay accurately quantifies HIV-1 subtype A/G, which is the predominant subtype in Côte d’Ivoire [24, 25].

*Lymphocyte subsets and T cell activation markers.* Phenotypic analyses of peripheral blood lymphocytes were performed on fresh whole blood within 4 h of collection in EDTA. Cells were stained with a combination of fluorochrome-conjugated anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (MAbs) for quantification of the lymphocyte subsets. Staining with the combination anti-CD8 and anti-CD38 and the combination anti-CD3, anti-CD8, and anti-HLA-DR was performed to quantify the level of CD4$^+$ and CD8$^+$ T cell activation. All MAbs were obtained from Becton Dickinson, San Jose, CA, USA. Aliquots of 100 µl peripheral blood were mixed with the appropriate MAb combinations and
incubated for 15 min in the dark at 4 °C. The red blood cells were lysed by adding 2 ml FACS lysing solution (Becton Dickinson, San Jose, CA, USA). The samples were centrifuged at 500 g for 5 min, resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% sodium azide (NaN₃), centrifuged again and fixed with PBS containing 1% paraformaldehyde (PFA). Cells were stored at 4 °C until flow cytometric analysis using a FACSScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). For analysis of percentages of CD38-positive cells in the CD8⁺ T cell subset, a gate was set around the bright CD8⁺ lymphocyte cluster. Percentages of HLA-DR-positive cells were analyzed within the CD4⁺ and CD8⁺ T cell subsets. With a gate set around the CD3⁺ T lymphocytes, anti-CD8 and anti-HLA-DR fluorescence intensities were displayed in a two-color dot-plot. The percentages of HLA-DR-positive CD3⁺CD8⁻ and CD3⁺CD8⁺ lymphocytes in the upper left and upper right quadrants of the dot-plot were recalculated in percentages of HLA-DR-positive cells in the CD4⁺ T cell subset and percentages of HLA-DR-positive cells in the CD8⁺ T cell subset, respectively. For all analyses, quadrant markers were set using negative isotype controls.

**Intracellular staining of β-chemokines.** Peripheral blood mononuclear cells (PBMC) were separated from fresh whole blood within 4 h of collection in EDTA by gradient centrifugation using lymphocyte separation medium (ICN Biomedicals, Aurora, OH, USA) and resuspended at 1 × 10⁶ cells/ml in RPMI 1640 containing L-glutamine (Life Technologies, Paisley, UK), 10% fetal calf serum (FCS) (Life Technologies, Paisley, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany). Cells were incubated overnight at 37 °C and 5% CO₂ in the presence of monensin (1.5 µg/ml) (Sigma, St. Louis, MO, USA) for the constitutive intracellular accumulation of RANTES and for 5 h at 37 °C and 5% CO₂ with phorbol myristate acetate (0.02 µg/ml) (Sigma, St. Louis, MO, USA) and ionomycin (1 µg/ml) (Sigma, St. Louis, MO, USA) in the presence of brefeldin A (10 µg/ml) (Sigma, St. Louis, MO, USA) for the stimulation-induced intracellular accumulation of MIP-1α, and MIP-1β. PBMC were subsequently washed (by resuspending in PBS containing 1% BSA and 0.05% NaN₃, centrifuging at 500 g for 5 min, and aspirating the supernatant). The T lymphocytes were stained by adding peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD3 and fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD8 MAbs (Becton Dickinson, San Jose, CA, USA) to the cell suspension for 15 min at room temperature. The cells were washed, fixed using Leucoperm reagent A (Serotec, Oxford, UK) for 15 min at room temperature, and washed again. Subsequently, cells were permeabilized using Leucoperm reagent B (Serotec, Oxford, UK) and intracellular β-chemokines were stained with anti-
RANTES PE, anti-MIP-1α PE (both from Pharmingen, San Diego, CA, USA) or carboxyfluorescein (CFS)-conjugated anti-MIP-1β (R&D Systems, Minneapolis, MN, USA) MAbs for 30 min at room temperature. The cells were washed,

Figure 1. Representative dot-plots for analyzing percentages of β-chemokine-positive CD4+ and CD8+ T cells in one control subject and one HIV-1-infected female sex worker (FSW). A. RANTES; B, MIP-1α; C, MIP-1β. All events shown are gated CD3+ T lymphocytes. Quadrant markers were set using negative isotype controls. Percentages of β-chemokine-positive CD3+ CD8+ and CD3+ CD8+ lymphocytes in the upper left and upper right quadrants were recalculated in percentages of β-chemokine-positive CD4+ T cells in the CD4+ T cell subset and β-chemokine-positive CD8+ T cells in the CD8+ T cell subset, respectively. Intracellular accumulation was constitutive for RANTES and mitogen-stimulated for MIP-1α and MIP-1β, see materials and methods section for details.
resuspended in PBS with 1% PFA, and stored at 4 °C until flow cytometric analysis using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). Percentages of β-chemokine-positive cells were analyzed within the CD4⁺ and CD8⁺ T cell subsets. With a gate set around the CD3⁺ T lymphocytes, anti-CD8 and anti-β-chemokine fluorescence intensities were displayed in a two-color dot-plot. The percentages of β-chemokine-positive CD3⁺ CD8⁻ and CD3⁺ CD8⁺ lymphocytes in the upper left and upper right quadrants of the dot-plot were recalculated in percentages of β-chemokine-positive cells in the CD4⁺ T cell subset and percentages of β-chemokine-positive cells in the CD8⁺ T cell subset respectively. For all analyses, quadrant markers were set using isotype controls. Figure 1A-C shows representative dot-plots for the analysis of the percentages of RANTES-, MIP-1α- and MIP-1β-positive CD4⁺ and CD8⁺ T cells, respectively, for one uninfected control and one HIV-1-infected FSW.

Statistical analysis. Differences between groups were tested for statistical significance using the non-parametric Mann-Whitney U test. Correlation analysis was performed with the non-parametric Spearman rank correlation test. The level of significance for all analyses was set at 0.05.

RESULTS

Characteristics of the study populations. Of the 28 HIV-1-infected FSWs, 4 (14%) were HIV-1 and HIV-2 dually seropositive. Median duration of reported sex work had been 24 months (range 1 – 156). Median age was 26 years (range 19 – 59) for the HIV-1-infected FSWs and 24 years (range 18 – 52) for the female uninfected control subjects. HIV-1-infected FSWs had a median cell count of 583 CD4⁺ T cells/µl (range 186 – 1487) and a median HIV-1 plasma viral load of 5.1 log₁₀ copies/ml (range 3.0 – 6.5).

HIV-1-infected FSWs had increased percentages of β-chemokine-positive T cells. Compared with uninfected controls, HIV-1-infected FSWs had significantly increased percentages of MIP-1α- and MIP-1β-positive CD4⁺ and CD8⁺ T cells (figure 2A and 2B). Percentages of RANTES-positive CD8⁺ T cells were also increased among HIV-1-infected FSWs, albeit not significantly. The relative increase of β-chemokine-positive T cells in HIV-1-infected FSWs compared with controls was more pronounced in the CD8⁺ T cell subset than in the CD4⁺ T cell subset. Overall, within the CD8⁺ T cell subset higher percentages of β-chemokine-positive cells were noted than within the CD4⁺ T cell subset among both controls and HIV-1-infected FSWs.
3.1. ASSOCIATION BETWEEN β-CHEMOKINES AND HIV-1 VIRAL LOAD / 75

Percentages of β-chemokine-positive T cells correlated with in vivo T cell activation. We found significant correlations between in vivo percentages of HLA-DR-positive cells and percentages of RANTES-, MIP-1α- and MIP-1β-positive cells in both the CD4+ and CD8+ T cell subsets of HIV-1-infected FSWs (table 1). Percentages of CD38-positive cells within the CD8+ T cell subset correlated significantly with percentages of MIP-1β-, but not of RANTES- or MIP-1α-, positive cells within the CD8+ T cell subset ($r = 0.443$, $P = 0.03$). Among controls, percentages of HLA-DR-positive cells correlated with percentages of MIP-1β-positive cells in the CD4+ ($r = 0.414$, $P = 0.04$) and CD8+ ($r = 0.508$, $P = 0.009$) T cell subset.

Figure 2. Percentages of β-chemokine-positive T cells in HIV-uninfected female controls (white dots) and HIV-1-infected FSWs (black dots). A. Percentages of RANTES-, MIP-1α- and MIP-1β-positive cells within the CD4+ T cell subset. B. Percentages of RANTES-, MIP-1α- and MIP-1β-positive cells within the CD8+ T cell subset. Horizontal lines indicate median values. Differences between groups were tested for statistical significance using the non-parametric Mann-Whitney U test. $P$, level of significance.
HIV-1 plasma viral load correlated with in vivo T cell activation. HIV-1 viral load was previously shown to correlate with T cell activation in the group of HIV-1-infected FSWs in Abidjan [26]. These results were confirmed in the present study but reached significant levels for percentages of CD38-positive CD8+ T cells only ($r = 0.47, P = 0.018$) and not for percentages of HLA-DR-positive cells among CD4+ T cells ($r = 0.348, P = 0.088$) or CD8+ T cells ($r = 0.355, P = 0.082$).

Percentages of β-chemokine-positive T cells correlated with HIV-1 plasma viral load. HIV-1 plasma viral load in HIV-1-infected FSWs correlated significantly with percentages of RANTES- and MIP-1β-positive cells in the CD8+ T cell subset. No statistically significant correlations were found between HIV-1 plasma viral load and percentages of MIP-1α-positive CD8+ T cells or percentages of β-chemokine-positive CD4+ T cells (table 1 and figure 3A).

Table 1. Correlations between percentages of β-chemokine-positive T cells and in vivo T cell activation, HIV-1 plasma viral load and absolute number of CD4+ T cells among HIV-1-infected FSWs in Abidjan.

<table>
<thead>
<tr>
<th>β-chemokine+ cells (%)</th>
<th>HLA-DR+ cells (%)</th>
<th>HIV-1 plasma viral load</th>
<th>CD4+ T cells/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4+ T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>$r = 0.581, P = 0.002, n = 25$</td>
<td>$r = 0.086, P = 0.697, n = 23$</td>
<td>$r = -0.218, P = 0.356, n = 20$</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>$r = 0.656, P = 0.001, n = 24$</td>
<td>$r = 0.214, P = 0.339, n = 22$</td>
<td>$r = -0.454, P = 0.051, n = 19$</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>$r = 0.683, P &lt; 0.001, n = 24$</td>
<td>$r = 0.268, P = 0.217, n = 23$</td>
<td>$r = -0.445, P = 0.049, n = 20$</td>
</tr>
<tr>
<td><strong>CD8+ T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>$r = 0.492, P = 0.012, n = 25$</td>
<td>$r = 0.545, P = 0.007, n = 23$</td>
<td>$r = -0.533, P = 0.011, n = 20$</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>$r = 0.480, P = 0.018, n = 24$</td>
<td>$r = 0.149, P = 0.510, n = 22$</td>
<td>$r = -0.546, P = 0.016, n = 19$</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>$r = 0.658, P &lt; 0.001, n = 24$</td>
<td>$r = 0.497, P = 0.016, n = 23$</td>
<td>$r = -0.693, P = 0.001, n = 20$</td>
</tr>
</tbody>
</table>

$r$, correlation coefficient; $P$, two-tailed level of significance; $n$, number of cases; MIP, macrophage inflammatory protein. Varying numbers of cases reflect missing data. $^a$Percentages of HLA-DR+ cells were correlated with percentages of β-chemokine+ cells within the same T cell subsets. $^b$Correlations performed with non-parametric Spearman rank correlation test. $P$ values $< 0.05$ in bold.

Percentages of β-chemokine-positive T cells correlated inversely with absolute numbers of CD4+ T cells. Absolute numbers of CD4+ T cells in HIV-1-infected FSWs correlated inversely with either percentages of β-chemokine-positive cells within the CD8+ T cell subset and with percentages of MIP-1α- and MIP-1β-positive cells within the CD4+ T cell subset (table 1 and figure 3B).
Inverse correlations with CD4⁺ T cell counts were more pronounced for percentages of β-chemokine-positive cells within the CD8⁺ T cell subset than within the CD4⁺ T cell subset. Among controls, no such correlations were detected.

*HIV-1 and HIV-2 dually seropositive subjects showed similar results compared to subjects seropositive for HIV-1 alone.* Compared to HIV-1 (alone) -infected subjects, the four HIV-1 and HIV-2 dually seropositive subjects had similar percentages of β-chemokine-positive CD4⁺ and CD8⁺ T cells. Their percentages of CD38⁻ and HLA-DR-positive T cells, CD4⁺ T cell counts and HIV-1 plasma viral load levels were also comparable.

![Figure 3](image.png)

**Figure 3.** Correlations between percentages of β-chemokine-positive cells and markers of disease progression among HIV-1-infected FSWs. *A*, Correlations between HIV-1 plasma viral load levels and percentages of RANTES-, MIP-1α- and MIP-1β-positive CD8⁺ T cells. *B*, Correlations between absolute numbers of CD4⁺ T cells and percentages of RANTES-, MIP-1α- and MIP-1β-positive CD8⁺ T cells. Correlation analysis was performed with the non-parametric Spearman rank correlation test. *r*, correlation coefficient; *P*, two-tailed level of significance. See table 1 for details.
DISCUSSION

In this study, we have shown that percentages of β-chemokine-positive T cells, in particular CD8+ T cells, are significantly increased in HIV-1-infected subjects, correlate positively with levels of T cell activation and HIV-1 plasma viral load, and correlate inversely with absolute numbers of CD4+ T cells. These data do not support the hypothesis that increased β-chemokine levels may constitute a correlate of protection against HIV. Our findings are consistent with the observations that increased β-chemokine levels in supernatants of mitogen-stimulated PBMC cultures [16, 17], or in serum [18-21], are associated with HIV disease progression rather than with long-term survival. Our findings also support and extend the studies of Tartakovsky et al. who found that high intracellular MIP-1β levels are correlated with high HIV-1 viral load levels and low CD4+ T cell counts [27, 28].

The positive correlation between percentages of β-chemokine-positive T cells and in vivo T cell activation is in line with the observation that increased serum and mRNA levels of RANTES are associated with elevated levels of activated CD38-positive CD8+ T cells [29].

Except for one patient, all HIV-1-infected subjects included in our study population had > 200 CD4+ T cells/µl. Therefore we were not able to compare percentages of β-chemokine-positive T cells between asymptomatic HIV-infected subjects and AIDS patients as has been done in previous studies [12-14]. Anyhow, in these and other studies, β-chemokine production levels were found to correlate inversely with HIV-1 viral load [12, 15], and positively with CD4+ T cell counts [14, 15], regardless of stage of HIV disease. These findings are at variance with our results. The effect of antiretroviral therapy, which is excluded in our study but not in all of the cited studies [14, 15], may have caused the discrepancy by maintaining low viral load levels and high CD4+ T cell counts and thus disturbing the natural course of the disease. Alternatively, the existence of an inverse relationship between intracellular accumulation and extracellular secretion of β-chemokines in HIV infection, as previously proposed [28], could provide an explanation.

Taken together, our findings show that percentages of β-chemokine-positive T cells, T cell activation and HIV-1 viral load are closely associated. Therefore, the increased frequency of β-chemokine-positive T cells in HIV-1-infected FSWs is likely the result of chronic T cell activation in these patients, which, in turn, may be driven by the HIV-1 viral load. This association is most clear for the CD8+ T cell subset which is also the subset that is most activated in HIV-1-infected patients [30]. The inverse association with CD4+ T cell counts suggests that increased percentages of β-chemokine-positive T cells more likely correlate with disease progression than with protection against HIV disease.
In most published studies, \( \beta \)-chemokine levels in HIV-infected subjects have been measured either in the supernatants of stimulated lymphocyte cultures [12-17], or directly in serum or plasma samples [18-22]. These studies provide little or no information about the identity of the cells that produced the chemokines. A novel aspect of our study is that we measured intracellular \( \beta \)-chemokine production at the level of a single T cell using flow cytometry. This allowed us to identify important differences between HIV-1-infected and -uninfected subjects on the one hand, and between CD4\(^+\) and CD8\(^+\) T cell subsets on the other. Although the experimental conditions used to detect MIP-1\(\alpha\) and MIP-1\(\beta\) (mitogen-stimulated intracellular accumulation) were different from those used for RANTES (constitutive intracellular accumulation), the results obtained for all three chemokines were similar and correlated with the ex vivo measurement of HLA-DR expression. This may indicate that the detection of MIP-1\(\alpha\) and MIP-1\(\beta\) after in vitro mitogenic stimulation, which is rather artificial, provides physiologically meaningful results.

MIP-1\(\alpha\) consists of two isoforms, LD78\(\alpha\) and LD78\(\beta\), which differ in only three amino acids. LD78\(\beta\) was shown to have 10- to 50-fold higher potency than LD78\(\alpha\) in inhibiting in vitro infection of PBMC with M-tropic HIV-1 [31]. The available antibodies to MIP-1\(\alpha\), including the one used in this study, cannot discriminate between the reported MIP-1\(\alpha\) isoforms. Should isoform-specific antibodies become available, it would be interesting to investigate whether or not the intracellular levels of both isoforms correlate with progression to HIV disease.

In summary, the results obtained here indicate that increased percentages of \( \beta \)-chemokine-positive T cells in treatment-naive HIV-1-infected subjects compared to controls are associated with increased in vivo T cell activation and HIV-1 viral load and decreased absolute numbers of CD4\(^+\) T cells. The increased frequency of \( \beta \)-chemokine-positive cells within the CD4\(^+\) and CD8\(^+\) T cell subsets of HIV-1-infected subjects is rather a sign of viremia-driven chronic T cell activation and disease progression than a correlate of protection against HIV disease.

