CCR5 promoter variants among Ugandan HIV-1 elite and viremic controllers: a laboratory ▯ based cross ▯ sectional study

Brian Nyiro
Makerere University College of Health Sciences https://orcid.org/0000-0001-7131-8137
Sharon Bright Amanya
Makerere and Lira University
Rose Nabatanzi
Makerere University College of Health Sciences
Alice Bayiyana
Makerere University College of Health Sciences
Linda Igumba Kalazane
Uganda Christian University
Francis Waswa
Makerere University College of Health Sciences
Eva Nabulime
Center For AIDS Research/ Joint Clinical Research Center
Daniel Karara
Makerere University College of Health Sciences
Joel Kabali
Medical Research Council/ Uganda Virus Research Institute
Gerald Mboowa
Infectious Disease Institute
Alex Kayongo
Makerere University Lung Institute
Immaculate Nankya
Center For AIDS Research/ Joint Clinical Research Center
David Patrick Kateete
Makerere University College of Health Sciences
Obondo James Sande (ojsande@gmail.com)
Makerere University College of Health Sciences https://orcid.org/0000-0002-2301-5980

Research article

Keywords: CCR5, HIV, Elite controllers, Viremic controllers and non-controllers

Posted Date: August 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-47544/v1

CCR5 promoter variants among Ugandan HIV-1 elite and viremic controllers: a laboratory-based cross-sectional study

Brian Nyiro[1], Sharon Bright Amanya[1,2], Rose Nabatanzi[1], Alice Bayiyana[1], Linda Igumba Kalazane[4], Francis Waswa[1], Eva Nabulime[1,3], Daniel Karara[1], Joel Kabali[3,6], Gerald Mboowa[1,7], Alex Kayongo[5], Immaculate Nankya[3], David Patrick Kateete[1] and Obondo James Sande[1]

Author Institutions

[1]Department of Immunology and Molecular Biology, Makerere University College of Health Sciences, Kampala, Uganda.

[2]Faculty of Health Sciences, Lira University, Lira, Uganda.

[3]Centre for AIDS Research Laboratory, Joint Clinical Research Centre, 10005, Wakiso, Uganda.

[4]Uganda Christian University, Plot 67-173, Bishop Tucker Rd, Mukono, Uganda.

[5]Makerere University Lung Institute, Kampala, Uganda.

[6]Medical Research Council, Uganda Virus Research Institute.

[7]The African Center of Excellence in Bioinformatics and Data Intensive Sciences, the Infectious Diseases Institute, McKinnell Knowledge Centre, Makerere University, Kampala, Uganda

***Corresponding author**: Obondo James Sande (ojsande@gmail.com)

[2] Department of Immunology and Molecular Biology, Makerere University College of Health Sciences, Kampala, Uganda

Abstract

Background

Mechanisms for HIV control among HIV-1 elite and viremic-controllers are not fully understood. In Uganda, Studies have reported individuals who without Antiretroviral therapy have the inherent ability to control HIV progression to AIDS for a period of greater than 5 years. However, reasons for this phenotype are not understood. The study objective was to determine the distribution of CCR5 co-receptor on CD4+ T-cells and its associated promoter variants among HIV-1 elite and viremic-controllers.

Methods

We isolated CD4+T-cells from PBMCs using EasySep CD4+ T-cell negative selection kit, and stimulated them with anti-CD3 and anti-CD28 for 48 hours. To quantify CCR5 expression, we performed immune-phenotyping using flow cytometry. CCR5 promoter polymorphisms were determined through sanger sequencing. The Kruskal–Wallis and the Mann-Whitney test were used to compare differences in the percentages of CCR5+ CD4+ T-cells and the differences in CCR5 densities on CD4+ T-cells respectively. p values < 0.05 were considered significant.

Results

The percentage of CCR5+CD4+ T-cells was higher among the non-controllers compared to the controllers although, the difference was not statistically significant; elite and viremic-controllers (p=0.9173), viremic and non-controllers (0.0702), elite and non-controllers (0.6010). Of significance was the CCR5 densities on CD4+ T-cells, which were significantly higher among non-controllers relative to the controllers; elite and viremic-controllers (p=3048), viremic and non-controllers (P=0.0312), elite and non-controllers (P=0.0210)

From the sequence analysis, the rs1799987A>G mutation was found among elite (71%) and viremic-controllers (61%), while the -2459A/A and rs41469351C>T mutation were among the non-controllers (57%). This study also identified two novel mutations 1070T>G and 785A>G among the elite controllers (14.3%).

