Postdoc Fellowships for non-EU researchers

Final Report

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Selection	Laboratory of Molecular Virology and Gene Therapy
Host institution	KU Leuven
Supervisor	Prof Zeger Debyser
Period covered by this report	from 01/07/2013.. to 31/12/2014
Title	HIV-1, Elite Controllers, host factors, role of cofactors Ethical issue on humans & privacy (Mol. virol.,gen.)

1. Objectives of the Fellowship (1/2 page)

Elite controllers (EC) are a rare group of human immunodeficiency virus type 1 (HIV-1) infected individuals who naturally control HIV-1 replication to levels below limit of detection without antiretroviral therapy (ART) and who rarely progress to AIDS. Understanding of how EC control viral replication could provide valuable clues to guide research on vaccine development and virus eradication. Although previous studies have implicated defective viruses [1], adaptive immune response [2, 3] and more recently possible cellular mechanisms [4, 5] as reasons of reduced HIV-1 replication in EC, none of the explanations has been exhaustive. Majority of reports suggest a block at the integration step in at least a subset of EC [6, 7].

The integration is a crucial step in the viral life cycle as proviral DNA copy gets stably inserted into the genome of the host cell a step catalyzed by the viral integrase. The observed block at the integration step in some EC could be due to cellular restriction factors impairing the integration process. However, no restriction factor has been identified thus far to act at this replication step. Alternatively, the expression or functionality of cellular cofactors involved in integration might be reduced in this subset of EC, resulting in reduced integrated provirus. Professor Zeger Debyser's research group has identified Transportin-SR2 [8], as a cofactor of HIV-1 nuclear import, and LEDGF/p75 [9], as a tethering factor of HIV integrase, that determines the sites of integration in the host genome. Both proteins are established as important cellular cofactors that are essential for efficient HIV replication and integration. However, little is known about expression levels, genetic polymorphisms and posttranslational modifications of these integrase cofactors within EC. In my postdoc project, we sought to identify the step at which HIV-1 replication is blocked and to study the possible role of cellular cofactors in modulating HIV-1 replication in a subset of EC. Specifically, I addressed the following questions:

1. Can I confirm the reduced *ex vivo* infection by laboratory HIV strains of primary $CD4^+$ lymphocytes obtained from selected EC?
2. Is reduced *ex vivo* infection associated with reduced integration and/or reduced nuclear import in selected EC? Are integration sites different in EC?
3. Is reduced integration due to reduced LEDGF/p75 expression levels or altered post translational modification of LEDGF/p75?
4. Is reduced nuclear import due to reduced levels in Transportin-SR2?

5. Is integration and/or nuclear import restricted by cellular proteins in some EC? Can I identify those restrictions to HIV replication in EC? Is the viral integrase itself subjected to altered modifications?

This project seeks to exploit the power of natural selection in humans to identify novel restriction mechanisms to HIV integration in a bedside to bench approach. Current data suggest that considerable heterogeneity exist among EC. Therefore, in a first step I did NOT aim at discovering generalized explanations for the whole group of EC. I took an individualized approach, evaluating in depth the mechanism of viral control within a selected group of EC. With Professor Zeger Debyser's laboratory track record and research tools related to nuclear import, integration and integration site selections we are excellently positioned to discover any mechanism of innate HIV replication control associated with early viral replication steps. Apart from a considerable increase in our basic understanding of HIV replication and innate control to human pathogens, insights could eventually lead to therapeutic and/or preventive strategies against HIV infection.

2. Methodology in a nutshell (1/2/ page)

Participants. Participants in this study were from two study cohorts. The first cohort is the Gasthuisberg Leuven (GHBL) cohort. The GHBL cohort is an ongoing observational natural history study of two disease progression-discordant HIV-1 infected couples established in Gasthuisberg University Hospital in Leuven, Belgium. In each couple one partner is an EC and the other is and HIV-1 progressor (PR). Participants in this cohort are followed up every 6 months where $CD4^+$ cell counts and viral loads are measured. Blood from the two disease progression-discordant couples is collected during follow up. Viral loads were determined using the automated Cobas Amplicor HIV-1 Monitor test (version 1.5; Roche Diagnostics). $CD4^+$ T cells were enumerated using the Multitest kit (CD4/CD3/CD8/CD45) on a FACSCalibur flow cytometer (Becton Dickinson). Blood from Belgian healthy volunteers used as controls (HC) in this study was collected at the visit of the two couples (Figure 1A). The second study cohort consisted of 5 ECs, 5 viremic controllers (VC), and 5 PRs with longitudinal follow-up data and available samples from the Sinikithemba chronic infection cohort. The Sinikithemba cohort comprises of 450 antiretroviral naïve, HIV-1 subtype C chronically infected adults enrolled from McCord Hospital (Durban, South Africa) from August 2003 to 2008 and followed longitudinally [10, 11]. Sociodemographic characteristics, plasma viral load and $CD4^+$ cell count measurements were obtained at baseline. $CD4^+$ cell counts and viral loads were measured every 3 and 6 months, respectively, from enrollment. Viral loads were determined using the automated Cobas Amplicor HIV-1 Monitor test (version 1.5; Roche Diagnostics). $CD4^+$ T cells were enumerated using the Multitest kit (CD4/CD3/CD8/CD45) on a FACSCalibur flow cytometer (Becton Dickinson). Samples from South African healthy volunteers used as controls (HC) in this study were also obtained (Figure 1B).