ACKNOWLEDGMENTS

We thank the community of female sex workers in Abidjan for their cooperation; Marie-Yolande Borget, Mathieu Maran, Daniel Yavo, and N’Depo Yenon for technical assistance; Emmanuel Abonga for providing control blood samples; Jef Braem and Serge Blockmans for logistical support in the field. This work was supported by the Belgian Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen, grant G.0396.99 and by the Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention (CDC), Atlanta, GA.
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3.1. ASSOCIATION BETWEEN β-CHEMOKINES AND HIV-1 VIRAL LOAD

Disrupted secretory capacity for β-chemokines in HIV-infected subjects

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Submitted

ABSTRACT

Contradictory findings concerning the role of β-chemokines in HIV infection have been reported probably as the result of different detection methods. Therefore, we compared frequencies of β-chemokine-positive lymphocytes detected by intracellular staining to effective β-chemokine secretion in culture supernatants in treatment-naive HIV patients, patients on highly active antiretroviral therapy (HAART) and uninfected controls. Positive correlations between β-chemokine-positive lymphocytes and β-chemokine secretion found among controls were not consistently present among naive and HAART patients. Compared to controls, the β-chemokine secretory capacity (supernatant secretion normalized for frequencies of positive lymphocytes) was decreased for macrophage inflammatory protein (MIP)-1α in HAART patients, decreased for MIP-1β in naive patients and in patients on HAART for less than three years, and increased for RANTES (regulated on activation, normal T cell expressed and secreted) in patients on HAART for more than three years. The data indicate that lymphocytes of HIV-infected subjects display a disrupted secretory capacity for β-chemokines.
INTRODUCTION

Although the β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES (regulated on activation, normal T cell expressed and secreted) have been shown potent HIV suppressor factors in vitro [1], their role in controlling HIV replication in vivo remains controversial. Obviously, the use of various detection methods have led to conflicting conclusions [2-6]. Higher β-chemokine levels in the supernatant of stimulated lymphocyte cultures were associated with asymptomatic HIV-1 infection [2, 3]. In contrast, increased intracellular β-chemokine levels were rather associated with advanced HIV disease [4, 5], and frequencies of β-chemokine-positive lymphocytes analysed this way correlated positively with HIV-1 load and inversely with CD4⁺ T cell counts [6]. These contradictory findings could suggest a discordant relationship between intracellular β-chemokine expression and levels of effectively secreted proteins in HIV-infected subjects.

To test this hypothesis, frequencies of β-chemokine-positive lymphocytes were compared to levels of β-chemokine supernatant secretion in treatment-naive HIV patients, in patients on highly active antiretroviral therapy (HAART) and in uninfected controls.

STUDY DESIGN

Study subjects. Peripheral blood was drawn on EDTA from 12 treatment-naive and 13 HAART-treated HIV patients, and from 13 HIV-negative controls. The study was approved by the institutional review board and written informed consent was given by all study subjects prior to enrolment.

Intracellular staining for β-chemokines. Intracellular β-chemokine detection was performed as described before [6]. Briefly, freshly isolated peripheral blood mononuclear cells (PBMC) were cultured for 5 h with phorbol myristate acetate, ionomycin and brefeldin A (Sigma, St. Louis, MO, USA) for the detection of MIP-1α and MIP-1β, and overnight with monensin (Sigma, St. Louis, MO, USA) for the detection of RANTES. PBMC were stained intracellularly using fluorochrome-labeled monoclonal antibodies against RANTES, MIP-1α (Pharmingen, CA, USA) and MIP-1β (R&D Systems, Minneapolis, MN, USA), and analysed using a FACSscan flow cytometer and CellQuest software (Becton Dickinson, CA, USA). Percentages of β-chemokine-positive cells were calculated within lymphocytes.
\( \beta \)-chemokine supernatant secretion. Fresh PBMC at \( 1 \times 10^6 \) cells/ml were cultured with 1 \( \mu \)g/ml phytohaemagglutinin (Abbott, Abbott Park, IL, USA) and 10 ng/ml recombinant human interleukin-2 (R&D Systems, Minneapolis, MN, USA) for three days. Supernatants were collected and stored at \(-80^\circ\)C. \( \beta \)-chemokine concentrations were measured by ELISA using antibody pairs for RANTES (Pharmingen, CA, USA), MIP-1\( \alpha \) and MIP-1\( \beta \) (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. The secretory capacity for \( \beta \)-chemokines was calculated as the \( \beta \)-chemokine supernatant secretion normalized for percentages of \( \beta \)-chemokine-positive lymphocytes, i.e. the estimated \( \beta \)-chemokine supernatant secretion by \( 1 \times 10^6 \) \( \beta \)-chemokine-positive PBMC. Differences between groups were tested for statistical significance by the Mann-Whitney U test. Correlation analyses were performed by the Spearman’s rank test. The level of significance was set at \( P = 0.05 \).

RESULTS AND DISCUSSION

HIV-1 load among treatment-naive HIV patients reached a median value of 4.2 \( \log_{10} \) copies/ml (range 3.4 – 5.9) and was below 2.6 \( \log_{10} \) copies/ml in all patients on HAART. Patients on HAART were arbitrarily subdivided in one group of 8 patients with < 3 years of treatment (median of 28 months, range 3 – 34) and one group of 5 patients with \( \geq 3 \) years of treatment (median of 73 months, range 46 – 89). Median CD4\(^+\) T cell count among patients on HAART < 3 years was 331/\( \mu \)l which was lower than among patients on HAART \( \geq 3 \) years (777/\( \mu \)l, \( P = 0.019 \)) and treatment-naive patients (555/\( \mu \)l, \( P = 0.025 \)).

Percentages of MIP-1\( \alpha \)-positive lymphocytes were increased among patients on HAART \( \geq 3 \) years. MIP-1\( \alpha \) supernatant levels were decreased in naive and < 3 year HAART patients but not in \( \geq 3 \) year HAART patients. As a result, HAART patients showed a decreased MIP-1\( \alpha \) secretory capacity (figure 1A-C). MIP-1\( \beta \)-positive lymphocytes were increased in patients on HAART < 3 years but not \( \geq 3 \) years, while supernatant levels were decreased in naive but not in HAART patients. This resulted in a decreased MIP-1\( \beta \) secretory capacity in naive and < 3 year HAART patients with restoration in \( \geq 3 \) year HAART patients (figure 1A-C). Naive and < 3 year HAART patients had increased RANTES-positive lymphocytes compared to controls. RANTES supernatant levels were increased in all patients on HAART, resulting in an increased RANTES secretory capacity in \( \geq 3 \) year HAART patients (figure 1A-C).
Figure 1. Secretory capacity for MIP-1α, MIP-1β and RANTES in controls, treatment-naive and HAART-treated HIV patients. A, Percentages of β-chemokine-positive lymphocytes detected by intracellular staining. B, β-chemokine secretion in PBMC culture supernatants detected by ELISA. C, β-chemokine secretion per unit of β-chemokine-positive lymphocyte (secretory capacity). Data are analyzed for 13 HIV-uninfected controls (white boxes), 12 treatment-naive HIV patients (light grey boxes), 8 patient on HAART < 3 years (dark grey boxes) and 5 patients on HAART ≥ 3 years (black boxes). Box plots represent the lowest value, 25th, 50th (median) and 75th percentiles and the highest value indicated by horizontal lines in ascending order. Differences between groups were tested for statistical significance by the Mann-Whitney U test. Abbreviations: $P$, level of significance.
Uninfected controls showed significant positive correlations between lymphocyte frequencies and secretion levels for MIP-1β and RANTES with a positive trend observed for MIP-1α (figure 2A). For MIP-1α, no correlations were found in any of the HIV patient groups. For MIP-1β, a positive correlation was present among naïve patients only. For RANTES, a positive correlation was present among ≥3 year HAART patients only (figure 2B-D). No correlations were found between β-chemokine positive lymphocytes, β-chemokine secretion levels or β-chemokine secretory capacity on the one hand and HIV-1 load on the other.

**Figure 2.** Correlations between percentages of β-chemokine-positive lymphocytes and levels of β-chemokine secretion. **A**, Controls; **B**, Treatment-naive HIV patients; **C**, HIV patient on HAART < 3 years; **D**, HIV patients on HAART ≥ 3 years. Percentages of β-chemokine-positive lymphocytes detected by intracellular staining (Y-axes) were correlated to β-chemokine secretion in PBMC culture supernatants detected by ELISA (X-axes) for RANTES, MIP-1α and MIP-1β. Correlation analyses were performed by Spearman’s rank test. Abbreviations: $r$, correlation coefficient; $P$, level of significance. Significant $P$ values are displayed in bold.
These findings are in line with previous studies showing decreased MIP-1α and MIP-1β supernatant levels in HIV patients and normalization by HAART [7, 8]. Similarly, we and others found increased RANTES levels in HIV patients compared to controls [9, 10]. However, a novel aspect of our study is that we measured intracellular β-chemokine expression in parallel with supernatant levels. In this way we were able to show, for the first time, a discordant relationship between frequencies of β-chemokine-positive lymphocytes and effective secretion in naive and HAART-treated HIV patients. The secretory capacity was decreased for MIP-1α and MIP-1β and increased for RANTES, and positive correlations between the two detection methods among controls were not always present among HIV patients. These findings confirm and extend previous data in HIV patients showing an inverse relationship between intracellular and serum levels of MIP-1β [5], and reduced IFN-γ bulk secretion together with an overexpanded IFN-γ-positive cell subset [11]. Also, these data are in agreement with HIV-induced impairment of other HIV-inhibitory substances. Patients with progressive HIV infection had decreased perforin and granzyme production by natural killer cells and CD8+ T cells [12-14], and decreased levels of the CD8 antiviral factor [15].

Interestingly, intracellular levels of MIP-1β and RANTES, supernatant levels of MIP-1α and MIP-1β, and MIP-1β secretory capacity were normalized in the group of HIV patients with ≥ 3 years of HAART. This may suggest that functional immune responses are improved in HIV patients after long periods of treatment only, as recently observed for immune parameters other than β-chemokine secretion [16].

A disrupted secretory capacity for β-chemokines may provide yet another mechanism of HIV pathogenesis. Increased percentages of β-chemokine-positive T cells have been associated with viremia-driven chronic immune activation [6]. Together with an increased activation state, a decreased capacity to effectively secrete MIP-1α and MIP-1β in the vicinity will render these cells even more susceptible to HIV infection.

Our data finally explain the contradictory conclusions concerning the role of β-chemokines in HIV infection. Previous data showing low MIP-1α and MIP-1β supernatant secretion [3] and high frequencies of cells producing these β-chemokines [6] in progressing HIV patients may be appropriately explained by a common disturbed secretory capacity for β-chemokines. As a result, non-progression of HIV disease may be characterized even better by high β-chemokine secretion levels per unit of β-chemokine-positive lymphocyte, defined here as β-chemokine secretory capacity. Although no correlations were found between β-
chemokine secretory capacity and HIV-1 load in this study, it would be interesting to investigate this in larger groups of selected progressing and non-progressing HIV patients.

ACKNOWLEDGEMENTS

We thank Guido Vanham and Robert Colebunders for critical remarks and discussion, and Christa Dreezen for help with statistical analysis. This work was supported by the Belgian ‘Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen’, grant G.0396.99.

REFERENCES


Chapter 4

HIV-specific T cell responses in HIV-exposed seronegative female sex workers

4.1. T helper cells in HIV-exposed seronegatives

4.2. Enhanced ELISPOT detection by addition of IL-7 and IL-15

4.3. Amplispot detection of Gag-specific T cells
HIV-specific T helper responses and frequency of exposure among HIV-exposed seronegative female sex workers in Abidjan, Côte d’Ivoire

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Journal of Infectious Diseases, 2004, in press

ABSTRACT

Background: HIV exposure characteristics that determine the induction of HIV-specific T cells, and in particular helper T cells, are not well understood.

Methods: HIV-1 Gag- and Env-specific T helper cells were analyzed by interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assay and by interferon-γ secretion flow cytometry in HIV-exposed seronegative (ESN) and HIV-infected female sex workers (FSWs) in comparison with HIV-seronegative female blood donors in Abidjan, Côte d’Ivoire.

Results: Low-level ELISPOT responses were detected in 8 (20%) of 40 ESN FSWs. All (100%) of 25 HIV-infected FSWs showed high-level ELISPOT responses. HIV-specific CD4+ T cells, and occasionally CD8+ T cells, were detected by secretion flow cytometry in three (38%) of eight ESN FSWs and in four (80%) of five HIV-infected FSWs. ESN FSWs with detectable HIV-specific T helper responses had more clients on the previous working day ($P = 0.02$) and more HIV exposures per month ($P = 0.02$), and tended to have a lower total duration of commercial sex work.

Conclusions: These findings demonstrate the presence of HIV-specific T helper cells in ESN FSWs in association with the frequency rather than the duration of HIV exposure. The data may have important implications for the evaluation of HIV vaccine efficacy.
INTRODUCTION

Some individuals appear to resist HIV infection despite frequent exposure to the virus [1-4]. These individuals constitute an ideal group for studying mechanisms of protection against HIV infection. To date, HIV-specific CD8⁺ cytotoxic T cells (CTL) probably constitute the best-documented correlate of HIV protection in HIV-exposed seronegative (ESN) subjects as they were detected in several populations with a variety of technical approaches [2, 5-9]. Furthermore, a positive association was found between the occurrence of HIV-specific CTL and the duration of prior sex work among ESN female sex workers (FSWs) in Nairobi [10, 11]. Unfortunately, a number of these ESN FSWs became HIV-infected despite pre-existing CTL responses. This was found to be associated with a break in sex work or a reduction in the number of clients per day which could have led to waning of CTL responses [12]. While crucial for the design of a protective HIV vaccine, the HIV exposure characteristics that are associated with the establishment of HIV-specific immunity in ESN FSWs remain largely unknown.

Several reports have consistently demonstrated the importance of HIV-specific CD4⁺ T helper cell function in HIV-infected subjects for induction and maintenance of host anti-HIV immunity, in particular HIV-specific CTL [13-16]. In agreement with this, one can anticipate an essential role for T helper cells in establishing an HIV-protective immune response in ESN subjects. Several studies have indeed reported the presence of HIV-specific T helper cells in ESN subjects [1, 4, 7, 17-20]. However, these studies used methods based on lymphocyte proliferation or bulk cytokine secretion that lack the specificity of more recent methods such as enzyme-linked immunosorbent spot (ELISPOT) and cytokine flow cytometry assays.

ESN FSWs studied in Abidjan, Côte d’Ivoire, were previously shown to have detectable anti-HIV IgA antibodies in cervicovaginal secretions [21], together with decreased levels of CXCR4 expression and increased levels of T cell activation [22]. In the present study, peripheral HIV-specific T helper responses were measured in ESN FSWs, for the first time to date, by the ELISPOT assay and by IFN-γ secretion flow cytometry. In addition, we verified whether HIV-specific responses in ESN FSWs were associated with estimations of sexual HIV exposure based on the duration of commercial sex work, number of clients on the previous working day and consistency of condom use.
MATERIAL AND METHODS

Study population. During the months May 2000, February-March 2001 and October 2001, 40 HIV-seronegative and 25 HIV-seropositive FSWs were enrolled consecutively as part of an ongoing surveillance study at a confidential clinic in Abidjan [23]. Blood samples were collected and information on socio-demographics and sexual behavior were obtained by a personal interview using standard questionnaires. None of the HIV-seropositive FSWs had received antiretroviral therapy prior to enrollment. Blood samples were also collected from 32 HIV-seronegative female blood donors at the national blood transfusion center in Abidjan. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA, USA and the Ethical Committees of the Ministry of Health, Côte d’Ivoire and the Institute of Tropical Medicine, Antwerp, Belgium. Informed consent was given by all study subjects before enrollment.

Laboratory methods. All laboratory analyses were performed in Abidjan using freshly obtained whole blood samples. Whole blood was drawn from FSWs and female blood donors into EDTA tubes (Becton Dickinson, San Jose, CA, USA). The HIV status of all subjects was determined in plasma by using a combination of ELISAs [24, 25]. The HIV-negative status of seronegative FSWs was confirmed by HIV-1 reverse transcriptase-polymerase chain reaction [26].

Interferon (IFN)-\(\gamma\) ELISPOT assay. Ninety-six-well polyvinylidenefluoride-bottom plates (Millipore, Molsheim, France) were coated with 10 µg/ml anti-IFN-\(\gamma\) monoclonal antibody (MAb) (Mabtech, Nacka, Sweden) overnight at 4 °C. Plates were washed with RPMI, blocked with 10% human serum for 1 h and washed again. Peripheral blood mononuclear cells (PBMC) were separated from whole blood within 4 h of collection and resuspended in RPMI containing 5% human serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Roche Diagnostics GmbH, Mannheim, Germany). As negative control, 5 \(\times\) 10⁵ PBMC were added to duplicate wells with medium alone. As positive control, 2.5 \(\times\) 10⁵ PBMC were added to duplicate wells with 0.5 µg/ml phytohemagglutinin (PHA) (Murex, Dartford, UK). 10 µg/ml baculovirus-expressed HIV-1 Gag p24 and Env gp120 proteins (National Institute for Biological Standards and Control, Potters Bar, UK) were added to 5 \(\times\) 10⁵ and 2.5 \(\times\) 10⁵ PBMC respectively in two wells each of quadruplicate wells. HIV-1 clade B proteins were used because recombinant proteins from clade A or clade A/G, the predominant HIV-1 strains in Côte d’Ivoire [27], were not available at the time the study was initiated. Addition of 10 µg/ml cytomegalovirus (CMV) lysate (BioWhittaker Europe, Verviers, Belgium) served as an additional positive control for measurement of virus-specific T cell
responses. Plates were incubated overnight at 37 °C and 5% CO₂, washed with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) and incubated with 1 µg/ml biotinylated anti-IFN-γ (Mabtech, Nacka, Sweden) for 3 h. Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech, Nacka, Sweden) for 2 h, washed again, and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Biorad, Hercules, CA, USA) for 30 minutes followed by rinsing in tap water.