Conclusion

Rs1799987 SNP highly detected among the elite and viremic controllers may be associated with reduced CCR5 densities on CD4+ T-cells while higher frequency of -2459 A/A and rs41469351 SNP among non-controllers may be associated with increased CCR5 densities on CD4+ T-cells. Thus Rs1799987 SNP may be responsible for the delayed HIV progression among elite and viremic controllers, while -2459A/A and rs41469351 SNP may be responsible for the rapid progression of HIV among non-controllers. *In vitro* studies are needed to study the effect of the two novel mutations 1070T>G and 785A>G among elite-controllers.

Key words

CCR5, HIV, Elite controllers, Viremic controllers and non-controllers

1. Background

HIV has claimed lives of more than 35 million people globally since its discovery, particularly in the WHO African region, home to 70% of the 36.7 million people currently living with HIV (1). Though no documented cure yet exists for HIV, those infected with the virus are enrolled on Antiretroviral Treatment (ART), which enables them to live long healthy lives.

One subset of individuals living with HIV, referred to as long term non progressors (LTNPs), are able to maintain their CD4+ T-cell count above 500 cells/µl for a period greater than five years prior to ART initiation (2). Among these, elite controllers have an undetectable viral load (HIV

plasma viral load < 50 viral RNA (vRNA) copies ml^{-1}) while others, viremic controllers, maintain their viral load between 50 and 2000 copies/ml (3). The existence of this population in Uganda has been reported in two previous prevalence studies. Laeyendecker reported a prevalence of 9.1% of LTNPs in Rakai district (2) while Kayongo, reported a prevalence of 0.26% among elite controllers in an Urban HIV ambulatory center in Kampala (2, 4). Reasons for intrinsic resistance to HIV in these controllers are not fully understood, especially in Uganda and other African populations with high genetic diversity (4).

Studies have identified mutations in *CCR5*, a major co-receptor for HIV infection that has been linked to delayed disease progression and resistance to HIV (5-8). The Δ32 mutation with a 32-base pair (bp) deletion in the open reading frame (ORF) of the *CCR5* gene confers resistance to HIV in homozygous individuals and retards disease progression in heterozygotes (9). While the *CCR5*Δ32 allele occurs at a variable frequency of 4–15% in Caucasian populations, with an average of 10% in Europe ((10, 11), it is rare to find this mutation among Asian or African populations (12, 13).

However, previous investigations have reported single nucleotide polymorphisms (SNPs) within the *CCR5* promoter region associated with altered *CCR5* expression (14) thereby positively or negatively influencing an individual's susceptibility and rate of disease progression to AIDS (15). Yet, despite the overwhelming evidence of *CCR5* promoter polymorphisms' influence on HIV susceptibility and disease progression, data on variations in the *CCR5* promoter region among elite and viremic controllers in Uganda is not known. Among different populations where it is found, the distribution of these *CCR5* promoter polymorphisms varies greatly (16-18). An example being HHC promoter haplotype that has been associated with faster disease progression among African Americans (16), although in the Thai population, this haplotype is reported with slower disease progression (19). The impact of these promoter variants on disease progression

are still unknown in Uganda. Exploring variations in this promoter region is essential to identify protective mutations among Ugandan HIV-1 elite and viremic controllers that can be associated with delayed HIV progression to AIDS. Therefore, this study determined the distribution of CCR5 and its associated promoter variants among the elite and viremic controllers in Uganda. Data generated provides insights into mechanisms that could be responsible for the different clinical HIV courses of disease seen among this study population.

2. Methods

2.1 The aim, design and setting of the study

The study aimed at identifying and characterizing genetic variations within the *CCR5* promoter region that may be responsible for the delayed progression of HIV among elite and viremic controllers in Uganda.

This was a laboratory-based cross-sectional study leveraging peripheral blood mononuclear cells (PBMC) samples collected by the Elite study between 2016 and 2018. The Elite study focused on the role of host genes in T-cell resistance among elite and viremic controllers in Uganda. Cryopreserved PBMC samples collected from ART naïve HIV infected individuals followed for a duration greater than 5 years were used in this study. Study participants were enrolled from Makerere University Joint AIDS Program (MJAP), Mulago ISS clinic.

The Immunology assays were conducted at Makerere University, College of Health Sciences Immunology Laboratories while the molecular Biology assays were conducted at the Center For AIDS Research (CFAR) laboratory, Joint Clinical Research Center in Kampala, Uganda.

2.2 Patient Characteristics

PBMCs were collected from three (3) patient groups, namely; a) elite controllers (undetectable viral load with > 5 years in care without ART), b) viremic controllers (viral load between 50 and 2000 viral RNA (vRNA) copies ml^{-1}) and c) non-controllers (HIV infected individuals well controlled on ART).