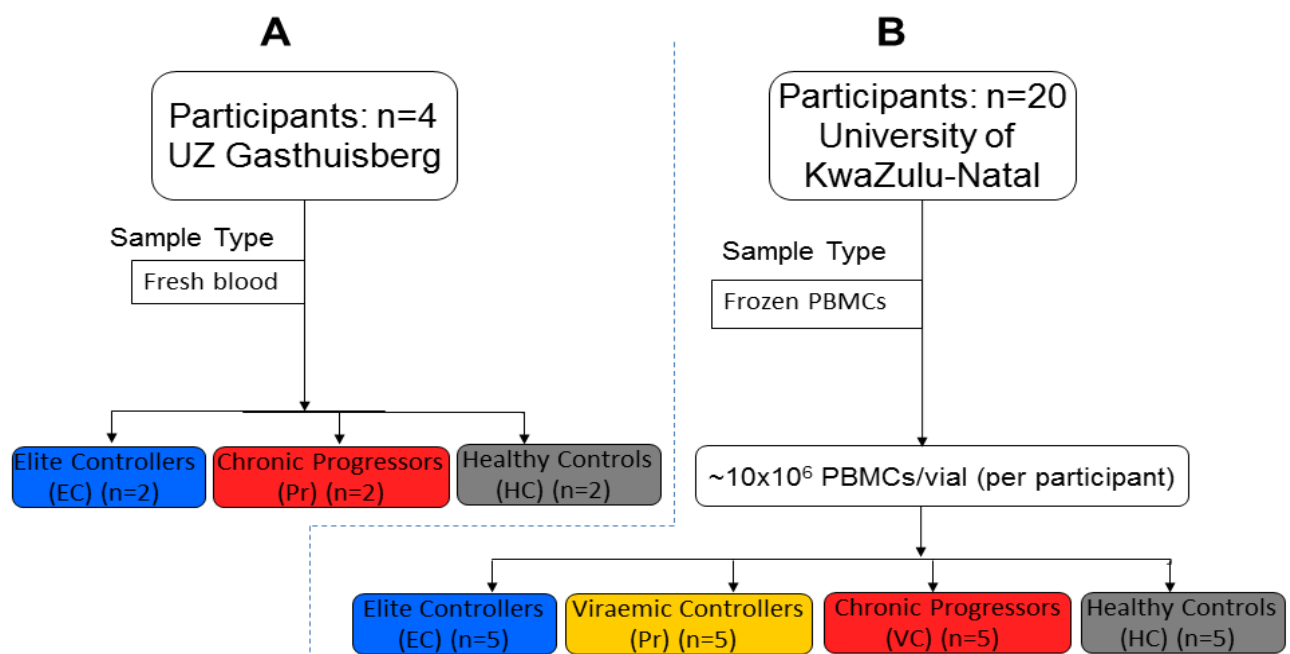

Figure 1. Design of HIV-1 cohorts used in this study and the number of participants in each group.

Ex vivo infection assays. $CD4^+$ T cells were activated in RPMI medium supplemented with 15% fetal bovine serum, recombinant IL-2 (100 U/ml), and 0.5 μg/ml anti-anti-CD3/CD8 bispecific antibody [12]. After 5 days in culture, a homogenous population of activated $CD4^+$ (97.3% purity) was detected by flow cytometry. Cells were infected on day 5 with R5-tropic HIV-1 strain 91US056 (MOI 0.001) and plated at 2×10^5 cells/well in a 96-well plate. At regular intervals, the cultures were fed by removing and replacing one-half of the culture supernatant with fresh medium. The removed supernatant was cryopreserved for later p24 antigen quantification by ELISA; control p24 levels from autologous cells without exogenous viral infection were subtracted from sample p24 levels.

In addition to R5-tropic HIV-1 isolates, activated cells were infected with yellow fluorescent protein (YFP)-encoding VSV-G–pseudotyped single-round HIV-1 vector for 2 hours. Following 2 hours transduction, cells were washed twice, plated in 24-well plates, harvested after 48 hours of incubation, and processed for flow cytometric analysis of YFP expression. The different viral DNA species formed during HIV infection were determined by quantitative PCR (qPCR).

Detection of HIV-1 DNA. For detection of different viral DNA species formed during HIV infection, cell lysates were harvested at different times. Cell lysates were harvested 18 hours after infection of activated cells with VSV-G–pseudotyped HIV-1 for the detection of HIV-1 LRTs. Amplification was performed with primers LRT1 and LRT2 and probe LRT-P, as previously described [13]. Cell lysates collected 48 hours (VSV-G–pseudotyped activated CD4+ T cells) were used for detection of integrated HIV-1 DNA, using nested PCR with Alu-Gag PCR. RNase P was used as a housekeeping gene for the quantification of input cell numbers.

Integration site selection. Integration site sequencing was performed as previously described [34,35]. Briefly, genomic DNA isolated from *ex vivo* transduced $CD4^+$ T cells was digested with MseI and linkers were ligated to this fragmented DNA and virus-host DNA junctions were amplified by nested PCR. Samples were barcoded using the second pair of PCR primers in order to generate 454 libraries. PCR products were purified and sequenced using 454/Roche pyrosequencing. Perfect matches to the long terminal repeat (LTR) linker and barcode were mapped to the human genome.