Spots were counted manually with a dissecting microscope. Average numbers of spots per well were normalized to spot forming cells (SFC)/10⁶ PBMC, negative control SFC were subtracted from antigen-stimulated SFC and negative values were scored as zero. HIV protein ELISPOT responses in ESN and HIV-infected FSWs were considered positive when the following conditions were met: (1) IFN-γ production was present in PHA-stimulated wells; (2) the number of spots in stimulated wells was at least two times higher than in negative control wells; and (3) the negative control-subtracted response exceeded a negative cut-off value of 6 SFC/10⁶ PBMC. The negative cut-off value was calculated as the average HIV protein response plus three times the standard deviation among 32 HIV-seronegative female blood donors at lower risk of HIV infection and values of 6 SFC/10⁶ PBMC were thus obtained for both p24 and gp120 proteins. For gp120, one blood donor sample was excluded in this analysis because it had high negative control and gp120 responses. This strategy allowed for increased ELISPOT sensitivity compared with previous studies that used pre-set cut-off values of 20 or 50 SFC/10⁶ PBMC [8, 28], and was justified by low SFC in negative control wells for all study subjects and low HIV protein responses among HIV-seronegative female blood donors. CMV lysate ELISPOT responses were considered positive when conditions (1) and (2) were met. Examples of ELISPOT wells for one HIV-infected FSW are shown in figure 1A.

IFN-γ secretion and enrichment flow cytometry. IFN-γ secretion flow cytometry was applied to identify HIV-specific CD4⁺ and CD8⁺ T cells according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, five million fresh PBMC were incubated overnight in 0.5 ml RPMI containing 5% human serum with medium alone (negative control), 10 µg/ml CMV lysate (positive control) and 10 µg/ml HIV-1 proteins p24 and gp120. The next day, cells were surface-stained with a CD45/IFN-γ bispecific MAb conjugate (Miltenyi Biotec), incubated for 45 min at 37 °C to permit surface capture of IFN-γ secretion, and stained with anti-IFN-γ phycoerythrin (PE) (Miltenyi Biotec), anti-CD69 fluorescein isothiocyanate (FITC), anti-CD8 peridinin chlorophyll protein (PerCP) and anti-CD3 allophycocyanin (APC) (all from Becton Dickinson, San
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Jose, CA) fluorochrome-labelled MAbs. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA). Gated CD4+ (as CD8-) and CD8+ T lymphocytes co-expressing CD69 and IFN-γ were considered as antigen-specific. Negative control percentages of CD69+/IFN-γ+ cells were subtracted from antigen-stimulated percentages and negative results were scored as zero. HIV-specific T cell responses in ESN and HIV-infected FSWs were considered positive when (1) percentages of stimulated CD69+/IFN-γ+ T cells were at least two times higher than negative control percentages and (2) negative control-subtracted percentages of CD69+/IFN-γ+ T cells exceeded negative cut-off values of 0.0090 and 0.0273 for p24-specific CD4+ and CD8+ T cells, respectively, and 0.0106 and 0.0144 for gp120-specific CD4+ and CD8+ T cells, respectively. Negative cut-off values were calculated as the average HIV protein response plus three times the standard deviation among 5 HIV-seronegative female blood donors. CMV-specific T cell responses were considered positive when condition (1) was met. Examples for the detection of antigen-specific CD4+ T cells are shown for one HIV-infected FSW in figure 1B.

**Statistical analysis.** The number of HIV exposures per month was estimated by multiplying the number of clients on the previous working day by the number of
days per month, the HIV-1 prevalence among male clients of FSWs, and the proportion of unprotected sex. The proportion of unprotected sex was based arbitrarily on the reported consistency in using condoms with ‘always’ = 0.1, ‘often’ = 0.35, ‘rarely’ = 0.7, and ‘never’ = 1. In 1998, HIV-1 prevalence among male clients of FSWs in Abidjan was 13.4 % [29]. The number of HIV exposures in lifetime was derived by multiplying the HIV exposures per month by the total duration of commercial sex work in months. Non-parametric tests were used for statistical analyses. Mann-Whitney U and Kruskal-Wallis H tests were used for comparing two and three groups respectively, Wilcoxon signed ranks test was used for comparing two variables in the same group. Spearman rank test was used for correlations. For all analyses, the level of significance was set at 0.05.

RESULTS

Characteristics of the study populations. Median age of the 40 ESN FSWs was 22 years (interquartile range (IQR) 21 – 28), compared to 24.5 (IQR 21 – 28) for the 32 female blood donors ($P = 0.319$) and 28 (IQR 23 – 36) for the 25 HIV-infected FSWs ($P = 0.01$). Median duration of commercial sex work reported by ESN FSWs was 14 months (IQR 6 – 36). Thirty three (82%) ESN FSWs reported to use condoms ‘always’ or ‘often’, and seven (18%) ‘rarely’ or ‘never’. The median reported number of clients during the previous working day was three (IQR 2 – 5). ESN FSWs were estimated to have a median of 4.3 (IQR 1.7 – 5.7) unprotected HIV exposures per month and a median of 51 (IQR 18 – 180) unprotected HIV exposures in lifetime. All 25 HIV-infected FSWs were HIV-1 seropositive and three (12%) were dually seropositive for HIV-1 and HIV-2.

Detection of low-level HIV-1-specific ELISPOT responses in ESN FSWs. Low-level HIV-1 Env- or Gag-specific ELISPOT responses above the female blood donor cut-off value were found in 8 (20%) of 40 ESN FSWs: in five (13%) against HIV-1 p24 protein, in six (15%) against HIV-1 gp120 protein and in three (8%) against both p24 and gp120 (figure 2). One additional ESN FSWs showed p24 and gp120 ELISPOT responses above the cut-off value but these responses did not exceed twice the number of spots in the negative control wells and were thus considered negative. Regardless the cut-off value, HIV-1-specific ELISPOT responses were higher among ESN FSWs than among female blood donors, reaching statistical significance for p24 but not for gp120. A direct correlation was found between of p24- and gp120-specific responses among ESN FSWs ($r = 0.464$, $P = 0.003$). No differences in magnitude were found between p24-specific and gp120-specific responses among ESN FSWs ($P = 0.215$).
Detection of high-level HIV-1-specific ELISPOT responses in HIV-infected FSWs. High-level ELISPOT responses were detected in all (100%) of 25 HIV-infected FSWs against Gag p24 protein. Env gp120 protein-specific ELISPOT responses were found in 11 (44%) of 25 HIV-infected FSWs and the magnitude of these responses was lower than for Gag p24 protein (\(P < 0.001\)) (figure 2). Four HIV-infected FSWs showed gp120 ELISPOT responses above the cut-off value that did not exceed twice the negative control response and were thus considered negative. Clearly, HIV-1-specific ELISPOT responses were significantly higher among HIV-infected FSWs than among ESN FSWs and female blood donors for both p24 and gp120. No significant correlation was found between p24- and gp120-specific responses among HIV-infected FSWs (\(r = 0.153, P = 0.464\)).

ESN FSWs had higher CMV-specific ELISPOT responses compared to female blood donors and HIV-infected FSWs. Stimulation with CMV lysate was included as a positive control for the detection of virus-specific T cell responses. 30 (94%) of 32 female blood donors, 39 (98%) of 40 ESN FSWs and 24 (96%) of 25 HIV-infected FSWs had detectable CMV-specific ELISPOT responses (figure 2). Significantly higher CMV responses were found among ESN FSWs compared with female blood donors and HIV-infected FSWs.
Table 1. IFN-γ secretion flow cytometry for the detection of HIV-1 protein- and CMV-specific CD4⁺ and CD8⁺ T cells in 5 female blood donors, 8 ESN FSWs and 5 HIV-infected FSWs, in comparison with the ELISPOT assay.

<table>
<thead>
<tr>
<th>IFN-γ secretion flow cytometry</th>
<th>ELISPOT assay</th>
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<tbody>
<tr>
<td></td>
<td>p24</td>
</tr>
<tr>
<td>T4 cells</td>
<td>T8 cells</td>
</tr>
<tr>
<td>Blood donors</td>
<td></td>
</tr>
<tr>
<td>LBDC00284</td>
<td>0.0038</td>
</tr>
<tr>
<td>LBDC00296</td>
<td>0.0044</td>
</tr>
<tr>
<td>LBDC00338</td>
<td>0.0044</td>
</tr>
<tr>
<td>LBDC00340</td>
<td>0.0008</td>
</tr>
<tr>
<td>LBDC00341</td>
<td>0.0000</td>
</tr>
<tr>
<td>ESN FSWs</td>
<td></td>
</tr>
<tr>
<td>LPXB06758</td>
<td>0.0000</td>
</tr>
<tr>
<td>LPXB06760</td>
<td>0.0125</td>
</tr>
<tr>
<td>LPXB06762</td>
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</tr>
<tr>
<td>LPXB06765</td>
<td>0.0000</td>
</tr>
<tr>
<td>LPXB06768</td>
<td>0.0018</td>
</tr>
<tr>
<td>LPXB06777</td>
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</tr>
<tr>
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</tr>
<tr>
<td>LPXB06786</td>
<td>0.0042</td>
</tr>
<tr>
<td>HIV⁺ FSWs</td>
<td></td>
</tr>
<tr>
<td>LPXB06756</td>
<td>0.0352</td>
</tr>
<tr>
<td>LPXB06767</td>
<td>0.0159</td>
</tr>
<tr>
<td>LPXB06772</td>
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</tr>
<tr>
<td>LPXB06779</td>
<td>0.0000</td>
</tr>
<tr>
<td>LPXB06782</td>
<td>0.1383</td>
</tr>
</tbody>
</table>

IFN-γ secretion flow cytometry data are negative control-subtracted percentages of CD69⁺/IFN-γ⁺ cells within CD4⁺ and CD8⁺ T cell subsets, ELISPOT data are negative control-subtracted spotforming cells per million PBMC. Values meeting criteria for positive responses are displayed in bold, see material and methods section for details.
Detection of HIV-1- and CMV-specific CD4\(^+\) and CD8\(^+\) cells by IFN-\(\gamma\) secretion flow cytometry. To assess the specificity of the ELISPOT responses in ESN FSWs, HIV- and CMV-specific CD4\(^+\) and CD8\(^+\) T cells were analyzed by IFN-\(\gamma\) secretion flow cytometry for eight ESN FSWs, five HIV-infected FSWs and five HIV-seronegative female blood donors in parallel with ELISPOT analyses (table 1). Three (38\%) out of eight ESN FSWs showed percentages of HIV-1-specific T cells above the female blood donor cut-off value. However, detection of HIV-specific T cells in ESN FSWs by IFN-\(\gamma\) secretion flow cytometry did not correlate with the ELISPOT assay: one tested positive in both assays, two tested negative in both assays, and for five ESN FSWs discordant results were noted. The one ESN FSW that tested positive in both assays showed a discrepancy in the magnitude of the responses: 0.01\% of p24-specific CD4\(^+\) T cells should correspond to 50 SFC per million PBMC if half the PBMC are CD4\(^+\) T cells, however only 10 SFC per million PBMC were detected. In contrast, among five HIV-infected FSWs, four (80\%) showed HIV-1-specific responses by IFN-\(\gamma\) secretion flow cytometry in concordance with the ELISPOT assay. Secretion flow cytometry failed to show HIV-specific T cells in one additional HIV-infected FSW that had been positive for p24 and gp120 in the ELISPOT assay. All subjects showed detectable percentages of CMV-specific CD4\(^+\) and in some cases CD8\(^+\) T cells, in concordance with the ELISPOT results.

Table 2. Association analysis between the presence of HIV-specific T helper responses and estimates of HIV exposure among ESN FSWs.

<table>
<thead>
<tr>
<th></th>
<th>ELISPOT assay</th>
<th>IFN-(\gamma) secretion flow cytometry</th>
<th>(\rho)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative ((n = 32))</td>
<td>Positive(^a) ((n = 8))</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>22 (20-27)</td>
<td>23 (21-32)</td>
<td>0.463</td>
</tr>
<tr>
<td>Duration of sex work (months)</td>
<td>15 (9-48)</td>
<td>11 (3-24)</td>
<td>0.215</td>
</tr>
<tr>
<td>Proportion of unprotected sex(^c)</td>
<td>0.35 (0.1-0.35)</td>
<td>0.35 (0.1-0.35)</td>
<td>0.346</td>
</tr>
<tr>
<td>Clients on last working day</td>
<td>3 (2-5)</td>
<td>4 (3-6)</td>
<td>0.143</td>
</tr>
<tr>
<td>Exposures to HIV per month</td>
<td>4.3 (1.7-5.7)</td>
<td>4.3 (1.8-6.8)</td>
<td>0.786</td>
</tr>
<tr>
<td>Exposures to HIV in lifetime</td>
<td>51 (17-265)</td>
<td>40 (10-67)</td>
<td>0.327</td>
</tr>
</tbody>
</table>

Data are displayed as median (interquartile range). \(^a\)Based on criteria for positive responses, see material and methods section for details. \(^b\)Two-tailed non-parametric Mann-Whitney \(U\) test. \(P\) values < 0.05 are in bold. \(^c\)The proportion of unprotected sex was based arbitrarily on the reported consistency in using condoms with ‘always’ = 0.1, ‘often’ = 0.35, ‘rarely’ = 0.7, ‘never’ = 1.
Detection of HIV-specific responses in ESN FSWs was associated with higher numbers of clients on the previous working day and higher numbers of HIV exposures per month. The detection of HIV-specific responses in the ELISPOT assay or IFN-γ secretion flow cytometry was compared to estimations of sexual exposure to HIV among ESN FSWs (table 2). Compared to ESN FSWs with a negative ELISPOT result, ESN FSWs with a positive ELISPOT result showed comparable estimated numbers of HIV exposures and a similar proportion of unprotected sex. However, total duration of sex work tended to be lower and number of clients on the previous working day tended to be higher among ESN FSWs with a positive ELISPOT result, although these differences were not statistically significant. ESN FSWs with a positive result by IFN-γ secretion flow cytometry had significantly more clients on the previous working day and significantly higher numbers of HIV exposures per month compared to ESN FSWs with a negative result. ESN FSWs with a positive result tended to have a shorter duration of sex work and an increased proportion of unprotected sex. Interestingly, the duration of sex work among ESN FSWs was inversely associated with the number of clients on the previous working day and directly with the proportion of unprotected sex (figure 4). No association was found between the duration of sex work and the number of exposures to HIV per month ($r = 0.027, P = 0.867$).

![Figure 3](image_url)

**Figure 3.** Sexual behavior characteristics of 40 ESN FSWs. **A.** Correlation between the duration of commercial sex work and the number of clients on the previous working day. **B.** Correlation between the duration of commercial sex work and the proportion of unprotected sex. The proportion of unprotected sex was based arbitrarily on the reported consistency in using condoms with ‘always’ = 0.1, ‘often’ = 0.35, ‘rarely’ = 0.7, and ‘never’ = 1. Correlation analyses were performed with the Spearman rank correlation test. $r$, correlation coefficient; $P$, level of significance.
DISCUSSION

The presence of HIV-specific T helper cells was assessed as a potential correlate of HIV exposure in a population of African ESN FSWs. Low-level Gag- or Env-specific T helper responses were detected in 20% of ESN FSWs by the ELISPOT assay and in 38% by IFN-\(\gamma\) secretion flow cytometry. The detection of HIV-specific T helper responses was associated with the frequency rather than with the duration of HIV exposure among ESN FSWs. These data indicate that HIV-specific T helper cells are primed in ESN FSWs, but that their persistence depends on frequent and continuous exposure to HIV.

Despite intensive prevention efforts and increasing condom use, seronegative FSWs in Abidjan are still frequently exposed to HIV and at high risk of acquiring infection [23]. Indeed, HIV-seronegative FSWs enrolled in this study were estimated to have a median of 4.3 unprotected exposures to HIV per month (or 52 per year) which is close to 60 or more HIV exposures per year reported for ESN FSWs in Nairobi [3].

ESN FSWs with a detectable HIV-specific T helper response had significantly more clients on the previous working day and more HIV exposures per month, and tended to have a lower total duration of commercial sex work. In concert, an inverse correlation between the total duration of commercial sex work and the number of clients on the previous working day was noted. Although we cannot exclude potential bias as the result of the self-reporting of sexual behavior, the extrapolation of these data, and the small number of subjects with detectable HIV-specific responses, these observations make sense in the light of previous reports. Among ESN FSWs in Nairobi, waning of CTL responses was detected in association with a break in sex work or a reduction in the number of clients per day [12]; HIV-specific responses in health care workers with occupational exposure to HIV rapidly fall below the limits of detection [17, 30]; reduced HIV-stimulated T helper cell activity was found in cord blood after short-course antiretroviral treatment for prevention of maternal-infant transmission [31]; the highest urethral concentrations of HIV-1-specific IgA were seen in ESN men with the most recent unprotected sexual episode [32]; and the majority of ESN women with HIV-specific T cells reported recent HIV exposure [33]. Together, these findings may suggest that frequent and continuous exposure to HIV is the prerequisite for the presence and maintenance of HIV-specific immunity in ESN subjects.