Elite and Viremic controllers were recruited basing on the following attributes; ART naïve, maintained their CD4 count ≥ 500 cells/µl, and the two controller phenotypes were differentiated by their viral loads with elite controllers having HIV plasma viral load of < 50 viral RNA (vRNA) copies ml^{-1}while viremic controllers maintained a viral load between 50 -2000 viral RNA (vRNA) copies $ml^{-1.}$ for a period, greater than 5 years. At enrollment, patients provided a peripheral blood sample, and HIV Viral Load was determined by qRT-PCR using Abbott Real Time HIV-1 assay (Abbott Molecular, USA). The time interval between initial viral load and enrollment viral load was determined and recorded in months. To confirm controller (elite/viremic controller) status, a follow-up VL was performed and the time interval between baseline and follow-up VL was also calculated. Any individuals with a hemoglobin of < 10g/dl and active opportunistic infection were excluded.

2.3 Laboratory Methods

2.3.1 Treatment of PBMCs before storage

PBMCs were isolated using the Ficoll gradient centrifugation method. The PBMCs were then washed with PBS and centrifuged at 1700 rpm for 5 min. The supernatant was discarded, and the pellet was re-suspended in 40 ml PBS. The cells were then washed twice before the cells were stained with trypan blue and counted using an automatic cell counter (Invitrogen, Carlsbad,

California, USA). The cells with viability ≥ 95% were prepared for storage by re-suspending them in 1 ml of freeze media, and each sample aliquoted and stored in 2 cryo-vials. The cryovials were immediately placed in Mr. Frosty storage container (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and later stored overnight in a freezer at − 80 °C. The cryovials were then transferred to liquid nitrogen for storage.

2.3.2 PBMC Thawing:

Cryopreserved PBMC samples were retrieved from liquid nitrogen at -196^{0}C and immediately transferred to a preset 37^{0}C water bath. Upon thawing, cells were washed with R10 media composed of RPMI 1640 medium (ThermoFisher Scientific, South America, catalogue no. 11875093), 1% Pen-Strep, 1% L-Glutamine, 1% Hepes buffer and 10% Fetal Bovine Serum (ThermoFisher Scientific, South America, catalogue no. 10270106) in a 15 ml centrifuge tube. We then determined cell yield where viability testing was done using Trypan blue solution. Cells were stained using 0.4% trypan blue solution at 1:1 dilution ratio. Samples with at least 80% viability were considered for CD4+ T cell isolation. A portion of cells harvested off in R10 media were used for DNA extraction and the rest for CD4+ T cell isolation.

2.3.3 CD4+ T cells Isolation

Following thawing, CD4+ T cell were isolated using the EasySep^{TM} Human Isolation Kit (Stem Cell Technologies, Catalogue no. 19052). The stem cell Isolation protocol was followed. But briefly, cells were centrifuged at 1500rpm for 10 minutes, decanted and the pellet re-suspended in 1ml of 2% FBS containing 0.5% EDTA. The samples were transferred into FACs tubes from where 50µl of the enrichment cocktail were added and then incubated at room temperature for 10 minutes. Thereafter, 100 µl of the magnetic beads were added and the sample incubated at room temperature for 5 minutes. The sample tube (lid removed) was then placed in the EasySep magnet and

incubated at room temperature for 5 minutes. In one continuous motion, the sample (isolated CD4+ T cells) was poured into a second tube after the 5 minutes' incubation. The isolated CD4+ T cells were washed in 1ml PBS, centrifuged at 1500rpm for 10 minutes. These were re-suspended in 2ml R-10 media, stained for counting with trypan blue and then incubated at 37^0C on a 24 well plate for 2 hours in a CO_2 incubator. The cells were also stained for purity using anti-CD3, and anti-CD4 and ran on a BD FACS Canto II (BD Biosciences, Franklin lakes, New Jersey, USA). Samples with an average purity of 98% and above determined after staining for flow cytometry were considered for stimulation. Prior to stimulation, the cells were rested in a 24-well-plate at 37^0C in a $C0_2$ incubator.

2.3.4 CD4+ T cell Stimulation

We prepared stimulatory antibodies; Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3) at a concentration of 5µg/ml each. A clearly mapped out 96-well plate was used. The plate was coated by adding 100 µl of anti-CD3 at a concentration of 5ug/ml and incubated at 37^0C in a CO_2 incubator for 2 hours. After incubation, the plate was blotted and 100,000 cells in 90 µl per sample were added. Using a pipette, 110 µl anti-CD28 was added to each well to make a total final volume of 200 µl at a concentration of 5ug/ml. For the negative control wells, 110µl of PBS was added to make volume of 200 µl per well. The plate was incubated for a total of 48 hours at 37^0C in a CO_2 incubator. After incubation, cells were washed with 200 µL staining buffer per well and then transferred to the 5 mm round bottomed polystyrene FACS tubes.