Cellular cofactor expression assay. *CDKN1A*, TRN-SR2 and LEDGF/p75 mRNA expression was analyzed by quantitative RT-PCR using the standard Taqman expression assay with primers and probes. *Actb* (encoding β-actin) was used as a housekeeping gene

3. Results

Reduced susceptibility of primary CD4$^+$ T-cells obtained from selected EC to HIV-1 infection. Previous studies have shown that CD4$^+$ T-cells from EC are susceptible to *ex vivo* HIV-1 infection albeit reduced susceptibility compared to cells from PR. Relative susceptibility of CD4$^+$ T cells from EC to HIV-1 infection was first was determined by performing *ex vivo* infection assays using CD4$^+$ T cells from EC (viraemia undetectable by commercial assays; median 973 CD4$^+$ T cells/µl, range 710–1.318 cells/µl) and reference cohorts of individuals with progressive HIV-1 infection (PR) but on treatment (viral load below limit of detection by commercial assays due to ART; median 217 CD4$^+$ T cells/µl, range 134–2000 cells/µl), or HC (with no HIV-1 infection at all) from the GHBL cohort. A population of activated of CD4$^+$ T cells was generated by 5 day *in vitro* culture with exogenous IL-2 and anti-CD3/CD8 bispecific antibodies, which stimulate CD4$^+$ T cells by TCR triggering while simultaneously deleting CD8$^+$ T cells resulting in a pure homogenous population of CD4$^+$ T cells (>97%). After 5 days of stimulation, cells were infected with R5-tropic HIV-1 (BAL) strain 91US056 (MOI 0.001). HIV-1 infection was monitored by p24 ELISA.

Under these activation and culture conditions, to multiple round HIV-1 (BAL) infection as measured by p24 production in EC CD4$^+$ T-cells was 8-10 fold lower than in CD4$^+$ T-cells of PR and HC (figure 2).

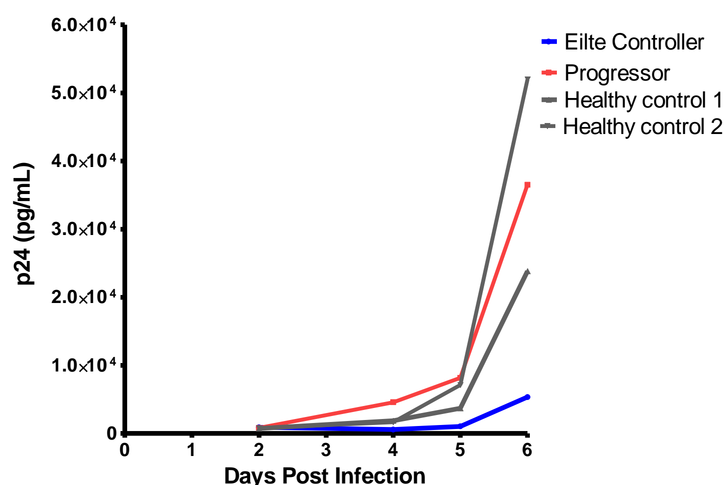

Figure 2. Reduced susceptibility of CD4+ T cells from elite controllers to HIV-1 infection. p24 antigen production in activated CD4$^+$ T cells from elite controller (EC) and control groups (HIV-1 progressor and HIV-1–negative persons) after exogenous HIV-1 infection with the R5-tropic HIV-1 strain 91US056 (MOI 0.001). One typical experiment is shown.

These data corroborate previous reports demonstrating lower susceptibility of EC cells compared to PR cells [4]. However, it is difficult to delineate at which step HIV-1 replication is blocked in this multiple round experiment.

Inhibition of early viral replication steps in CD4+ T cells from elite controllers. In order to identify the steps of the viral replication cycle that may be inhibited in CD4$^+$ T cells from elite controllers, we subsequently conducted ex vivo infection experiments with a yellow fluorescence protein–encoding (YFP-encoding), VSV-G–pseudotyped HIV-1 vector that bypasses viral coreceptor-mediated entry steps and causes single cycle infection without supporting production of new viral progeny during the viral post integration phase. Using ex vivo activated CD4$^+$ T cells, we

observed about that the proportion of YFP$^+$ CD4$^+$ T cells from EC was smaller than that of cells from PR and HC (Figure 3A).