In contrast to many other studies [5, 8, 33], including this one, some studies failed to detect HIV-specific T cell responses in ESN subjects [34, 35]. First of all, absence of detectable HIV-specific T cells may have resulted from the applied
methodology, including the use of pre-set negative cut-off values leading to decreased ELISPOT sensitivity (discussed below). In addition, the HIV exposure rate among HIV-1 discordant couples and other populations of ESN subjects is most probably to be manifold lower and also less continuous compared with African FSWs [5, 35], in such a way that the frequency of pre-existing HIV-specific T cells in these subjects may have decreased below the detection limit by the time of blood sampling. Lastly, it is very conceivable that other immune responses contribute to protection against infection with HIV in these populations, such as HIV-specific IgA [21, 36], or noncytolytic CD8+ T cell mediated HIV inhibition [37, 38].

Detectable ELISPOT responses to HIV-1 proteins in our group of ESN FSWs were relatively weak as they ranged from seven to twenty SFC/10⁶ PBMC. Instead of using a pre-set cut-off value of 20 or 50 SFC/10⁶ PBMC like in other studies [8, 28], HIV protein ELISPOT responses in our study were considered positive if they exceeded the average response plus three times the standard deviation in a large group of HIV-negative female blood donors at lower risk of HIV infection, statistically limiting 99.7% of negative ELISPOT responses. Given the fact that assay conditions such as the origin and quality of the samples, operators, composition of the media, etc. may vary for every study, this approach may be more justifiable than using pre-set cut-off values. In the present study, a high ELISPOT sensitivity was obtained as a consequence of low background responses among all study subjects and very low HIV protein responses among HIV-seronegative female blood donor controls. The use of freshly isolated rather than cryopreserved PBMC, and human serum instead of fetal calf serum in the culture medium could have been instrumental, together with the fact that baculovirus-expressed p24 and gp120 proteins selected for this study showed significantly lower background stimulation in low-risk HIV-negative laboratory workers compared with a set of HIV proteins from other sources (authors’ unpublished data). Thus, although of low level, HIV-specific T helper responses among ESN FSWs fell within the detectable range of this sensitive ELISPOT assay. Moreover, experiments are being conducted with a highly sensitive modified ELISPOT method to confirm the presence of HIV-specific T helper cells in ESN FSWs [39].

Whether such low-level HIV-specific responses are of significance with respect to protection against infection with HIV remains uncertain. In that regard, the in vitro responses to HIV proteins observed in a subgroup of ESN FSWs in this study may also be coincidental markers of increased exposure to HIV. Alternatively, they may have resulted from infection with cross-reactive pathogens, as recently proposed [40, 41], or from exposure to cross-reactive allo-antigens [42, 43], both of which
may be more experienced by ESN FSWs than by female blood donors. In fact, the protective role of T helper responses in ESN subjects can only be addressed in a longitudinal seroconversion study, for which, unfortunately, the present study was not designed.

ELISPOT and IFN-\(\gamma\) secretion flow cytometry assays showed concordant results for the detection of HIV-specific responses in HIV-infected subjects, and for the detection of CMV-specific responses in all study subjects. Among ESN FSWs, however, detection of HIV-specific T cells by IFN-\(\gamma\) secretion flow cytometry did not correlate with the ELISPOT assay, which may have been the consequence of the low-level responses in these subjects close to the detection limit for both assays. In addition, discrepancies were noted in the magnitude of the responses detected by the two methods. Indeed, IFN-\(\gamma\) secretion flow cytometry may be prone to magnification of responses, since IFN-\(\gamma\) secreted by stimulated HIV-specific T cells may be taken up by non-stimulated T cells in their vicinity, as previously suggested [44].

Classically, exogenous viral proteins are taken up by antigen-presenting cells, processed and presented on MHC class II molecules to stimulate CD4\(^+\) T cells. In this study, however, some ESN and HIV-infected FSWs also showed HIV-1 protein-stimulated CD8\(^+\) T cells in addition to CD4\(^+\) T cells. This may have been the result of an alternate pathway for exogenous antigen processing and presentation on MHC class I molecules to CD8\(^+\) T cells, referred to as antigen cross-presentation [43, 44]. Occurrence of this pathway in ESN FSWs in vivo could indicate that HIV-specific CTL are not necessarily the result of an extraordinary controlled HIV infection in these subjects as recently proposed [45], but that CD8\(^+\) T cells may be primed directly with defective non-replicating particles or exogenous viral proteins. Interestingly, antigen cross-presentation has been suggested to play a role in protection against HIV disease progression [46, 47], and it should therefore be studied more in ESN subjects as well.

Among HIV-infected FSWs, T helper responses directed against Gag p24 were much higher than against Env gp120. This may be explained by the fact that HIV-1 Gag is more conserved than HIV-1 Env in such a way that the clade B Gag p24 protein used in our analyses has more immunodominant epitopes in common with the circulating HIV-1 clade A/G viruses in Côte d’Ivoire than the clade B Env gp120 protein. Interestingly, these differences between Gag and Env responses were not present among ESN FSWs, suggesting that ESN FSWs recognize as many conserved clade B epitopes in Env as they do in Gag. This may be in line with the previous observation of a differential HIV-1 epitope recognition in ESN and HIV-
infected subjects [11]. In that respect it can be hypothesized that ESN FSWs in our study respond to the rare conserved epitopes within in the highly variable Env that may also be more protective against HIV, while HIV-infected subjects rather respond to newly emerging but less protective Env epitopes.

In conclusion, low levels of HIV-specific T helper cells in a subgroup of ESN FSWs in Abidjan, Côte d’Ivoire were associated with the frequency of HIV exposure rather than with the duration of sex work. These data may have important implications for HIV vaccine design and monitoring of immune responses in vaccinees.

ACKNOWLEDGMENTS

We thank the community of female sex workers in Abidjan for their cooperation, Souleymane Sawadogo, Kabran N’Guessan and N’Depo Yenon for technical assistance, Emmanuel Abonga for providing female blood donor samples, Jef Braem and Serge Blockmans for logistical support in the field, Ward Schrooten for critical remarks and discussion. This work was supported by the Belgian Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen, grant G.0396.99 and by the Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention (CDC), Atlanta, GA. The following HIV-1 recombinant proteins were obtained through the EU Program EVA/MRC Centralised Facility for AIDS reagents, NIBSC, UK (grant number QLK2-CT-1999-00609 and GP828102): p24 (EVA620) and gp120 (EVA607) from Dr I Jones.

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Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15 – the Amplispot assay

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ABSTRACT

The importance of the enzyme-linked immunosorbent spot (ELISPOT) assay as a tool for studying immune responses in vitro is becoming increasingly apparent. However, there remains a need for enhanced sensitivity for the detection of low frequency antigen-specific T cell responses. We reasoned that the addition of a combination of the cytokines interleukin (IL)-7 and IL-15 would selectively increase interferon (IFN)-γ production from antigen-stimulated CD4+ and CD8+ effector memory T cells. Freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMC) from four healthy donors were analysed by ELISPOT for the frequency of purified protein derivative (PPD)-specific CD4+ T cells or cytomegalovirus (CMV) peptide-specific CD8+ T cells. Addition of IL-7 and IL-15 increased the number of PPD-specific CD4+ T cells up to 2.4-fold in fresh PBMC and up to 18-fold in cryopreserved PBMC. The cytokines also increased the number of CMV peptide-specific CD8+ T cells in fresh PBMC up to 7.5-fold. No additional increases were seen when antibodies to co-stimulatory molecules CD28 and CD49d were applied together with the cytokine combination. These data demonstrate that the sensitivity of the ELISPOT assay may be significantly augmented by addition of the cytokines IL-7 and IL-15 to antigen-stimulated cells. This method will be particularly useful for the assessment of antigen-stimulated cytokine production by T cells in cryopreserved biological specimens.
INTRODUCTION

The enzyme-linked immunosorbent spot (ELISPOT) assay has become widely utilised as a tool to study cellular and humoral immune responses in vitro [1, 2]. ELISPOT has been used to determine the frequency of specific CD4+ and CD8+ T cell responses to self [3, 4], tumor [5, 6], viral [7-9], bacterial [10, 11] and other antigens [12, 13]. Secretion of a range of cytokines (interferon (IFN)-γ, TGF-β, TNF-α, interleukin (IL)-4, IL-5, IL-6, IL-10, IL-12) [14-17], β-chemokines (MIP-1α, MIP-1β, RANTES) (these authors, unpublished) and cytotoxins (granzymes) [18] may be measured. The sensitivity of the ELISPOT assay for detecting CD8+ T cell responses has been estimated to be at least 1 log10 higher than traditional limiting dilution analysis or bulk 51Cr release assay [7, 8, 19, 20].

Although the sensitivity and technical ease of the ELISPOT assay make it a useful alternative to traditional analytical methods, there remains a need for increased sensitivity in detection of low frequency antigen-specific T cell responses. In addition, cytokine production may be compromised in cryopreserved samples (these authors, unpublished) [21, 22]. Use of autologous dendritic cells as antigen-presenting cells may augment the sensitivity of ELISPOT [21, 23, 24]. However, this method requires large amounts of fresh peripheral blood and in vitro maturation of monocytes over a period of 5 to 7 days. Thus, it is desirable to identify a combination of cytokines and/or co-stimulatory molecules that might substitute for the potent antigen-presenting capacity of mature dendritic cells.

Naive T cells require interactions with self-MHC molecules in order to survive and proliferate under lymphopenic conditions [25-27]. Memory T cells do not require these signals, but depend upon cytokines including IL-7 and IL-15 for homeostatic proliferation [28-34], and can be expanded in vitro with addition of these cytokines [35-38]. IL-7, which may be produced in vivo in response to T cell depletion [39], can enhance proliferation of both naive and memory CD8+ T cells [40]. IL-15 is produced by a wide variety of cells, including monocytes and dendritic cells, and induces CD4+ and CD8+ T cell proliferation [41-45]. If the TCR is activated by antigen, IL-15 can augment cytokine production [46]. We reasoned that a combination of antigen, IL-7 and IL-15, would selectively increase cytokine production from CD4+ and CD8+ effector memory T cells, leading to increased sensitivity of detection in the ELISPOT assay.
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MATERIAL AND METHODS

Peripheral blood mononuclear cells (PBMC) and donors. Heparinised blood samples were obtained, with informed consent, from healthy donors among some were known to be vaccinated with the Bacillus Calmette-Guérin (BCG) vaccine (a live vaccine prepared from an attenuated strain of Mycobacterium bovis), or had a known cellular response against cytomegalovirus (CMV). PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were either used fresh or were kept frozen at –140 °C (vapour phase of liquid nitrogen) in foetal calf serum (FCS) (Gemini Bioproducts, Woodland, CA, USA) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

Protein and peptide preparations. IFN-γ production by CD4+ or CD8+ T cells was assessed in response to stimulation with protein antigen or synthetic peptides, respectively. Purified protein derivative (PPD) (Staten Serum Institute, Copenhagen, Denmark) was used as protein antigen. Synthetic peptides included HLA-A*0201-restricted immunodominant epitopes from cytomegalovirus (CMV) matrix protein pp65, (495-503, NLVPMVATV) and human immunodeficiency virus type-1 (HIV-1) Gag (77-85, SLYNTVATL), and an HLA-B*35-restricted epitope from HIV-1 Pol (329-337, HPDIVIYQY) (peptides provided by G. Ogg, Oxford University). As a negative control, PBMC were stimulated with media alone. As a positive control, PBMC were stimulated with staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, MO, USA).

ELISPOT assay. For ELISPOT assays, 96-well nitrocellulose-bottom plates (Multiscreen-HA, Millipore, Molsheim, France) were coated with 50 µl/well of anti-human IFN-γ at a concentration of 5 µg/ml (1-D1K, Mabtech, Nacka, Sweden) and incubated at 4 °C overnight. The following day, plates were washed four times in PBS and blocked with 50 µl/well of culture medium (RPMI containing 15% FCS, 1% L-glutamine, 1% penicillin-streptomycin and HEPES buffer). Cytokine cocktails were prepared containing IL-7 and IL-15 (R&D Systems, Minneapolis, MN, USA) in concentrations ranging from 1 to 100 ng/ml. For some experiments, co-stimulatory antibodies anti-CD28 and anti-CD49d were added at 1 µg/ml (Becton Dickinson, San Jose, CA, USA). One hundred microliters of medium, with or without cytokines and co-stimulatory antibodies, was distributed to replicate wells of the ELISPOT plate. Antigens were added such that final concentrations were 10 µg/ml for PPD and 10 µg/ml for synthetic peptides. PBMC (2 × 10^5) were then added in a volume of 50 µl, bringing the total volume in each well to 200 µl. Incubation was continued overnight (16-18 h) at 37 °C, 5% CO₂. ELISPOT plates were developed as previously described [7]. Briefly, plates were washed four times
with PBS containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 h with 50 µl/well of biotinylated anti-human IFN-γ at a concentration of 1 µg/ml (7-B6-1, Mabtech, Nacka, Sweden) at 37 °C. Plates were then washed four times in PBS with 0.1% Tween-20 and incubated for 1 h with avidin-bound biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) at room temperature. ELISPOT plates were washed again four times in PBS with 0.1% Tween-20 and developed by incubating for 5 minutes with stable diaminobenzadine substrate (DAB, Research Genetics, Huntsville, AL, USA), followed by rinsing in tap water. Spots were counted using an AID ELISPOT reader (Cell Technology Inc., Jessup, MD, USA), totals for duplicate or triplicate wells were averaged and normalised to numbers of IFN-γ spotforming cells per $1 \times 10^6$ PBMC. Average values for negative medium control wells were subtracted from the average values from antigen-stimulated wells.

RESULTS

Enhanced ELISPOT detection of CD4+ T cell responses in fresh and cryopreserved PBMC by addition of both IL-7 and IL-15.

Frequencies of CD4+ T cells reactive against PPD were analysed by ELISPOT in fresh and cryopreserved PBMC from two donors (donors 1 and 2) known to be vaccinated with BCG. PBMC were incubated overnight in duplicate wells in the presence of media alone, PPD, media supplemented with 50 ng/ml of both IL-7 and IL-15, and PPD supplemented with 50 ng/ml of both IL-7 and IL-15 (figure 1, panels A-D for fresh PBMC from donor 1, panels F-I for cryopreserved PBMC from donor 1).

For fresh PBMC, addition of both IL-7 and IL-15 to the PPD-stimulation resulted in an increase in the number of PPD-specific IFN-γ secreting cells per million PBMC by a factor of 1.6 for donor 1 (mean ± standard error of the mean (S.E.M.): $2635 \pm 77.5$ vs. $1670 \pm 155.0$, figure 1E) and by a factor of 2.4 for donor 2 ($605 \pm 122.5$ vs. $250 \pm 25.0$, data not shown). Addition of IL-7 and IL-15 enhanced spontaneous IFN-γ release in medium control wells by a factor of 9.7 ($145 \pm 60.0$ vs. $15 \pm 7.5$, figure 1E) for donor 1 and by a factor of 19 ($95 \pm 40.0$ vs. $5 \pm 5.0$, data not shown) for donor 2.

In the absence of IL-7 and IL-15, the number of PPD-specific IFN-γ secreting cells was significantly lower in cryopreserved PBMC compared to fresh PBMC ($420 \pm 27.5$ vs. $1670 \pm 155.0$ for donor 1, factor 0.25, figure 1J and figure 1E; $55 \pm 25.0$ vs. $250 \pm 25.0$ for donor 2, factor 0.22, data not shown). Addition of IL-7 and IL-15 to PPD in cryopreserved PBMC augmented the number of PPD-specific IFN-γ secreting cells by a factor of 4.7 for donor 1 ($1970 \pm 150.0$ vs. $420 \pm 27.5$, figure
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1J) and by a factor of 18 for donor 2 (980 ± 242.5 vs. 55 ± 25.0, data not shown), restoring these responses to levels near those observed in fresh PBMC. The combination of IL-7 and IL-15 also enhanced spontaneous IFN-γ release by cryopreserved PBMC in medium control wells, by a factor of 77 (765 ± 362.5 vs. 10 ± 2.5, figure 1J) for donor 1 and by a factor of 38 (190 ± 72.5 vs. 5 ± 5.0, data not shown) for donor 2.

For both donors, PPD-specific IFN-γ-producing lymphocytes were CD4+ T cells as demonstrated by intracellular cytokine flow cytometry using monoclonal antibodies specific for IFN-γ and CD3, and CD4 cell depletion (data not shown).

Figure 1. ELISPOT detection of PPD-specific CD4+ T cell responses in fresh and cryopreserved PBMC from donor 1 with addition of both IL-7 and IL-15. A-D, For fresh PBMC, pictures are shown from ELISPOT wells containing 2 × 10⁵ PBMC stimulated with medium alone (A), 10 µg/ml of PPD (B), medium supplemented with 50 ng/ml of IL-7 and IL-15 (C) and 10 µg/ml of PPD supplemented with 50 ng/ml of IL-7 and IL-15 (D). E, Averaged values from duplicate wells normalised to numbers of IFN-γ spot-forming cells per 1 × 10⁶ PBMC for the different stimulation conditions. Error bars represent the standard error of the mean. F-J, For cryopreserved PBMC, idem.
Enhanced ELISPOT detection of CD8⁺ T cell responses in fresh PBMC by addition of IL-7 and IL-15. Frequencies of CD8⁺ T cells reactive against the HLA-A*0201-restricted immunodominant epitope from CMV matrix protein pp65 (NLVPMVATV) were analysed by ELISPOT in freshly isolated PBMC from an HLA-A*02-positive donor (donor 3). PBMC were incubated overnight in triplicate wells in the presence of media alone, CMV peptide, media supplemented with 50 ng/ml of IL-7 and IL-15 (C) and 10 µg/ml of CMV peptide supplemented with 50 ng/ml of IL-7 and IL-15 (D). E, Bars represent averaged values from triplicate wells normalised to numbers of IFN-γ spotforming cells per 1 × 10⁶ PBMC for the different stimulation conditions. Error bars represent the standard error of the mean.