2.3.5 Cell Surface Staining

Subsequently, cells were surface stained and incubated for 30 minutes with the following monoclonal antibodies; CD3 $^{\text{Percyp cy5.5}}$, CD4 $^{\text{APC}}$, CCR5 $^{\text{PE}}$, and Zombie Aqua (BD bioscience, San

Jose, CA, USA) (Table 1). The cells were acquired on an eight-color FACS CANTO II (BD Biosciences, San Jose, CA, USA). At least 50,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one controls (FMOS). Data obtained were analyzed using FlowJo version 10.1 (San Carlos, CA, USA) and GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA).

Table 1. Cell surface markers used as parameters to define CCR5 expressing T cell phenotypes

Cell Marker	Phenotype Function
CD3	T cell lineage marker
CD4	CD4+ T lineage
CCR5	Chemokine receptor

2.3.6 DNA Extraction

DNA was extracted using the QIAamp DNA mini Kit (Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions as used in the previous studies (20). 200µl of sample containing $2x10^6$ cells was added to micro-centrifuge tube together with 20µl of Qiagen protease. 200µl of buffer AL was added to the sample which was then mixed thoroughly to ensure efficient lysis and then incubated at 56^0c for 10 minutes. 200µl of ethanol was added to the sample and then mixed by pulse vortexing. After vortexing, the mixture was added to span column (in a 2ml collection tube) and centrifuged at 8000rpm for 1 minute. The mini spin column was later placed into a clean 2 ml collection tube. The extracted DNA was washed using AW1 and AW2 and spun at 8000 rpm for 1 minute and 14000 for 3 minutes respectively. The empty column was span to prevent possible buffer AW2 carry over and later DNA was eluted using AE buffer into a new 1.5ml micro-centrifuge tube.

2.3.7 PCR and Sequencing

PCR amplification of CCR5 promoter region was carried out using the following cycling conditions; Initial denaturation at 95°C for 3 minutes; 31 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2.40 minutes; followed by 68°C for 7 min. The PCR master mix contained High fidelity Super script III platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) in the presence of 2X reaction buffer, 5Mm MgCL2 with primers shown in table 2 developed using GenBank sequence with accession number U95626. as described in a similar study (21). The promote amplicon size was 2189 base pairs (21).

Table 2. Primers used in the amplification of Promoter 1 region of the CCR5 gene

Primer	Binding position	Amplicon size	Annealing temperature
Forward 5′CCAAGCACCAGCAATTAGC3′	58105 – 58122	2189	60^0C
Reverse 5′TGCCACCACAGATGAATGTC3′	60293 – 60274		60^0C

2.3.8 PCR clean up

From all samples that gave a single band after Gel electrophoresis, 10µl was aliquoted and added into a PCR tube followed by 2µl of ExoSAP IT. The samples were transferred into a thermocycler (Applied Biosystems, California, United States) and ran under the conditions: 37^0 C for 45 seconds, 800C for 45 seconds (inactivate ExoSAP-IT) and held at 4^0C.

2.3.9 Cycle sequencing

Sequencing was performed using an ABI version 3.1 BigDye Kit (Applied Biosystems, Catalogue no. 4337456) and ABI3500xl Genetic Analyzer. Briefly, a master mix was prepared as follows; 0.5µl Big Dye terminator, 1.75µl 5X sequencing buffer, 2.5 µl primer as shown in the primer map (Fig 1) and primer sequences are shown in table 3 below (18) and 4.25µl water. 9µl of sequencing master mix was added into each well where 1µl of DNA was added.

Briefly, PCR amplifications was subjected to thermal cycling as follows: 96°C for 1 minute; 30 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2.40 minutes; followed by 68°C for 7 min.

Table 3 Primers used in sequencing of the CCR5 promoter 1 region

Primer	Binding position
Forward F 5′CCAAGCACCAGCAATTAGC3′	58105 – 58122
Reverse R 5′TGCCACCACAGATGAATGTC3′	60293 – 60274
Forward IFS 5′TTGCTGTTTGGGGTCT3′	58471 – 58486
Forward F1 5′GAGTGGAGAAAAAGGGGG3′	59013 – 59030
Reverse R1 3′AGAATAGATCTCTGGTCTGAAA5′	59375 – 59354

Fig 1. The CCR5 promoter primer map for the primers used in sanger sequencing.