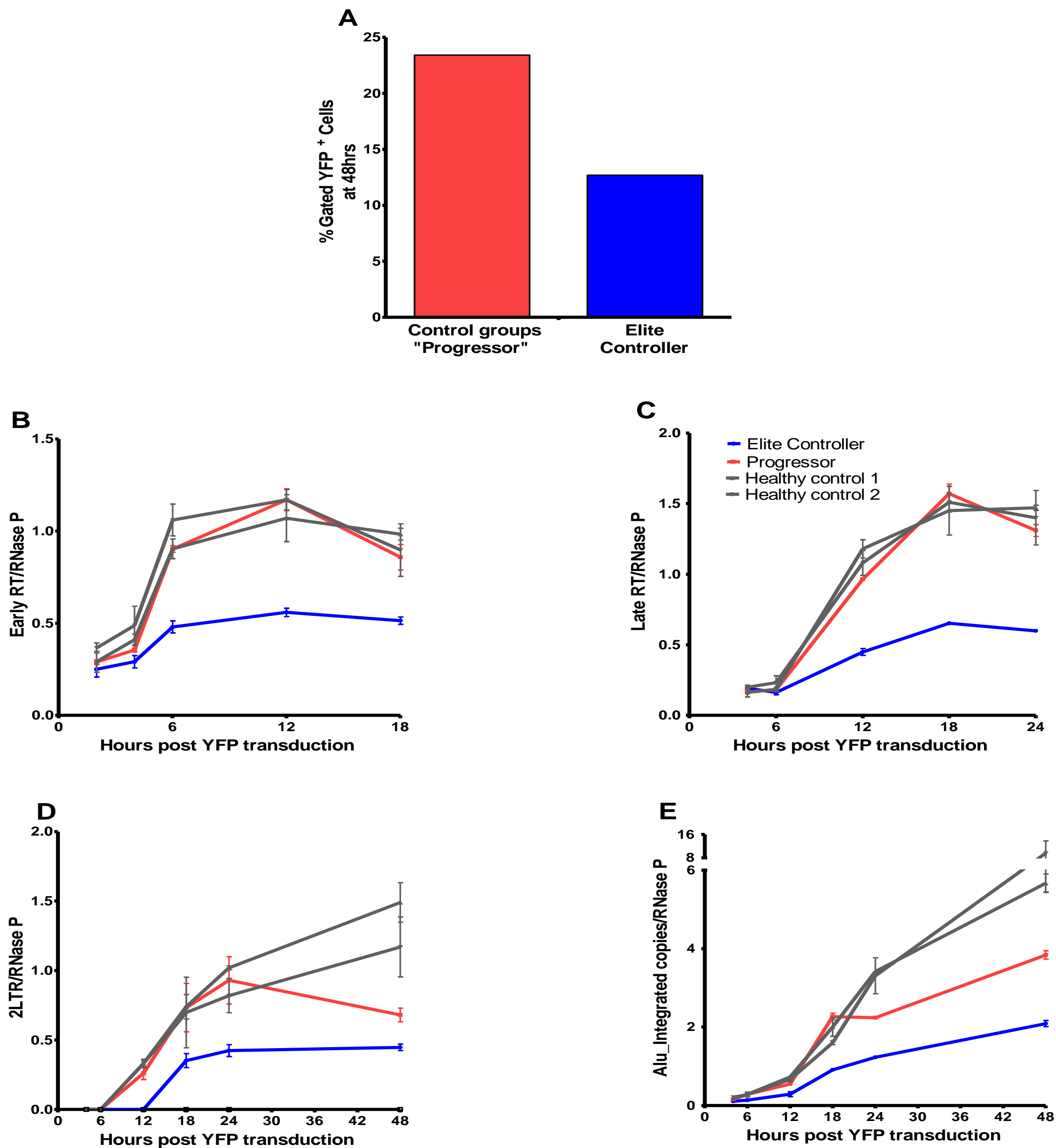

Figure 3. Analysis of early HIV-1 replication steps in CD4+ T cells after infection with a single cycle YFP-encoding, VSV-G–pseudotyped HIV-1 vector. (**A**) Proportion of YFP$^+$ CD4$^+$ T cells from the indicated participants following *ex vivo* transduction with HIV-1 YFP single round vector. (**B, C, D, and E**) Quantitative analyses of early reverse transcripts, late reverse transcripts, 2LTR circles and integrated HIV-1 DNA. Typical experiments are shown.

To more precisely pinpoint the block to HIV-1 replication in CD4$^+$ T cells from EC, we used real-time PCR to quantitate different HIV-1 DNA species. Early reverse transcripts, late reverse transcripts, and integrated provirus were quantitated by using specific primer and probe

combinations. The results show that the replication block in CD4+ T cells from EC occurs immediately following cell entry as evidenced by the 2-fold reduction in early reverse transcripts (Figure 3B) and late transcripts (Figure 3C).

2-LTR circles, which are a marker of nuclear import of the preintegration complex, were reduced in CD4+ T cells from EC compared to PR and HC (Figure 3D). As previously demonstrated [6, 7], quantitation of the proviral copy number using an Alu primer/probe set at 48 hr postinfection demonstrated a 2-fold reduction on HIV-1 integrated copies in CD4+ T cells from EC compared to the control groups PR and HC (Figure 3E). Taken all together these data suggest a replication block at a step between entry and reverse transcription.

Integration site distribution in EC compared to PR. Integration of retroviral DNA into the host genome is a defining feature of the retroviral replication. HIV-1 integration is known to be favoured in active transcription units, which promotes efficient transcription of the viral genes. Although our data demonstrate that CD4+ T-cells from EC are less susceptible compared to progressors due to a block between reverse transcription and integration, we also investigated the integration site distribution (Figure 4).