Figure 2. ELISPOT detection of CD8⁺ T cell responses specific for an immunodominant HLA-A*0201-restricted epitope from CMV matrix protein pp65 in fresh PBMC from donor 3 with addition of both IL-7 and IL-15. A-D, Pictures are shown from ELISPOT wells containing 2×10⁶ PBMC stimulated with medium alone (A), 10 µg/ml CMV peptide (B), medium supplemented with 50 ng/ml of IL-7 and IL-15 (C) and 10 µg/ml of CMV peptide supplemented with 50 ng/ml of IL-7 and IL-15 (D). E, Bars represent averaged values from triplicate wells normalised to numbers of IFN-γ spotforming cells per 1 × 10⁶ PBMC for the different stimulation conditions. Error bars represent the standard error of the mean.

Addition of both IL-7 and IL-15 to CMV peptide resulted in a 7.5-fold increase in the number of IFN-γ secreting cells per million PBMC as compared to CMV peptide alone (mean ± S.E.M.: 2285 ± 675.5 vs. 305 ± 1.7, figure 2E). Addition of both IL-7 and IL-15 enhanced the spontaneous release of IFN-γ in medium control wells by a factor of 10 (200 ± 58.5 vs. 20 ± 1.7, figure 2E).

As opposed to the combined addition of the cytokines, the individual addition of IL-15 to CMV peptide only resulted in a marginal increase in the number of IFN-γ secreting cells compared to CMV peptide alone (mean ± S.E.M.: 370 ± 117.6 vs. 305 ± 2.9, factor 1.2), while the addition of IL-7 individually did not have any effect at all (300 ± 70.1 vs. 305 ± 2.9, factor 0.98) (data not shown).
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Combination of both IL-7 and IL-15 with co-stimulatory antibodies provides no additional enhancement. Addition of co-stimulatory antibodies anti-CD28 and anti-CD49d to PPD stimulation, in the absence of cytokines, resulted in increased numbers of PPD-specific IFN-γ secreting cells compared to PPD stimulation alone, by a factor of 1.5 for fresh PBMC from donor 1 (mean ± S.E.M.: 2550 ± 70 vs. 1670 ± 155, figure 3A and figure 1E) and by a factor of 1.5 for cryopreserved PBMC from donor 1 (625 ± 120 vs. 420 ± 27.5, figure 3B and figure 1J). For fresh PBMC from donor 1, the enhancement of PPD-specific IFN-γ secretion provided by co-stimulatory antibodies equalled that induced by IL-7 and IL-15 (factor 1.5 vs. 1.6), but was much lower for cryopreserved PBMC from donor 1 (factor 1.5 vs. 4.7). The same conclusions were valid for donor 2 (data not shown).

When both cytokines (IL-7 and IL-15) and co-stimulatory antibodies (anti-CD28 and anti-CD49d) were combined with PPD stimulation of PBMC from donor 1, no significant additive effects were observed. Although the total number of IFN-γ secreting cells was increased compared to stimulation with PPD alone (2470 ± 107.5 vs. 1670 ± 155 for fresh PBMC, factor 1.5, figure 3A and figure 1E; 805 ± 270 vs. 420 ± 27.5 for cryopreserved PBMC, factor 1.9, figure 3B and figure 1J), the magnitude of the increase was comparable to or lower than that observed when cytokines or co-stimulatory antibodies were added individually to PPD. This effect was explained by a sharp increase in background release of IFN-γ in medium control wells treated with both cytokines and co-stimulatory antibodies (935 ± 10
vs. 15 ± 7.5 for fresh PBMC, factor 62, figure 3A and figure 1E; 1340 ± 2.5 vs. 10 ± 2.5 for cryopreserved PBMC, factor 134, figures 3B and figure 1J). The same conclusions were valid for donor 2 (data not shown).

Optimal cytokine concentrations. The cytokines IL-7 and IL-15 were added to PPD in the ELISPOT assay using cryopreserved PBMC from donors 1 and 2, in concentrations ranging from 1 ng/ml to 100 ng/ml for both cytokines. For donor 1, addition of IL-7 and IL-15 at a concentration as low as 1 ng/ml resulted in a 7.3-fold increase in the number of PPD-specific IFN-γ secreting cells compared to PPD alone (mean ± S.E.M.: 1315 ± 107.6 vs. 180 ± 10, figure 4). The magnitude of enhancement was not significantly increased when higher concentrations of cytokines were added. The maximal enhancement observed was 7.9-fold, at IL-7 and IL-15 concentrations of 50 ng/ml (figure 4). However, augmenting the concentrations of IL-7 and IL-15 in medium control wells also resulted in increased spontaneous release of IFN-γ, thereby reducing the net enhancement of PPD-specific responses. Concentrations in the 1-5 ng/ml range appeared to provide maximal enhancement of antigen-specific responses with minimal increases in background spontaneous IFN-γ release. The same results were obtained for donor 2 (data not shown).

Induction of primary responses against HIV-1 peptides by addition of both IL-7 and IL-15. Frequencies of CD8⁺ T cells reactive against an HLA-A*0201-
restricted human immunodeficiency virus type-1 (HIV-1) Gag peptide epitope (77-85, SLYNTVATL) and an HLA-B*35-restricted HIV-1 Pol peptide epitope (329-337, HPDIVIYQY), were analysed by ELISPOT in cryopreserved PBMC from two donors (donors 3 and 4) without known exposure to HIV. Both donors were HLA-A*02-positive, donor 3 was HLA-B*35-negative and donor 4 was HLA-B*35 positive. PBMC were incubated overnight in duplicate wells in the presence of media alone, HIV-1 peptides, media supplemented with 50 ng/ml of both IL-7 and IL-15, and HIV-1 peptides supplemented with 50 ng/ml of both IL-7 and IL-15.

In the absence of IL-7 and IL-15, neither donor mounted a response against the Gag peptide or the Pol peptide (mean ± S.E.M.: 15 ± 2.5 and 15 ± 2.5, respectively, for donor 3; 25 ± 15 and 15 ± 5, respectively, for donor 4). In the presence of IL-7 and IL-15, however, a significant increase was observed in the response of donor 3 against the Gag peptide (120 ± 5 vs. 15 ± 2.5, factor 8). No significant increases were seen for the Pol peptide in donor 3, or for either peptide in donor 4 (data not shown).

DISCUSSION

In this report, we demonstrate that simultaneous addition of the cytokines IL-7 and IL-15 enhances detection of antigen-specific CD4+ and CD8+ T cells in the IFN-γ ELISPOT assay by factors ranging from 1.6- to 7.5-fold for fresh PBMC and 4.7- to 18-fold for cryopreserved PBMC. Antibodies to co-stimulatory molecules CD28 and CD49d applied together with the cytokine combination increased background IFN-γ release and did not result in additional enhancement. Optimal enhancement of antigen-specific responses with minimal background IFN-γ release was provided at cytokine concentrations as low as 1 ng/ml.

This method may prove particularly useful for the quantification of antigen-specific CD4+ and CD8+ T cells in the setting of longitudinal clinical studies, in which only cryopreserved PBMC samples are available. Detection of antigen-specific cells by ELISPOT using cryopreserved PBMC samples has shown variable sensitivity [21, 22]. This is probably the result of decreased cell viability [47, 48], and the selective depletion of monocytes after freezing and thawing [49, 50]. The presence of viable antigen-presenting cells (i.e., monocytes and/or dendritic cells) is believed to be critical for efficient induction of antigen-specific T cell responses in ELISPOT assays [21, 51]. In addition, the method described here may be relevant for subjects with low-level HIV-specific T cell responses that have been difficult to detect by standard methods. These subjects include individuals exposed to HIV but who remain uninfected [52-54], HIV-infected patients on HAART with suppressed viral
load and subsequent diminished T cell responses [21, 55, 56], and patients with very low CD4+ T cell counts resulting in antigen unresponsiveness in both CD4+ and CD8+ T cell subsets [57]. This method will also be useful for measuring responses to experimental HIV vaccines. Recently, a phase III efficacy trial testing the canarypox-based ALVAC vCP1452 vaccine with gp120 boost was postponed because of low-level T cell responses against HIV Env and Gag in the phase II trial [58]. Finally, the method may be applied to detect T cell responses to other infectious agents (viral, bacterial or other), as well as tumor or “self” antigens, in instances where low-level responses may be overlooked by standard methods [21].

Fresh and cryopreserved PBMC samples from all four donors included in the study showed elevated background IFN-\(\gamma\) release in the presence 50 ng/ml of both IL-7 and IL-15. In addition, 50 ng/ml of both IL-7 and IL-15 induced an apparently non-specific response against the HIV-1 Gag peptide in donor 3, suggesting that primary in vitro responses may be generated in a proportion of donors by this cytokine combination. Accordingly, it will be important to establish a statistically valid range of positive and negative responses by testing irrelevant peptides in the presence of the cytokine combination in HIV-unexposed, seronegative individuals. However, for cryopreserved PBMC from two donors, it was shown that cytokine concentrations as low as 1 ng/ml can preserve the enhancement of antigen-specific responses in the absence of significant background IFN-\(\gamma\) release. Accordingly, the use of lower concentrations of cytokines (i.e., 1-5 ng/ml rather than 50 ng/ml, as shown in figure 4) might also prevent the amplification of primary responses.

The results presented in this study demonstrate that combined addition of the cytokines IL-7 and IL-15 to antigen-stimulated PBMC can significantly enhance IFN-\(\gamma\) production. Thus, the presence of these two cytokines significantly enhances the sensitivity of the ELISPOT assay. This method will be particularly useful for the assessment of antigen-stimulated cytokine production by CD4+ and CD8+ T cells in cryopreserved specimens and in subjects with low-level antigen-specific T cell responses that have been difficult to detect by standard methods.

ACKNOWLEDGEMENTS

We thank Einar Martin Aandahl and Patrick A. Haslett for performing cytokine flow cytometry analyses, Graham S. Ogg for providing peptides, John Carroll and Chris Goodfellow for preparing the figures, Guido van der Groen for helpful discussion. Funding for this research was provided by the National Institutes of Health (R21-AI47746 to B.L.S. and R01-AI46254 to D.F.N.). W.J. was supported by a research grant (G.0396.99) and a travel grant from the Nationaal Fonds voor
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Wetenschappelijk Onderzoek (NFWO) - Vlaanderen. D.F.N. is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

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Enhanced detection of HIV-1 Gag-specific T cells in HIV-exposed seronegative and HIV-infected female sex workers by the Amplispot assay

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In preparation

ABSTRACT

T cell responses directed against Gag proteins and overlapping matrix Gag peptide pools were analysed in African ESN FSWs, HIV-infected FSWs and HIV-seronegative female blood donors by the highly sensitive Amplispot assay. Gag protein-specific responses were detected in 5 of 20 (25%) ESN FSWs compared to 9 of 10 (90%) of HIV-infected FSWs; two ESN FSWs showed Gag protein responses within the range of responses observed among HIV-infected FSWs. In addition, 5 of 20 (25%) ESN FSWs showed detectable Gag peptide pool responses, compared to 9 of 10 (90%) of HIV-infected FSWs. Three (15%) ESN FSWs showed responses against both Gag protein and peptide pools. ESN FSWs and HIV-infected FSWs preferentially responded to the same peptide pools containing the last part of p17 and the first part of p24, but showed discordant responses for p15 of which the first part was more often targeted by ESN FSWs and the last part more often by HIV-infected FSWs. Together, these data provide further evidence for the presence of HIV-specific T cell responses in ESN FSWs which have been difficult to detect by standard methods. Further analysis of Amplispot responses against single peptides may lead to the identification of novel HIV-1 Gag epitopes that are preferentially recognised by ESN FSWs and which may be of importance for future HIV vaccine design.
INTRODUCTION

Characterising of the immune responses which are associated with non-transmission of HIV among HIV-exposed seronegative (ESN) individuals is of great importance for designing a successful HIV vaccine [1]. HIV-specific T cells have been well-documented as a correlate of protection against infection with HIV in several ESN populations [2-6]. Recently, a clinical trial has been initiated to test a vaccine that is based in part on the HIV epitopes that were recognised by HIV-specific cytotoxic T cells in a population of African ESN female sex workers (FSWs) [7].

However, for several reasons, the presence of HIV-specific T cells in ESN subjects remains a controversial issue. HIV-specific T cells are generally detected at low levels, 10 to 100-fold lower than in HIV-infected subjects and near the limits of detection for ELISPOT and intracellular cytokine flow cytometry assays, putting their physiological relevance into question [8-10]. In most ESN populations, the majority of subjects did not show detectable HIV-specific T cells [6, 9-12], indicating that protection could be due to other immune responses such as HIV-specific IgA [13, 14], or noncytolytic CD8+ T cell mediated HIV inhibition [15, 16], or that HIV-specific T cell responses are below the limit of detection of the present methods. In addition, the recent suggestion that ESN FSWs contain extraordinary low levels of replicating virus may indicate that HIV-specific T cells in ESN FSWs, as in HIV-infected subjects, are in fact effector cells combating infection rather than circulating memory cells which have to prevent infection [17]. Also, a protective role for HIV-specific T cells may be best proven in prospective follow-up studies or when their presence can be clearly associated with the extent of HIV exposure of ESN subjects. However, the sexual behaviour characteristics that correlate with HIV-specific T cells in ESN FSWs are just beginning to be defined [18, 19]. Therefore, more studies are needed that unambiguously prove the presence and the role of HIV-specific cellular immunity in protection against infection with HIV.

Two previous studies have led to the conception of the present study. Firstly, we have detected HIV-specific T helper responses in ESN FSWs by stimulation of fresh peripheral blood cells with HIV-1 Gag protein in the ELISPOT assay. Although the detected responses were statistically above the background responses in a large group of HIV-seronegative female blood donors at lower risk for HIV exposure, responses among ESN FSWs were essentially of low-level [10]. Secondly, in an effort to enhance the sensitivity of detection of antigen-specific T cell responses, we designed the Amplispot assay as a modification of the standard ELISPOT assay by the combined addition of the cytokines IL-7 and IL-15. Both
cytokines significantly increased the number of antigen-specific T cells in fresh peripheral blood mononuclear cells (PBMC) (up to 7.5-fold), and even more so in cryopreserved PBMC (up to 18-fold) [20].

In the present study, T cell responses directed against Gag proteins and overlapping Gag peptide pools were analysed in cryopreserved PBMC from ESN and HIV-infected FSWs and compared to those of HIV-seronegative female blood donors, using the sensitive Amplispot assay. We were able to detect low-level Gag protein-specific responses in a subset of ESN FSWs and, more importantly, for some subjects, the magnitude of the responses fell within the range of responses observed among HIV-infected FSWs. A subgroup of ESN FSWs also showed detectable Gag peptide pool responses. These preliminary findings will allow the identification of specific HIV-1 Gag epitopes that are specifically recognised by ESN FSWs in Abidjan.

MATERIAL AND METHODS

Study population. 20 HIV-seronegative and 10 HIV-infected FSWs were enrolled consecutively in a confidential clinic in Abidjan as part of a multiple-centre trial testing the efficacy of the HIV microbicide nonoxynol-9 [21]. Blood samples and standard questionnaires with information on socio-demographics and sexual behaviour were collected. None of the HIV-infected FSWs had received antiretroviral therapy prior to enrolment. Blood samples were also collected from 10 HIV-seronegative female blood donors at the national blood transfusion centre in Abidjan. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA, USA, and the Ethical Committees of the Ministry of Health, Côte d’Ivoire and the Institute of Tropical Medicine, Antwerp, Belgium. All study subjects gave their informed consent before enrolment.

Laboratory methods. Whole blood was drawn from FSWs and female blood donors in EDTA tubes (Becton Dickinson, San Jose, CA). Within 4 h of collection, plasma was separated from whole blood by centrifugation, and stored at −70 °C. The HIV status of all subjects was determined in plasma by using an HIV testing algorithm based on a combination of ELISAs [22, 23]. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by gradient centrifugation using lymphocyte separation medium (ICN Biomedicals, Aurora, OH, USA), resuspended in RPMI containing 50% foetal calf serum (Life Technologies, Paisly, UK) and 10% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and stored in liquid nitrogen.
Proteins, peptides and peptide pools. Recombinant baculovirus-expressed HIV-1 subtype B p24 Gag protein was obtained from the Centralised Facility for AIDS Reagents (National Institute for Biological Standards and Control, Potters Bar, UK). Recombinant HIV-1 subtype B p55 Gag protein expressed in yeast was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Rockville, MD, USA). Proteins were used in ELISPOT assays at a final concentration of 10 µg/ml. Forty-nine peptides of 20 amino acids in length, with 10-amino acid overlaps between sequential peptides, comprising the entire Gag region of HIV-1 subtype A (reference strain 92UG037) were also obtained from the AIDS Research and Reference Reagent Program. Peptides were dissolved in water or in 50% DMSO and mixed together in 14 pools each containing seven peptides according to the algorithm acquired by a matrix (table 1). Peptide pools were used in ELISPOT assays at a final concentration of 2 µg/ml. Staphylococcal Enterotoxin B (SEB) (Sigma-Aldrich, Bornem, Belgium) was used as a positive control at a final concentration of 5 µg/ml.

Table 1. Fourteen matrix pools A-G and 1-7 each consisting of seven 20-amino acid peptides with 10-amino acid overlaps spanning the Gag region of HIV-1 subtype A (reference strain 92UG037).