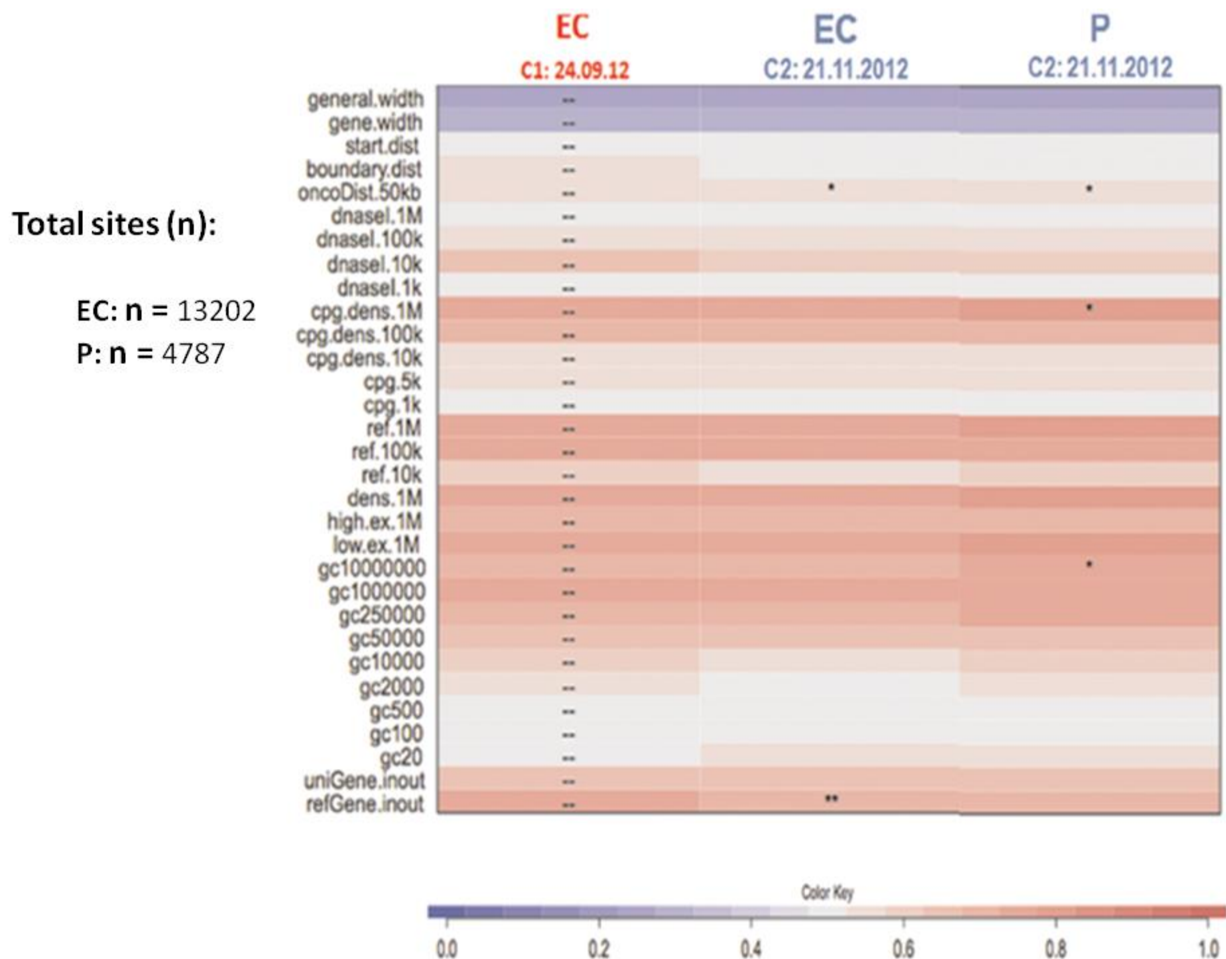

Figure 4. HIV-1 Integration site distribution. Integration site profile of HIV-1 in CD4+ T-cells from elite controllers (EC) and progressor (denoted as P). ***n*** denotes the number of integration sites that were sequenced in each group.

Our results show no distortion in HIV-1 integration site selection between CD4+ T cells obtained from EC versus CD4+ T cells from PR. The lack of differences in the integration site selection

implies that integration site selection is not responsible for reduced susceptibility in CD4+ T cells from EC compared to PR at least in the Belgian EC subset.

High expression of p21 is associated with reduced susceptibility of EC CD4+ T cells to HIV-1 infection. Next we reasoned that reduced HIV-1 susceptibility in CD4+ T cells from elite controllers might further be attributed to altered expression levels of cellular cofactors (LEDGF/p75 and TRN-SR2) involved in integration at least in this subset of EC. In addition to cellular cofactors, LEDGF/p75 and TRN-SR2 known to be involved in integration, we measured *CDKN1A* mRNA in CD4+ T cells by quantitative RT-PCR. Cyclin-dependent kinase inhibitor p21 encoded by CDKN1A gene has been shown to modulate HIV-1 reverse transcription in CD4+ T cells from EC [4, 5].

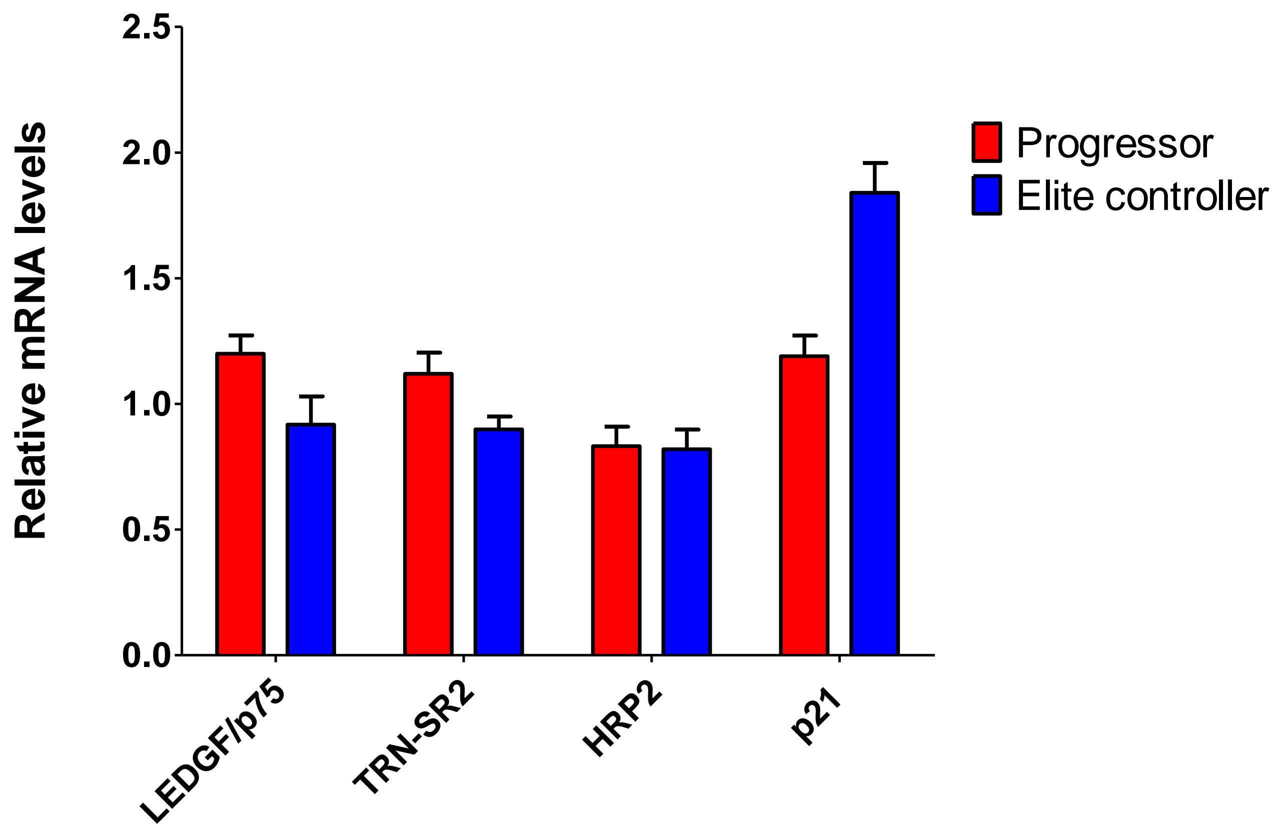

Figure 5. Relative mRNA expression levels of the cellular cofactors.

Consistent with the previous report [4], our data demonstrate elevated levels of *CDKN1A* mRNA expression in CD4+ T cells from EC compared to PR (Figure 5). However, there was no differential expression of LEDGF/p75 and TRN-SR2 in CD4+ T cells from EC and PR (Figure 5) which could of cause be attributed to small number (n=2) of participants studied per group.

Taken all together, the data from the EC from Leuven Belgium suggest that reduced permissiveness to HIV-1 infection in the CD4+ T-cells is mediated by a block in HIV-1 replication at a step between cell entry and reverse transcription.

To further confirm the relative susceptibility of EC CD4+ T-cells to HIV-1 infection, additionally we performed the same *ex vivo* infection assay using CD4+ T-cells from South African elite controllers (viral load below detection levels by commercial assays; median 701 CD4+ T cells/µl,

range 516–834 cells/µl) and from control groups of individuals with viremic control (median VL 265 copies/ml, range 28–1690 copies/ml; median 555 CD4+ T cells/µl, range 424–1060 cells/µl), progressive HIV-1 infection (median VL 156,000 copies/ml, range 78962–322000 copies/ml; median 427 CD4+ T cells/µl, range 117–727 cells/µl), or no HIV-1 infection at all.

***Inhibition of early viral replication steps in CD4$^+$ T cells from elite controllers* in Sinikithemba cohort.** Consistently with the data obtained from GHBL cohort, data from the Sinikithemba cohort confirms reduced susceptibility of CD4$^+$ T-cells from the EC ass reflected by fewer YFP+ CD4$^+$ T cells compared to CD4$^+$ T from control groups, VC, PR and HC (Figure 6A).