<table>
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Interferon (IFN)-γ Amplispot assay. The Amplispot assay is a modified ELISPOT assay with enhanced sensitivity for detection of antigen-specific T cells by the combined addition of the cytokines IL-7 and IL-15, as described elsewhere [20]. In brief, ninety-six-well polyvinylidene-fluoride-bottom plates (Millipore, Molsheim, France) were prewetted with sterile water containing 70% ethanol,
washed, and coated with 5 µg/ml anti-IFN-γ monoclonal antibody (MAb) (Mabtech, Nacka, Sweden) overnight at 4 °C. Cryopreserved PBMC were thawed, washed twice, counted, and resuspended in RPMI containing 10% foetal calf serum (BioWhittaker, Verviers, Belgium), 100 U/ml penicillin, and 100 µg/ml streptomycin (Roche Diagnostics GmbH, Mannheim, Germany) (further referred to as culture medium) at a concentration of 2 × 10⁶ cells/ml, and incubated overnight at 37 °C and 5% CO₂. The next day, plates were washed with phosphate buffered saline (PBS), and blocked with 50 µl/well of culture medium for 1 hour at 37 °C and 5% CO₂. Cells were added to ELISPOT plates at concentrations ranging from 50,000 to 200,000 cells/well, depending on viable cell recovery. HIV proteins, peptide pools and SEB were added to duplicate wells in a final volume of 200 µl of culture medium containing 1 ng/ml of the cytokines IL-7 and IL-15 (R&D Systems, Minneapolis, OH, USA). Culture medium containing 1 ng/ml of the cytokines IL-7 and IL-15 alone, and together with 0.08% DMSO, was added in duplicate wells as negative controls for HIV protein and HIV peptide pool stimulation, respectively. Plates were incubated overnight at 37 °C and 5% CO₂, washed with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO) and incubated with 1 µg/ml biotinylated anti-IFN-γ (Mabtech, Nacka, Sweden) for 2 hours at 37 °C. Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech, Nacka, Sweden) for 1 hour at room temperature, washed again, and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Biorad, Hercules, CA, USA) for 30 minutes at room temperature followed by rinsing in tap water. Spots were counted with an automated ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Average numbers of spots per well were normalised to spot forming cells (SFC) per million PBMC, negative control SFC were subtracted from stimulated SFC and negative values were scored as zero.

HIV-specific responses in ESN and HIV-infected FSWs were considered positive when the following conditions were met: (1) IFN-γ production was present in SEB-stimulated wells; (2) the number of spots in stimulated wells was at least two times greater than in negative control wells; and (3) the negative control-subtracted response exceeded negative cut-off values of 28, 29, and 70 SFC per million PBMC for p24 protein, p55 protein, and Gag peptide pools, respectively. In addition, HIV peptide pool responses were considered positive when (4) responses above the cut-off value for pools A-G were mirrored with at least one other positive response for pools 1-7, and vice versa. Negative cut-off values for p24 protein, p55 protein, and for all the peptide pools together, were calculated as the average ELISPOT response plus three times the standard deviation among 10 HIV-seronegative female blood donors at lower risk for HIV exposure.
Statistical analysis. Non-parametric tests were used for statistical analyses. Mann-Whitney U and Fisher’s exact tests were used for comparing continuous and categorical variables, respectively, between two groups. Spearman rank test was used for correlations. For all analyses, the level of significance was \( P < 0.05 \).

RESULTS

Characteristics of the study populations. Median age of HIV-seronegative female blood donors was 24 years (interquartile range (IQR) 22 – 27), compared to 25 (IQR 23 – 31) of ESN FSWs \( (P = 0.169) \) and 27 (IQR 24 – 33) of HIV-infected FSWs \( (P = 0.149) \). Median duration of commercial sex work among ESN FSWs was 3.2 years (range 0.5 – 20) compared to 1.9 years (range 0.3 – 16.8) among HIV-infected FSWs. Nine ESN FSWs had a sex work duration of less than two years, six ESN FSWs between two and five years, and five ESN FSWs had a sex work duration of more than five years. ESN FSWs had a significantly higher median CD4+ T cell count than uninfected healthy female blood donors (median of 1289 vs. 954, \( P = 0.043 \)), while this of HIV-infected FSWs was significantly lower (median of 511 vs. 954, \( P = 0.001 \)).

HIV-1 subtype B Gag protein Amplispot responses. Amplispot responses against HIV-1 p24 protein remained below 20 SFC per million PBMC among 10 HIV-seronegative female blood donors, and a negative cut-off value of 28 SFC per million PBMC was calculated (figure 1A). Five (25%) of 20 ESN FSWs showed detectable p24-specific Amplispot responses above this cut-off value. Two additional ESN FSWs showed p24 responses above the cut-off value but less than twice the number of spots in the negative control wells and were thus considered negative. Two ESN FSWs showed relatively high responses of 180 and 200 SFC per million PBMC respectively. Nine (90%) out of 10 HIV-infected FSWs had detectable p24-specific Amplispot responses: five had relatively low responses ranging from 75 to 192 SFC per million PBMC, four showed high responses ranging from 494 to more than 7000 spots per million PBMC. One HIV-infected FSW showed p24 responses above the cut-off value but less than twice the number of spots in the negative control wells and was classified as negative p24 responder.

HIV-seronegative female blood donors showed responses of up to 66 SFC per million PBMC when stimulated with HIV-1 p55 protein in the Amplispot assay (figure 1B). This resulted in an increased negative cut-off value of 92 SFC per million PBMC. None of the ESN FSWs showed Amplispot responses exceeding this cut-off value. Seven (70%) out of ten HIV-infected FSWs showed detectable p55 responses ranging from 100 to 1489 SFC per million PBMC. One HIV-
infected FSW showed p55 responses above the cut-off value but less than twice the number of spots in the negative control and was thus considered negative.

Positive correlations between p24 and p55 protein responses were found among HIV-infected FSWs (r = 0.806, P = 0.005), but not among ESN FSWs (r = -0.239, P = 0.310) or female blood donors (r = 0.090, P = 0.805), indicating that p55 can be used for the detection of high-level HIV-1 Gag-specific responses, but lacks specificity for the detection of the low-level responses among ESN FSWs.

**HIV-1 subtype A Gag peptide pool Amplispot responses.** A negative cut-off value of 70 SFC per million PBMC for all peptide pools was calculated based on Amplispot responses of 10 HIV-negative female blood donors. One female blood donor showed irrelevant responses above the cut-off value for Gag pools B and C that were not mirrored by one or more responses in Gag pools 1-7 (figure 2A). Five (25%) out of 20 ESN FSWs showed detectable peptide pool Amplispot responses ranging from 71 to 277 (average of 115) SFC per million PBMC (figure 2B). The number of Gag peptide pools recognised ranged from three to eleven (median value of 6), indicating the potential presence of a minimum of two and a maximum of
In order to determine the number and the identity of the peptides responsible for the pool responses, potential single peptides have to be retested in the Amplispot assay. This work is currently in progress.

Of ten HIV-infected FSWs, nine (90%) showed detectable peptide pool Amplispot responses ranging from 75 to 9630 (average of 1145) SFC per million PBMC. Five HIV-infected FSWs showed responses remaining below 1200 SFC per million PBMC (figure 3A). Four others had high Amplispot responses of more than 1800.
4.3. Amplispot Detection of Gag-Specific T Cells

The number of Gag peptide pools recognised ranged from six to fourteen (median value of 10). Like for ESN FSWs, retesting of the potential single peptides needs to be done to determine the exact number and identity of the peptides involved.

For both ESN and HIV-infected FSWs, regions of HIV-1 subtype A Gag that were most frequently recognised were the second part of p17 protein and the first one third of p24 protein, included in Gag peptide pools B and C (figure 4A). Also, the average magnitude of the responses among ESN and HIV-infected FSWs was
highest in these Gag regions (figure 4B). In similar bell-shaped curves, responses among both ESN and HIV-infected FSWs decreased towards the end of p24 (figure 4B). Differences between ESN and HIV-infected FSWs were more pronounced in the p15 region of Gag. Here, ESN FSWs responded more to the first part of p15, while HIV-infected FSWs responded more to the last part (figure 4A). The magnitude of responses to p15 was lower than to other regions of Gag among both ESN and HIV-infected FSWs (figure 4B).

Figure 4. Comparison of Gag regions recognised by ESN FSWs and HIV-infected FSWs. A. Percentage of subjects with a detectable Amplispot response for the different Gag regions contained within peptide pools A-G. B. Average values of detectable Amplispot responses for the different Gag regions contained within peptide pools A-G. Error bars represent the standard error of the mean.
Correlations between Gag protein and Gag peptide pool responses. Of five ESN with detectable peptide pool Amplispot responses, three also showed detectable p24 protein-specific responses, and one other showed p24 responses above the cut-off value but less than twice the negative control response. Two ESN FSWs (LPIC00104 and LPIC00573) with high peptide pool responses of up to 200 SFC per million PBMC (pools B and D, respectively), showed the highest p24 responses with a similar magnitude. Overall, among ESN FSWs, p24 protein-specific responses correlated with responses against peptide pools A through E individually, and with the sum of the responses against peptide pools A-G ($r = 0.671, P = 0.001$). No such correlations were found for p55 protein.

Eight of nine HIV-infected FSWs with detectable peptide pool Amplispot responses also showed detectable p24 protein responses whereas seven out of nine also showed detectable p55 protein responses. Two HIV-infected FSWs (LPIC50007 and LPIC50195) with the highest peptide pool responses also showed the highest p24 and p55 responses. Overall among HIV-infected FSWs, p24 protein-specific responses correlated with responses to the individual peptide pools A through F, and with the sum of the responses against peptide pools A-G ($r = 0.867, P = 0.001$). The p55 protein-specific responses correlated with peptide pools A through G individually, and with the sum of the responses against peptide pools A-G ($r = 0.770, P = 0.009$).

Table 2. Association analysis between the presence of Gag-specific T cell responses measured by Amplispot and estimates of HIV exposure among ESN FSWs, and CD4+ T cell counts.

<table>
<thead>
<tr>
<th></th>
<th>Gag p24 protein or Gag peptide pool Amplispot Negative result (n = 13)</th>
<th>Positive result (n = 7)</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 (23 – 32.5)</td>
<td>25 (23 – 30)</td>
<td>0.659</td>
</tr>
<tr>
<td>Duration of sex work (months)$^b$</td>
<td>43 (17 – 70.5)</td>
<td>19 (16 – 49)</td>
<td>0.405</td>
</tr>
<tr>
<td>General number of clients per day$^c$</td>
<td>3 (3 – 4)</td>
<td>4 (3.25 – 4.75)</td>
<td>0.188</td>
</tr>
<tr>
<td>Number of clients during previous week$^d$</td>
<td>15 (7 – 21)</td>
<td>26 (9 – 62)</td>
<td>0.392</td>
</tr>
<tr>
<td>Sex with regular partners during previous week (%)$^e$</td>
<td>25</td>
<td>40</td>
<td>1.000$^c$</td>
</tr>
<tr>
<td>Proportion of unprotected sex$^{cd}$</td>
<td>0.35 (0.35 – 0.35)</td>
<td>0.35 (0.35 – 0.6)</td>
<td>0.439</td>
</tr>
<tr>
<td>CD4+ T cell count/µl</td>
<td>1252 (998 – 1488)</td>
<td>1348 (1086 – 1492)</td>
<td>0.782</td>
</tr>
</tbody>
</table>

Data are median (interquartile range). $^a$ Mann Whitney U test. $^b$ Based on eleven ESN FSWs with a negative result and four with a positive result. $^c$ Fisher’s exact test. $^d$ The proportion of unprotected sex was based on the reported consistency in using condoms with ‘always’ = 0.1, ‘often’ = 0.35, ‘rarely’ = 0.7, ‘never’ = 1.
Correlations between Gag-specific responses and sexual behaviour characteristics among ESN FSWs. ESN FSWs with a positive Amplispot response against either Gag p24 protein or against Gag peptide pools had a similar age and consistency in using condoms (proportion of unprotected sex) compared with ESN FSWs with a negative Amplispot response (table 2). However, the total duration of commercial sex work tended to be shorter, and the numbers of clients per day and during the previous week as well as the proportion of ESN FSWs having had sex with their regular partners during the previous week tended to be higher among ESN FSWs with a positive result. However, these differences were not statistically significant. No differences were noted in the number of peripheral blood CD4$^+$ T cells among the two groups.

DISCUSSION

T cell responses directed against HIV-1 Gag proteins and overlapping Gag peptide pools were analysed in African ESN FSWs by the highly sensitive Amplispot assay. A subgroup of ESN FSWs showed detectable Gag peptide pool responses against the same p17 and p24 regions that were recognized by T cells from HIV-infected FSWs. In addition, some ESN FSWs had gag protein-specific responses of similar magnitude as seen in HIV-infected FSWs. These data provide further evidence for the presence of HIV-specific T cell responses in ESN FSWs that have been difficult to detect by conventional methods.

Our findings are in agreement with studies from others that found detectable ELISPOT responses in Kenyan ESN FSWs by stimulation with HIV peptides [2, 8, 11]. In these studies, however, responses were measured against HIV-1 subtype B CTL epitopes that were previously defined in Caucasian HIV-infected subjects. Our study systematically used peptides spanning the entire Gag region of HIV-1 subtype A, which is more relevant because of the dominant subtype A/G circulating recombinant form in Abidjan which has subtype A Gag [24]. This approach may lead to the identification of T cell epitopes that are specifically recognized by ESN subjects and that may be associated with protection against infection with HIV.

ESN FSWs in our study showed Amplispot T cell responses against about half the number of Gag peptide pools recognised by HIV-infected FSWs, and the average magnitude of the responses was about 10-fold lower, suggesting that both breadth and magnitude of HIV-specific responses are lower in ESN FSWs than in HIV-infected FSWs. Still, both ESN FSWs and HIV-infected FSWs preferentially responded to the same peptide pools contained within the last part of p17 and the
first part of p24 within HIV-1 subtype A Gag. Interestingly, discordant responses were observed for the p15 region of Gag; the first part contained T cell epitopes that were predominantly recognized by ESN FSWs whereas the last part of p15 was targeted more often by HIV-infected FSWs. This could indicate that some immunogenic Gag T cell epitopes may be shared by ESN and HIV-infected FSWs while others are not, potentially leading to HIV-1 Gag regions that are associated with protection against HIV infection.

The finding that T cells of ESN FSWs specifically recognise other HIV-1 epitopes than T cells of HIV-infected FSWs [11], could not yet be confirmed. Identification of single peptides responsible for the peptide pool responses is currently in progress in our laboratory. However, by preliminary single peptide prediction based on the matrix peptide pool configuration, we found that an HLA-B18 restricted HIV-1 Gag p24 epitope FRDYVDRFY solely recognised by ESN and not HIV-infected FSWs in Nairobi [11], elicited dominant responses as part of the 20-mer peptide present in pools E and 2 (peptide 30, EPFRDYVDRFFKTLRAEQAT, table 1) in three of nine HIV-infected FSWs but only subdominant responses in one of five ESN FSWs. Therefore, it is unlikely that it represents an HIV-protective epitope in Abidjan.

The Amplispot assay detected Gag p24 protein responses in ESN FSWs ranging from 29 to 200 SFC per million PBMC, with an average response of 96 SFC. This is a marked increase (approximately 10-fold) in comparison with our previous ELISPOT study detecting p24 protein responses in ESN FSWs ranging from 10 to 20 SFC per million PBMC with an average response of 13 SFC [10]. Also, for HIV-infected FSWs, Gag peptide pool responses detected with the Amplispot assay were generally higher than in most other studies applying the ELISPOT assay but otherwise similar reagents [25, 26]. Together, these data confirm the high sensitivity of the Amplispot assay and underscore its validity for application in HIV epitope mapping, screening of HIV-specific T cell responses in HIV-exposed seronegative subjects, in HIV vaccine studies, and in other instances where T cells are difficult to detect by standard methods [20].

Amplispot T cell responses were considered positive if they exceeded the average response plus three times the standard deviation among 10 HIV-negative female blood donors who were analysed together with ESN and HIV-infected FSWs. In this way, cut-off values of 28, 92 and 70 SFC per million PBMC were obtained for Gag proteins p24, p55, and Gag peptide pools respectively. Given the fact that assay conditions and samples may vary for every study, this approach may be more accurate than using pre-set cut-off values [8, 27]. The high cut-off values may be
explained by the use of cryopreserved rather than fresh PBMC, and by the addition of the cytokines IL-7 and IL-15 which, despite increased background responses, improve the noise to signal ratio [20].

ESN FSWs with detectable ELISPOT responses against either Gag p24 protein or Gag peptide pools showed a lower sex work duration, and reported slightly higher numbers of clients per day and per week. These differences were not statistically significant probably because of the small sample size of 20 ESN FSWs and because of lack of sexual behaviour characteristics for 3 ESN FSWs with a positive result and 2 ESN FSWs with a negative result. Additional bias may have come from the self-reporting of the numbers of clients and use of condoms. In a previous study, a negative association was found between the duration of sex work and the number of clients per day [10]. All together, these findings may still support the hypothesis that HIV-specific immunity is essentially short-lived and requires persisting antigenic exposure [18, 19, 28].

In summary, we have detected T cell responses directed against Gag proteins and overlapping Gag peptide pools in a subgroup of African ESN FSWs by the highly sensitive Amplispot assay. These data provide further evidence for the presence of HIV-specific T cells in ESN FSWs that have been difficult to detect by standard methods. Further analysis of Amplispot responses against potentially reactive single peptides will allow us to identify the specific HIV-1 Gag epitopes recognised by ESN FSWs which may be relevant for HIV vaccine design.

ACKNOWLEDGEMENTS

We thank the community of female sex workers in Abidjan for their cooperation, Emmanuel Abonga for providing blood samples from blood donors, Doug Nixon and Barbara Shacklett for useful discussion. This work was supported by the Belgian Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen, grant G.0396.99 and by the Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention (CDC), Atlanta, GA. The following HIV-1 recombinant proteins were obtained through the EU Program EVA/MRC Centralised Facility for AIDS reagents, NIBSC, UK (grant number QLK2-CT-1999-00609 and GP828102): p24 (EVA620). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 subtype B p55 protein from Chiron Corporation and the DAIDS, HIV-1 subtype A Gag peptide set from the DAIDS.
REFERENCES


Chapter 5
Discussion

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5.1. MAIN FINDINGS OF THIS THESIS

In an effort to identify correlates of protective immunity against HIV, a number of consecutive studies were carried out to compare innate and adaptive immune responses with potential anti-HIV activity in HIV-exposed seronegative (ESN) female sex workers (FSWs) at high risk for HIV infection and HIV-seronegative female blood donors at lower risk for HIV infection in Abidjan, Côte d’Ivoire.

ESN FSWs showed increased in vivo activation of CD8⁺ T cells, which could be a sign of ongoing anti-HIV activity by CD8⁺ cytotoxic T lymphocytes. ESN FSWs had decreased in vivo expression of the HIV coreceptor CXCR4, but not of CCR5, on memory and naïve CD4⁺ T cells, and this decrease was associated with a prolonged duration of commercial sex work (§2.1). Intracellular β-chemokine levels in CD4⁺ and CD8⁺ T cells were comparable among ESN FSWs and controls, suggesting no role for the in vitro HIV-inhibitory β-chemokines in protection against infection with HIV in vivo. Cellular activation and supernatant secretion of β-chemokines and cytokines was decreased after allogeneic stimulation in ESN FSWs compared with female blood donors, indicating suppressed alloimmune reactions among ESN FSWs (§2.2).

The potentially protective role of β-chemokines was studied further in HIV-infected FSWs from Abidjan. Increased intracellular β-chemokine levels among HIV-infected FSWs were a sign of HIV disease progression since they correlated directly with T cell activation and HIV-1 viral load and inversely with CD4⁺ T cell counts (§3.1). Intracellular and secreted β-chemokine levels were analysed in parallel and were found to correlate directly among HIV-negative controls but not among HIV-infected subjects. These data indicate that the effective secretion of β-chemokines, and not the intracellular accumulation, may constitute a correlate of protection against HIV disease progression. The data also show that the secretory capacity for β-chemokines may be disturbed in HIV infection (§3.2).

HIV-specific CD4⁺ and CD8⁺ T cells responding to HIV-1 Env- and Gag proteins and HIV-1 Gag overlapping peptide pools were detectable in subsets of ESN FSWs. HIV-specific responses had lower frequencies and were more limited in breadth in ESN FSWs than in HIV-infected FSWs. However, they were potentially targeted against different HIV-1 Env and Gag peptides that may constitute important HIV-protective epitopes (§4.1 and 4.3). Remarkably, these responses correlated rather with the frequency than with the total duration of HIV exposure among ESN FSWs (§4.1). ESN FSWs also showed higher numbers of cytomegalovirus (CMV)-specific T helper cells than female blood donors and HIV-infected FSWs (§4.1).
5.2. CORRELATES OF PROTECTIVE IMMUNITY AGAINST HIV

CXCR4 downregulation correlated with a prolonged (> 12 months) duration of commercial sex work among ESN FSWs (§2.1), decreased alloimmune responses were more characteristic for ESN FSWs with a commercial sex work duration of < 5 years (§2.2), and HIV-specific T cell responses were detected in association with higher numbers of clients and shorter sex work duration (§4.1 and §4.3). ESN FSWs with the shortest duration of commercial sex work reported the highest numbers of clients per day, but also the most consistent condom use (§2.1 and §4.1). Based on the reported sex work duration and numbers of clients per day alone, dose-response associations between the extent of sexual exposure and CXCR4 expression, allo-stimulated CD69 expression and HIV-specific T cell responses may be postulated (figure 1). High frequency of sexual exposure among ESN FSWs with a short duration of commercial sex work may induce HIV-specific T cell responses, downregulation of CXCR4 expression, and possibly tolerance towards alloantigens (data not available because of limited range of sex work duration, see §2.2). Together with increasing duration of commercial sex work, the number of clients per day and the frequency of sexual exposure decreases, resulting in waning of HIV-specific T cell responses, and restoration of the alloimmune responses and possibly also of CXCR4 expression (data not available because of limited range of sex work duration, see §2.1).

Figure 1. Schematic presentation of the observed associations between potential correlates of protection against HIV and sexual exposure among ESN FSWs. Association for CXCR4 expression was derived from §2.1, for allostimulated CD69 expression from §2.2, and for HIV-specific T cells from §4.1 and §4.3 (black lines). The association between duration of commercial sex work and number of clients per day (grey surface) was derived from §2.1 and §4.1. Dotted lines indicate missing data as the result of limited ranges of duration of commercial sex work in the different studies.
Unprotected sexual exposure among ESN FSWs results in exposure to HIV, to alloantigens from sexual partners, and to other microorganisms. In an attempt to investigate the causality between the observed immunological differences between ESN FSWs and female blood donors and the nature of sexual exposure, the relevant interactions that have been described in the literature were summarised (table 1). Exposure to HIV may explain all of the observed immunological characteristics of ESN FSWs, albeit that several HIV interactions were described for chronic HIV infection that may not be comparable with the low-level mucosal HIV exposure experienced by ESN FSWs. Exposure to alloantigens may also be associated with the observed immune status of ESN FSWs, however also here the majority of studies concerned systemic alloimmunisation or blood transfusion that may not be fully comparable with mucosal alloantigen exposure. Some of the immune characteristics of ESN FSWs could also be explained by exposure to other viruses or sexually transmitted infections. Taken together, this suggests that the observed immune status of ESN FSWs does not necessarily constitute a specific anti-HIV response, but may also be explained by exposure to alloantigens or other microorganisms.

**Table 1.** Literature-based causality between the immunological differences between ESN FSWs and female blood donors and sexual exposure to HIV, alloantigens or other microorganisms.

<table>
<thead>
<tr>
<th>Immune status of ESN FSWs in Abidjan</th>
<th>Unprotected sexual exposure among ESN FSWs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo T8 cell activation; increased PHA-induced expression of CD69 and cytokines/β-chemokines</td>
<td>Exposure to HIV</td>
<td>Exposure to alloantigens</td>
</tr>
<tr>
<td>Higher levels of CMV-specific CD4⁺ T cells</td>
<td>CMV reactivation in HIV patients [20, 21]</td>
<td>CMV reactivation by allogeneic blood transfusion [22]</td>
</tr>
</tbody>
</table>
The HIV-protective capacity of these immune responses can only be addressed in longitudinal studies that follow-up on the seroconversion status of ESN FSWs, for which, unfortunately, the studies in this thesis were not designed. Many of the ESN FSWs in Abidjan had not Ivorian but Nigerian, Ghanaian or other West African nationalities. Especially as a result of the recent political instability in Côte d’Ivoire, many women tended to move to other cities in West Africa and were not available for follow-up at the confidential clinic in Abidjan. In addition, some ESN FSWs got enrolled in other studies so that results would no longer be comparable.

Without evidence from prospective studies, the meaning of the observed immune correlates remains speculative. In fact, it cannot be excluded that they constitute simple epiphenomena of the sexual exposure by itself, rather than being specific mechanisms by which the immune system protects against HIV infection. Indeed, some of the immune responses among ESN FSWs appeared to correlate with the frequency and the duration of the sexual exposure. In addition, the observed immunological correlates could be explained by exposure to alloantigens or by infection with microorganisms other than HIV (table 1).

However, it is plausible to hypothesise that the immune correlates constitute a dynamic HIV-protective condition that is multifactorial in nature. In that regard, mucosal HIV-specific IgA antibodies, previously detected in ESN FSWs from Abidjan [23], may ward of infection by preventing transcytosis of HIV through the mucosal epithelia [24]. In the event of productive viral infection despite HIV-specific IgA, HIV-specific memory CD4+ and CD8+ T cells (§4.1 and §4.3) are ready to proliferate and kill HIV-infected cells [25], thereby limiting viral dissemination, possibly clearing HIV from the body, or containing the infection to extremely low levels [26]. The increased in vivo CD8+ T cell activation among ESN FSWs (§2.1) may be a sign of this ongoing HIV-specific cytolytic T lymphocyte (CTL) activity [1]. Supported by a growing body of scientific evidence, repeated and continuous unprotected sexual contacts may be necessary to maintain and boost protective HIV-specific humoral and cellular responses [27-29], in agreement with the dose-response association between sexual exposure and the obtained immune status among ESN FSWs in Abidjan (§2.2, §4.1).

HIV-specific humoral and cellular responses are rather well-established potential correlates of protection against HIV. The notion of tolerance towards alloantigens among ESN FSWs constitutes a totally new idea in our understanding of the anti-HIV immune mechanisms. Ten years ago, alloimmunisation was proposed as a putative AIDS vaccine [30]. The hypothesis that activated alloimmune responses could protect against HIV infection was supported by several independent lines of
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evidence. These included the presence of HLA molecules on the viral envelope [31], cross-reactive immune responses against HLA and gp120 as the result of a high degree of homology [32], and rare and particular HLA polymorphisms among ESN FSWs in Nairobi [33]. However, ESN FSWs in Abidjan did not have increased alloantibody levels, and they showed decreased rather than increased cellular alloimmune responses compared with female blood donors (§2.2). If protection against HIV infection really occurs among ESN FSWs, than this cannot be explained by increased alloimmune responses. In that regard, our data cannot support alloimmunisation as a vaccine strategy to prevent HIV transmission. Still, based on our findings it may be possible that decreased alloimmune responses play a role in protection against HIV infection. Indeed, tolerance towards alloantigens could prevent CD4-bearing cells from becoming susceptible HIV targets as the result of allogeneic activation [34]. Extremely relevant to this complex problem is the unanswered question whether natural (e.g. sexual intercourse or pregnancy) or artificial (e.g. blood transfusions in women with recurrent spontaneous abortions) alloimmunisation results in tolerance rather than activation towards alloantigens. Although we could not control for pregnancy or multiparity of the women, our data suggest that natural alloimmunisation by sexual contacts among ESN FSWs results in tolerance towards alloantigens. Further research in this domain is needed before any application in HIV vaccine development can be investigated.

Downregulation of CXCR4 is unlikely to play a role in the scenario where HIV-specific immunity and possibly suppressed alloimmune reactions contribute to protection against infection with HIV. The majority of HIV-1 strains that are sexually transmitted use CCR5 as a coreceptor [35], and the CXCR4 coreceptor is only used by HIV strains that emerge during later stages of the infection. Remarkably, decreased CXCR4 expression has also been achieved by alloimmunisation [3], and it may therefore rather be an epiphenomenon of sexual exposure to alloantigens among ESN FSWs than a correlate of protection against HIV infection. In the same way, the increased levels of CMV-specific T cells observed among ESN FSWs may be the result of reactivation of latent CMV infection and are unlikely to be associated with protection against HIV.

5.3. IMPLICATIONS FOR HIV VACCINE DESIGN

A protective HIV vaccine may prove the best hope for controlling the world-wide HIV epidemic. Although many candidate vaccines are being developed on an empirical basis, the knowledge of the immune correlates of protection against HIV infection would greatly support ongoing efforts in vaccine design. Previous studies of the ‘Pumwani cohort of HIV-resistant sex workers’ in Nairobi have highly
contributed to our understanding of HIV-protective immune responses, including their findings of the importance of HIV-specific CTL [15, 27, 36]. Recently, a DNA/modified vaccinia virus vaccine has been developed, partially based on the HIV CTL epitopes targeted by ESN FSWs in Nairobi, and phase I clinical trials in healthy low-risk volunteers from Oxford and Nairobi have commenced [37].

First of all, the consistent detection of HIV-specific cellular responses in the population of ESN FSWs in Abidjan (§4.1 and §4.3) confirms this vaccine design approach. Indeed, HIV neutralising antibodies have been difficult to achieve by immunisation, and it is now generally believed that HIV vaccines aiming at the induction of cell-mediated immunity, or at the combination of humoral and cellular responses, may have higher chances of success. However, in addition to CTL, ESN FSWs from Abidjan also showed detectable levels HIV-specific T helper cells, which are not targeted by the Nairobi vaccine. The importance of HIV-specific T helper responses in protection against HIV disease progression has become well-accepted [38]. However, their potentially important role in protection against HIV transmission and in HIV vaccine design should be emphasised more.

Another aspect of our studies that may contribute to vaccine development is the comprehensive screening for HIV-1 subtype A Gag epitopes in ESN FSWs. Although this work is still in progress, preliminary results indicate that several ESN FSWs display detectable T cell responses to distinct HIV-1 Gag peptides (§4.3). These peptides may constitute ‘long sought after’ HIV epitopes to which protective T cell immunity is primed, and that should be included in future HIV vaccines. Indeed, ESN FSWs in Nairobi responded to different HIV-1 epitopes compared with HIV-infected FSWs. However, since the screening was limited to HIV-1 subtype B epitopes previously identified in Caucasian HIV-infected subjects, protective HIV epitopes in ESN FSWs may have been missed [15].

The latter findings were obtained by using the highly sensitive Amplispot assay, which is a modified ELISPOT assay with up to 18-fold increased sensitivity of detection (§4.2). Importantly, the specificity of this modified assay is unchanged, as antigen responses in uninfected/unexposed subjects remain low to undetectable. This method may prove particularly useful for the detection of T cell responses that have been difficult to detect by standard methods. In that regard, a phase III efficacy trial testing the ALVAC vCP1452 vaccine was postponed because of low ELISPOT responses in vaccinees [39], and one wonders what would have been the outcome if T cell responses were measured with the Amplispot assay instead. Thus, the Amplispot assay may be a method of preference for testing the immunogenicity of future HIV vaccines in phase II and phase III clinical trials.
Together, the data provided in this thesis suggest that protection against HIV infection among ESN FSWs is multifactorial in nature, consisting of HIV-neutralising IgA antibodies, HIV-specific T helper cells and CTL, and possibly suppressed alloimmune responses. In addition, these putative correlates of HIV protection appear to be dependent on frequent and continuous sexual exposure to HIV. Induction of this HIV-protective multifactorial condition by currently used vaccination strategies may likely prove to be difficult. Specific solutions should be found to address the need for persisting antigen and immune responses at the site of viral challenge, which are, in 90% of the cases world-wide, the vaginal mucosa. A recent study has started to address the problem of persisting antigen by using orally administered lactic acid bacteria expressing HIV proteins as an HIV vaccine [40]. In a more appropriate approach, vaginal administration of a genetically modified *Lactobacillus acidophilus* strain with strong capacity to colonise the vaginal mucosa may provide the persisting antigen exposure and mucosal immune responses that are needed to protect against HIV infection.

5.4. RECOMMENDATIONS FOR FURTHER RESEARCH

A multifactorial mechanism of protection against infection with HIV may be a plausible interpretation of the results described in this thesis. Still, based on our results and on reports from others, HIV-specific T cell responses on their own probably constitute the best-documented correlate of protective immunity defined in ESN populations to date. An interesting new lead described in this thesis constitutes the enhanced detection of HIV-specific T cells in ESN FSWs by the highly sensitive Amplispot assay, and the possibility to comprehensively map HIV-protective epitopes in these subjects by the use of matrix peptide pools. Further research should aim at identifying these potentially protective epitopes within all HIV proteins and for all available ESN populations in the world. ESN subjects in different regions of the world will likely target different HIV-protective epitopes depending on the circulating HIV strains and on the local HLA allele distribution. The result would be that ESN subjects around the world represent key populations necessary for the design of region-specific HIV vaccines consisting of sets of HIV-protective epitopes matched to the prevalent HLA alleles in that location.

However, although HIV-specific T cell responses could be consistently detected in subgroups of ESN individuals in our and other studies, large proportions of persons in these populations actually failed to show HIV-specific T cells. While this could mean that HIV-specific responses in these persons were below the limit of detection of the applied assays, it could also indicate that HIV-specific T cells were
actually not present and that other, yet unknown, single- or multiparameter factors are associated with the ESN status.

The innate immune system offers an attractive area for further research on HIV-protective factors. Innate immune responses form the first line of defense by acting in a rapid and broadly specific manner against invading pathogens, and several soluble innate factors with potential HIV-protective activity have been described [41]. For years, researchers have tried to identify the components that make up the CD8 antiviral factor (CAF), also referred to as noncytotoxic CD8$^+$ T cell activity (CNAR), by which CD8$^+$ T cells inhibit HIV replication in CD4$^+$ T cells in vitro. Recently, the $\alpha$-defensins 1, 2, and 3 were proposed to contribute to CAF in long-term non-progressor patients [42], however this observation could not be confirmed [43, 44]. Although their contribution to the ESN status has also been suggested [45], further studies are needed to establish the role of $\alpha$-defensins in protection against HIV transmission. Another cellular factor called CEM-15, a DNA deaminase shown to render HIV non-infectious [46, 47], may also be studied in the context of ESN populations. Similarly, dominant inhibitors of HIV reverse transcription were shown in simian cells, and elucidation of these factors in humans may prove a promising new area of research [48]. Innate natural killer cell cytotoxicity results from the interaction of killer immunoglobulin-like receptors (KIRs) with HLA class I molecules on infected target cells. The presence of a specific KIR/HLA combination correlated with non-progression of HIV disease [49]. Further challenges may lie in analysing KIR/HLA combinations that are associated with protection against infection with HIV.

In contrast with HIV vaccines that aim at inducing complex and multifactorial memory responses against HIV, single innate factors with potent anti-HIV activity may be more easily translated into successful therapies or HIV-preventive microbicides. The innate immune system has not been extensively studied in the context of protection against infection with HIV and merits renewed attention.