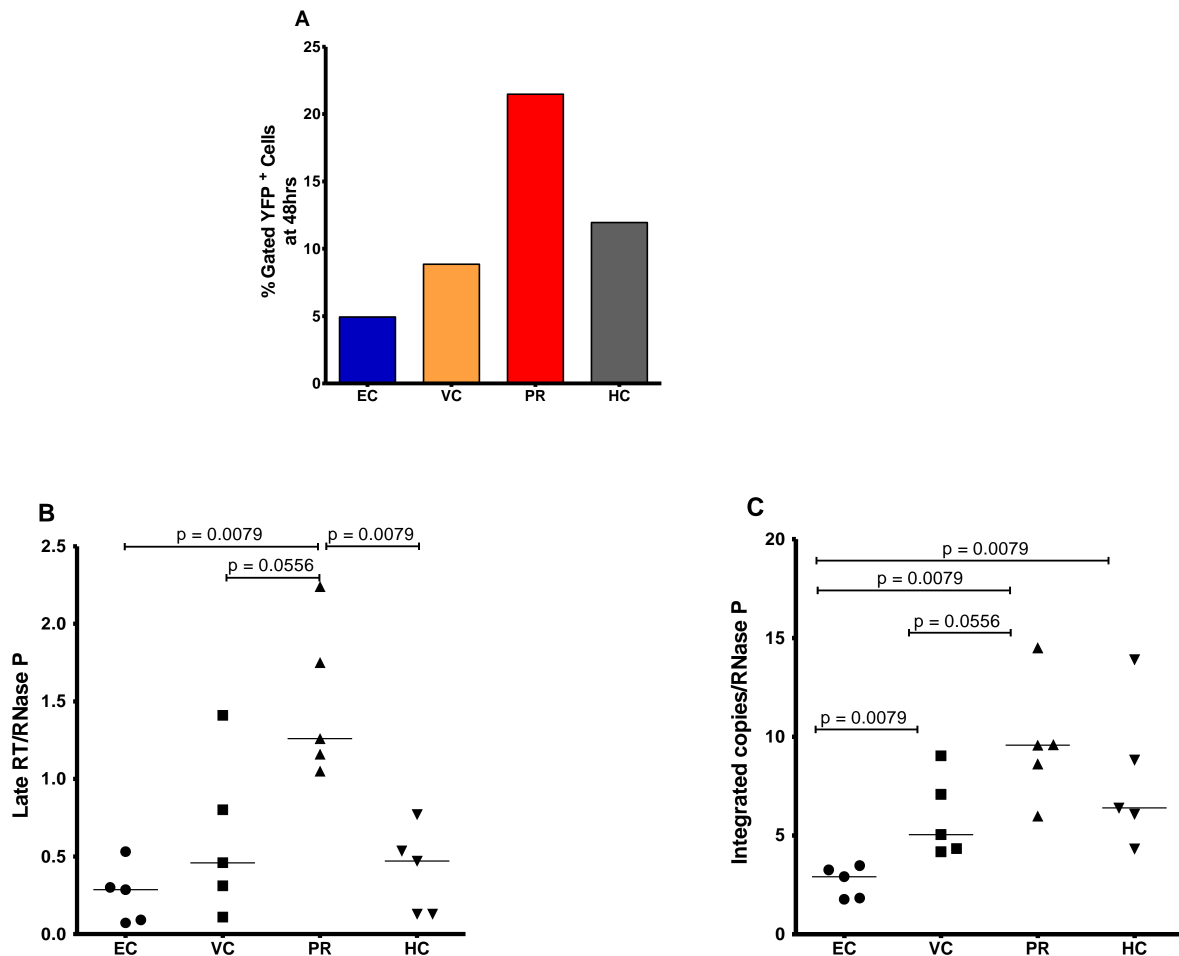

Figure 6. Analysis of early HIV-1 replication steps in CD4$^+$ T cells from South Africa after infection with a single cycle YFP-encoding, VSV-G–pseudotyped HIV-1 vector. (**A**) The proportion of YFP$^+$ CD4$^+$ T cells from the indicated participants following *ex vivo* transduction with HIV-1 YFP single round vector. (**B**) Quantitative analysis of late reverse transcripts. (**C**) Quantitative analysis of integrated copies using Alu PCR.

Next we performed quantitative PCR to measure different HIV-1 DNA species following *ex vivo* infection with VSVG pseudotyped HIV-1 YFP single round vector. Our data demonstrate that CD4$^+$ T cells from EC had significantly reduced late reverse transcripts compared to PR (Figure 6B). Consistent with the number of reverse transcripts, EC had fewer numbers of integrated copies

compared to the control groups VC, PR and HC ($p = 0.0079$) (Figure 6C). Although the differences were modest, cells from PR contained higher numbers of integrated copies compared to VC (Figure 6C).

The data obtained from the Sinikithemba cohort of EC confirm the data obtained from GHBL cohort. Although no specific host restriction factor has been reported to restrict HIV-1 replication in $CD4^+$ T cells from EC, our data suggest a replication block between entry and reverse transcription.

High expression of p21 is associated with reduced susceptibility of EC $CD4^+$ T cells to HIV-1 infection in the Sinikithemba cohort. To delineate the possible mechanism of restriction in $CD4^+$ T-cells we measured the expression of cellular cofactors reported to play a significant role in HIV-1 replication.