5.5. REFERENCES

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Thesis publications

PUBLISHED OR IN PRESS MANUSCRIPTS


SUBMITTED OR IN PREPARATION MANUSCRIPTS


Summary

Twenty years after its discovery, HIV has become the world’s number one killer among infectious diseases. An HIV vaccine may prove the best hope for controlling the HIV pandemic, especially in developing countries where antiretroviral therapy is unlikely to become fully available in the near future. An effective, broad-spectrum HIV vaccine is needed that generates long-term immune memory and protection at the site of infection, particularly at the mucosal sites where the virus gains entry into the body. Understanding of the immunological mechanisms that protect against infection with HIV is of great importance for the design of such a vaccine. However, despite numerous research efforts, a clear correlate of protective immunity against HIV infection has yet to be identified.

Subjects who are frequently exposed to HIV without becoming infected (HIV-exposed seronegative or ESN) are being identified in several at-risk populations, including regular partners of HIV-infected subjects, female sex workers (FSWs) in high-prevalence areas, intravenous drug users exposed to HIV by needle-sharing, and hemophiliacs that have repeatedly been infused with HIV-contaminated blood products. These subjects constitute ideal populations to study potential mechanisms of protection against HIV. In this thesis, in an effort to identify the correlates of protective immunity, innate and adaptive immune responses with potential anti-HIV activity were studied in ESN FSWs at high risk for HIV infection in comparison with HIV-seronegative low-risk female blood donors in Abidjan, Côte d’Ivoire.

ESN FSWs showed increased in vivo activation of CD8+ T cells, which could be a sign of ongoing anti-HIV activity by CD8+ cytotoxic T lymphocytes (CTL). Decreased in vivo expression of the HIV-coreceptor CXCR4 on the major HIV target cells, CD4+ T cells, was noted. Although CXCR4 downregulation was associated with a prolonged duration of sex work, it may likely not be HIV-protective since HIV-1 strains that are sexually transmitted use CCR5 and not CXCR4 as a coreceptor for cell entry. No differences were found in CCR5 expression, and in frequencies of T cells expressing the HIV-protective β-chemokines or Th1 and Th2 cytokines. ESN FSWs showed a downregulated rejection response against foreign cells, since in vitro allostimulation resulted in decreased lymphocyte activation and decreased secretion of β-chemokines and Th1 cytokines. Tolerance against alloantigens may prevent CD4-bearing cells from becoming susceptible HIV targets by mucosal allogeneic activation.
While β-chemokines did not show a protective effect among ESN FSWs, their potential role in protection against HIV disease progression was studied further in HIV-infected FSWs in Abidjan, Côte d’Ivoire. Increased intracellular β-chemokine levels in HIV-infected FSWs were rather a sign of HIV disease progression since they correlated directly with T cell activation and HIV-1 viral load, and inversely with CD4+ T cell counts. Intracellular and secreted β-chemokine levels were found to correlate positively among HIV-negative controls but not among HIV patients. Secreted β-chemokine levels per unit of β-chemokine-positive lymphocyte were decreased in HIV patients for macrophage inflammatory protein (MIP)-1α and MIP-1β and increased for RANTES (regulated upon activation, normal T cell expressed and secreted), suggesting a disrupted β-chemokine secretory capacity, in addition to increased intracellular β-chemokine levels, as a sign of HIV disease progression. These data explain the contradictory conclusions concerning the role of β-chemokines in HIV infection, depending on the applied methodology of detection.

HIV-specific CD4+ helper T cells and CD8+ CTL responding to HIV-1 Env and Gag proteins and to HIV-1 Gag peptide pools were detectable in a subset of ESN FSWs. CD4+ and CD8+ T cell responses among ESN FSWs showed a lower magnitude than among HIV-infected FSWs, but were potentially targeted against different regions of HIV-1 Gag. Future identification of the single peptide responses among ESN FSWs may reveal specific HIV epitopes to which protective T cell immunity is primed, and which should be included in candidate HIV vaccines. Remarkably, detection of HIV-specific T cell responses correlated with the frequency rather than with the duration of HIV exposure among ESN FSWs, suggesting the need for persistent antigenic exposure for the induction and maintenance of HIV-specific T cells among ESN FSWs.

HIV-specific CD4+ and CD8+ T cell responses among ESN FSWs were generally of low level and difficult to detect with standard methods like the enzyme linked immunosorbent spot (ELISPOT) assay. In order to increase the sensitivity of detection of antigen-specific T cells, the Amplispot assay was developed as a modification of the ELISPOT assay by the addition of the recombinant cytokines IL-7 and IL-15. In comparison with the ELISPOT assay, the Amplispot assay showed up to 7-fold increased sensitivity for freshly isolated white blood cells, and up to 18-fold for cryopreserved white blood cells. This method may prove particularly useful for the detection of low-level HIV-specific T cell responses in cryopreserved samples, in ESN subjects, and in subjects participating in future trials testing the immunogenicity and efficacy of experimental HIV vaccines.
At present, the meaning of the observed immunological correlates among ESN FSWs can only be hypothesised, as definite proof of HIV-protective capacity will require longitudinal studies that follow-up on the seroconversion status of ESN FSWs. Although it cannot be excluded that these immune factors constitute epiphenomena of sexual exposure by itself, the data provided in this thesis may indicate that protection against HIV infection among ESN FSWs is multifactorial in nature, consisting of HIV-neutralising IgA antibodies, HIV-specific T helper cells and CTL, and possibly suppressed alloimmune responses. Inducing these protective conditions by currently used vaccination strategies may likely prove to be difficult, and specific solutions should be found to address the need for maintenance of humoral and cellular immune responses at the site of viral challenge by persisting antigen.
Samenvatting

Twintig jaar na de ontdekking van het human immunodeficiency virus (HIV) als de oorzaak van het acquired immunodeficiency syndrome (AIDS), heeft het virus de status van meest dodelijke ziekteverwekker verworven. De ontwikkeling van een beschermend HIV vaccin betekent wellicht de beste garantie om ooit de wereldwijde AIDS epidemie in te kunnen perken. Een vaccin tegen HIV zal vooral zijn nut kunnen bewijzen in ontwikkelingslanden waar zowel de beschikbaarheid als de toepasbaarheid van dure antivirale therapie in de nabije toekomst beperkt zal blijven. In deze landen is er dringend nood aan een efficiënt HIV vaccin dat langdurige immunologische bescherming tegen HIV infectie kan opwekken. Een van de obstakels die de ontwikkeling van dergelijk vaccin in de weg hebben gestaan, is de beperkte kennis van de immunologische mechanismen die HIV infectie kunnen afweren. Ondanks de reeds geleverde inspanningen op dit gebied, ontbreekt het ons nog steeds aan duidelijke correlaten van protectieve immuniteit.

In verscheidene hoog-risico populaties worden mensen gevonden die ondanks een frequente blootstelling aan het virus niet met HIV besmet worden. Deze personen worden HIV-exposed seronegatives (ESN) genoemd en zijn reeds gevonden bij HIV-seronegatieve vaste partners van seropositieve personen, bij prostituees in gebieden met een hoge HIV seroprevalentie, bij intraveneuze druggebruikers die worden blootgesteld via besmette naalden, en bij hemofiliepatiënten die herhaaldelijk behandeld werden met bloedfactoren besmet met HIV. Deze personen zijn van groot belang voor de studie van potentiële factoren die beschermend kunnen werken tegen HIV infectie. Deze thesis heeft als hoofdobjectief potentiële correlaten van protectieve immuniteit te bestuderen in een populatie van ESN personen. Aangeboren en verworven immuunresponsen werden bestudeerd in een groep van ESN prostituees met een hoog-risico gedrag voor infectie met HIV in vergelijking met HIV-seronegatieve vrouwelijke bloed donoren met een laag-risico gedrag in Abidjan, Ivoorkust.

ESN prostituees vertoonden een verhoogde in vivo activatie van CD8⁺ T cellen, wat kan wijzen op de activiteit van HIV-specifieke CD8⁺ cytotoxische T lymfocyten (CTL). Een verminderde membraanexpressie van de HIV coreceptor CXCR4 op CD4⁺ T cellen, de voornaamste doelwitcellen van HIV, werd ook vastgesteld. Deze verlaging stond in rechtstreeks verband met een langere werktermijn van de prostituees. De mate van cellulaire expressie van CXCR4 speelt wellicht geen rol in de bescherming tegen HIV infectie, aangezien HIV stammen die worden overgedragen via seksueel contact gebruik maken van een andere coreceptor, namelijk de CCR5 coreceptor, om CD4 cellen binnen te
SAMENVATTING

Er werden geen verschillen vastgesteld in de cellulaire expressie van CCR5, noch in de proporties CD4^+ of CD8^+ T cellen die de HIV-inhiberende β-chemokines of Th1 en Th2 cytokines tot uitdrukking brengen. ESN prostituées vertoonden wel een felle daling van de afstotingsreacties tegen vreemde menselijke cellen. In vitro stimulatie van de lymfocyten van de ESN prostituées met het vreemde celmateriaal van mannelijke bloed donoren resulteerde in een lagere cellulaire activatie en een verminderde secretie van β-chemokines en cytokines in vergelijking met vrouwelijke HIV-seronegatieve bloed donoren met een laag HIV risicogedrag. Deze tolerantie tegenover alloantigenen zou kunnen voorkomen dat CD4^+ T cellen geactiveerd worden door mucosale allogene stimulatie tijdens onbeschermd seksuele blootstelling en daardoor meer vatbaar zouden worden voor infectie met HIV.


ESN prostituées vertoonden HIV-specifieke CD4^+ helper T cellen en CD8^+ CTL gericht tegen HIV Env en Gag proteïnen, en tegen overlappende reeksen van Gag peptiden van HIV-1 subtype A. De T cel responsen in ESN prostituées vertoonden een lagere frequentie in vergelijking met HIV-geïnfecteerde prostituées, maar bleken gedeeltelijk andere gebieden van het Gag proteïne te herkennen. De verdere identificatie van de enkelvoudige Gag peptiden in ESN prostituées kan leiden tot de ontdekking van nieuwe HIV epitopen die HIV-beschermende T cel responsen kunnen uitlokken. Zulke HIV-beschermende HIV epitopen kunnen van groot
belang zijn voor de ontwikkeling van toekomstige HIV vaccins voor de stimulatie van celgemedieerde HIV responsen. Dit onderdeel van het onderzoek is echter nog niet voltooid. De detectie van HIV-specifieke T cel responsen in ESN prostituées correleerde met de frequentie (het aantal klanten per dag), maar niet met de totale duur (de werktermijn) van de blootstelling aan HIV. Dit kan erop wijzen dat een aanhoudende en frequente blootstelling aan HIV noodzakelijk is voor de inductie en het behoud van HIV-specifieke T cellen in de ESN prostituées.

ESN prostituées vertoonden in het algemeen erg lage aantallen HIV-specifieke T cellen die moeilijk detecteerbaar waren met traditionele technieken zoals de enzyme linked immunosorbert spot (ELISPOT) methode. Daarom werd de Amplispot methode op punt gesteld als een modificatie van de ELISPOT methode met een verhoogde gevoeligheid door het toevoegen van recombinante cytokines interleukine (IL)-7 en IL-15. De gevoeligheid voor de detectie van antigene specifieke cellen door middel van de Amplispot methode was tot 7-maal hoger voor vers geïsoleerde witte bloedcellen, en tot 18-maal hoger voor ingevroren witte bloedcellen in vergelijking met de ELISPOT methode. Deze nieuwe methode kan van belang zijn voor de detectie van HIV-specifieke cellen in ingevroren stalen, in ESN personen, en in personen die deelnemen aan toekomstige klinische trials om de immunogeniciteit en werkzaamheid van experimentele HIV vaccins na te gaan.

Op dit moment kan de betekenis van de aangetoonde immunologische correlaten in ESN prostituees wel gepostuleerd maar niet bewezen worden. Een definitief bewijs voor de beschermende werking van de immunologische responsen kan enkel bekomen worden in longitudinale studies die opvolgen welke ESN prostituees uiteindelijk beschermd blijven tegen HIV infectie, en welke niet. Deze informatie dient dan in verband te worden gesteld met de blijvende aanwezigheid van de immunologische correlaten van bescherming tegen HIV. Hoewel we niet kunnen uitsluiten dat de aangetoonde immunologische factoren een eenvoudig gevolg kunnen zijn van de onbeschermende seksuele blootstelling, het lijkt aannemelijk dat bescherming tegen HIV infectie in ESN prostituees veroorzaakt wordt door het samengaan van verschillende factoren. Deze factoren kunnen HIV-specifieke IgA antilichamen, HIV-specifieke T helper cellen en CTL, en mogelijk ook tolerantie tegenover alloantigenen omvatten. Het opwekken van zulk een complexe HIV-beschermende toestand in toekomstige vaccinees kan moeilijk blijken met de huidige beschikbare vaccinatiestrategieën. Specifieke oplossingen dienen gezocht te worden om deze potentiële HIV-beschermende humorale en cellulaire immunoresponsen op te wekken en te behouden door strategieën toe te passen met een continue mucosale antigeenvrijstelling.
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AWARDS AND GRANTS


2002. Travel grant from the Fonds voor Wetenschappelijk Onderzoek – Vlaanderen for a research visit at the Gladstone Institute of Virology and Immunology, UCSF, San Francisco, 3 February - 3 March 2002.

2003. Scholarship from the National Institute of Allergy and Infectious Diseases, Bethesda, USA, for the Keystone Symposium “Twenty Years of HIV Research: From Discovery to Understanding”, Banff, Canada, 29 March - 4 April 2003.
RESEARCH MISSIONS


JOURNAL PUBLICATIONS


INVITED SPEAKER PRESENTATIONS

Study on the endogenous mechanisms that protect HIV-Exposed Uninfected Female Sex Workers (EU FSWs) against infection with HIV in Abidjan. Biomedical Seminars, Institute of Tropical Medicine, Antwerp, 20 April 2000.


HIV-exposed but uninfected female sex workers in Abidjan, Côte d’Ivoire: the quest for correlates of protection against HIV. Biomedical seminars, Institute of Tropical Medicine, Antwerp, 15 November 2001.


Study of endogenous mechanisms that protect HIV-exposed seronegative female sex workers against infection with HIV in Abidjan, Côte d’Ivoire: focus on HIV-specific T cell responses. Immunology lab meetings, Institute of Tropical Medicine, Antwerp, 8 February 2003.

ORAL PRESENTATIONS AT CONFERENCES


POSTER PRESENTATIONS AT CONFERENCES


Acknowledgements

The work presented in this thesis could never have been completed without the invaluable contributions of many colleagues, collaborators, family members and friends who deserve some words of gratitude.

At the Institute of Tropical Medicine, Antwerp. First of all, I want to thank my promoter Luc Kestens for having given me the opportunity to work on the project and for his personal and scientific guidance and advice throughout my time in his laboratory.

I am indebted to Guido Vanham who was always found ready to help with both the theoretical and practical considerations that come with new projects. The many critical contributions made by Guido van der Groen were always perfectly timed so to keep my work on the straight path. Bob Colebunders, my co-promoter, also consistently showed interest in the progress of my research.

In the immunology lab, it was first Chris, Luc Boel and Lieve who introduced me to the practicalities of HIV research. Without the technical assistance of Chris, many projects would never have been completed. Together with Janet, Kim, Pascale, Kevin, Harr, Jo, Zehra and Jeannine, they contributed to the cosy atmosphere inside and outside the lab.

It was nice to experience the camaraderie of Helen, Yven, Eric and Ward, who faced the same challenges and frustrations that come with publications and with the “10 things not to do during a PhD”. I enjoyed our shared moments of decompression after work, on conferences and on the occasional beer-brewing workshop. I am thankful to Ward for his efforts in improving the statistical analyses in my research.

I was extremely lucky to get helping hands from my students Beatrice (alias Davina), Kirsten and An. I liked their enthusiasm and the moments of team spirit that came with it. I admit I did not always appreciate their nuisance, however most of the time they were just perfect excuses for escaping the solitary job in the office.

At the Projet RETRO-CI, Abidjan, Côte d’Ivoire. I am thankful to John Nkengasong whose support was unconditional by providing excellent field laboratory facilities, and important scientific help from the beginning until the end.
Thanks to Bea Vuylsteke, I could dispose of the samples and the curious behaviour of our study subjects, and I got thoroughly introduced to Abidjan during evening and weekend expeditions. I am also grateful to Chantal Maurice, Thierry Roels, Terence Chorba, Odette Tossou, and Monica Nolan for their help with the science.

I especially need to acknowledge my friend Souleymane Sawadogo who prove to be a talented helping hand in the lab and an experienced guide to African life. Stéphania, Marie-Yolande, Christiane, Bile, Emmanuel, Mireille and Fred made the laboratoire de virologie all the more a nice place to work. I am also thankful to Mathieu, N’Depo, Kabran, and Daniel for several hundreds of HIV tests and megabytes of flow data. Peter and James, thank you for the driving: 10 years without a scratch!

In the Ernst & Young house, Abidjan, Côte d’Ivoire. Thanks to Jef’s and Serge’s hospitality, I had a place to call home (with a pool!) every time I came down. Together with Jef, Harry, Clinton, Angus, Isabella, Bryan, Liezel, Sonja, Abou and Agostin, I spent some glorious time in Abidjan.

At the Gladstone Institute of Virology and Immunology, San Francisco. I am thankful for Doug Nixon’s generosity and for the support he gave me. I enjoyed the scientific collaboration but also the parties and mountain expeditions with Barbara, Karl, Catherine, Sophie, Kim, Martin, Johan, Annika and Walter.

At home. Geert en Carpi, bedankt voor de bezoekjes op verplaatsing en Rudi voor het nalezen van mijn schrijfwerk. Caroline, je steun en vertrouwen hebben veel voor me betekend. Speciale dank gaat uit naar mijn ouders.

And anyone I may have forgotten.

Wim,
December 2003