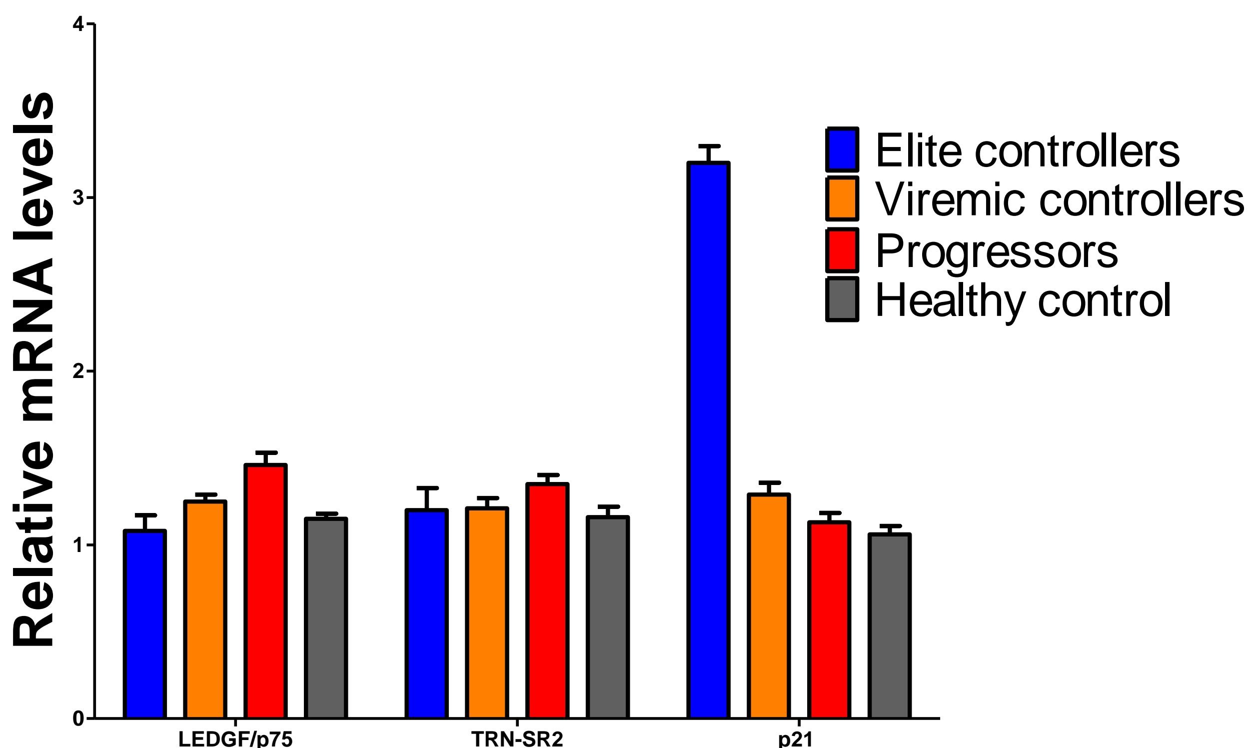

Figure7. Relative mRNA expression levels of the cellular cofactors in Sinikithemba cohort.

Consistent with the data from GHBL cohort and previous report [4], our data demonstrate elevated levels of *CDKN1A* mRNA expression in $CD4^+$ T cells from EC compared to the control groups VC, PR and HC (Figure 7). Taken together these data suggest a correlation between high *CDKN1A* (p21) mRNA expression levels and reduced susceptibility in $CD4^+$ T cells from EC.

4. Perspectives for future collaboration between units (1 page)

Integration of HIV-1 DNA into the host cell genome is a critical step during replication. The sites of HIV-1 integration are not randomly placed in chromosomes, but instead are strongly influenced by

local features. HIV-1 favors integration in transcription units, and analysis of gene activity in infected target cells revealed that integration was particularly favored in active genes [14-16]. Integration site selection of autologous virus in $CD4^+$ T cells from EC might be different from that of control groups VC, PR and HC.

In collaboration with the Laboratory of Molecular Virology and Gene Therapy in KU Leuven, we want to investigate the integration profile of autologous HIV-1 in CD4+ T cells compared to the control groups. In future collaborative experiments we will investigate the role of p21 in this process in more detail.

5. Valorisation/Diffusion (including Publications, Conferences, Seminars, Missions abroad...

5.1 Peer-reviewed publication

5.1.1 Jonas Demeulemeester, Sofie Vets, Rik Schrijvers, **Paradise Madladla**, Thumbi Ndung'u, Marc De Maeyer, Zeger Debyser, Rik Gijsbers. Integrase variants retarget viral integration on both a local as well as on a global scale and are associated with disease progression. *Cell Microbe* 2014; 16 (5) 651-62

5.1.2 Sara El Ashkar, Jan De Rijck, Jonas Demeulemeester, Sofie Vets, **Paradise Madlala**, Katerina Cermakova, Zeger Debyser and Rik Gijsbers. BET-independent MLV-based Vectors integrate Efficiently and Associate Less with Proto-oncogene Transcription Start Sites. *Moil Ther Nucleic Acids* 2014, 3, e179 dci

5.1.3 **Paradise Madlala**, Rik Gijsbers, Annaleen Hombrouck, Lise Werner, Koleka Milisana, Ping An, Salim Abdool Karim, Cheryl Winkler, Zeger Debyser and Thumbi Ndung'u. Association of polymorphisms in the LEDGF/p75 gene (*PSIP1*) with susceptibility to HIV-1 infection and disease progression. *AIDS* 2011; 25 (14): 1711-9.

5.2 . Publications submitted to journals or in preparation

Paradise Madlala, Ravesh Singh, Lise Werner, Koleka Mlisana, Salim S. Abdool Karim, Ping An, Cheryl A. Winkler, and Thumbi Ndung'u. Association of genetic variant A1650G of Cyclophilin A Gene (*PPIA*) with accelerated disease progression among black South Africans (**manuscript under revision**).

5.3 Presentation

Paradise Madlala, Rik Gijsbers, Sofie Vets, Erik Van Wijngaerden, Rik Schrijvers, Zeger Debyser. Reduced susceptibility of CD4+ T-cells from elite controllers to HIV-1 infection reveals a block at a step between reverse transcription and integration. Selected oral presentation at *Second annual meeting Belgian Society for Virology Monday, December 8th, 2014 The Royal Academies for Science and the Arts Hertogstraat/Rue Ducale 1, Brussels*.

I returned to South Africa during my stay on several occasions to organize shipment of the samples from South Africa to Belgium for the purposes of this project.

6. Skills/Added value transferred to home institution abroad (1/2 page)

During my postdoc I established a collaboration between the lab in Leuven and my home University of Kwazulu-Natal. During my postdoc in Leuven I learned all necessary techniques to continue this study in South Africa in collaboration with the lab of Prof. Debyser. Long term goal is to identify cellular factors that explain the elite control phenotype in African patients.

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