2.3.10 Data Analysis

Flow cytometry data were analyzed using FlowJo version 10.5.2 software. CD4+ T cells were distinguished by their surface expression of CD3 and CD4. $CD4^+$ T cells, we identified CCR5+ T cells and determined both the percentage CD4+CCR5+ T cells and CCR5+ MFI (to ascertain the CCR5 density on CD4+ T cells). Statistical analysis was performed using GraphPad Prism 7. The Mann Whitney and Kruskal Wallis tests for non-parametric variables facilitated comparison of differences among groups. P values < 0.05 indicated a significant difference.

Sanger Sequence data analysis was performed using mutation surveyor Mutation version 5.5 (SoftGenetics; Pennsylvania, USA). **U95626** and **NT_022517 r**eference sequences were used in assembly as used in other studies (18). A search of the GenBank NCBI SNP database (dbSNP) determined whether polymorphisms detected in this study had been previously reported. The

CCR5 numbering system was used where the first nucleotide of the translational start site is designated as +1 and the nucleotide immediately upstream from that is −1 (22).

3. Results

3.1 Participant characteristics

This was a cross-sectional study conducted among 30 HIV-1 chronically infected individuals. These included 14 elite controllers [HIV plasma viral load < 50 viral RNA (vRNA) copies ml^{-1}], 9 viremic controllers [HIV plasma viral load between 50 -2000 viral RNA (vRNA) copies ml^{-1}] and 7 non-controllers (ART controlled) whose demographic characteristics are summarized in Table 4.

257 **Table 4: Characteristics of elite, viremic and non-controllers derived per group (n=30)**

Participant	Age	Sex	CD4 count	Duration in Care (Years)	Viral Load	Months between VLs	[1]BMI
Elite Controllers							
1	53	F	1245	10	UD	8	33.9
3	32	F	1008	5	UD	9	31.8
4	38	F	919	9	UD	12	18.9
7	36	F	1188	7	UD	8	38.5
13	56	M	833	7	UD	9	17.2
36	41	M	778	9	UD	12	25.2
37	37	F	1063	6	UD	8	26.1
40	45	F	1036	6	UD	11	25.6
42	28	F	653	8	UD	9	23.5
15	42	F	909	5	UD	12	31.8
16	30	F	1050	5	UD	10	29.3
21	37	F	728	6	UD	9	23.9
6	38	F	1162	6	UD	8	25.3
22	39	F	650	9	UD	5	30.7
Viremic Controllers							
24	32	F	698	5	280	14	30
26	56	F	652	8	1380	8	25.3
5	51	F	895	10	1299	10	29.5
12	37	F	805	5	155	15	24.5
38	40	M	732	6	388	9	28.9
31	41	F	852	10	285	7	30.2
20	56	M	897	11	1220	14	25.6
32	37	F	772	10	243	9	29
14	54	F	669	8	782	8	25.3
Non controllers							
NC28	38	F	589	5	10500	6	21.4
NC30	42	F	1021	8	2840	10	21.3
NC 2	40	M	920	6	10800	15	27.2
NC 11	40	F	940	5	14800	8	19.1
NC 18	29	F	747	5	2310	8	25.3
NC025	43	F	781	8	5250	10	32.7
NC 8	41	F	1192	6	2840	6	37.5

258 UD denotes undetectable; [1]BMI denotes body mass index

3.2 CCR5 expression on CD4+ T cells among elite, viremic and non-controllers

Because CCR5 is expressed only on activated CD4+ T cells (23, 24), we activated CD4+ T cells *in vitro* using Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3). We then performed flow cytometry to study CCR5 expression. Flow cytometry data was analyzed using Flow Jo and the gating strategy used to determine CCR5 expression (Fig. 2A).

3.3 Percentage CCR5+CD4+ T cells and their CCR5 densities among elite, viremic and non-controllers

Considerable evidence suggests that CCR5+CD4 T cells are needed during early stages of HIV infection (25, 26). To elucidate the distribution of CCR5+CD4 T cells among elite, viremic and non-controllers, we stimulated CD4 T cells from participants with Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3). We later carried out flow cytometry to ascertain the percentage of CCR5+CD4 T cells. The Kruskal–Wallis test was used to compare differences in the percentage of CCR5 expressing CD4+ T cells among elite, viremic and non-controllers. p values < 0.05 were considered significant. We found that elite and viremic controllers had lower percentage of CCR5+CD4+ T cells compared to non-controllers, although the differences were not statistically significant; elite controllers and viremic controllers (P=0.9173), viremic controllers and non-controllers (P=0.0702), elite controllers and non-controllers (p=0.6010) (Fig 2B). These results suggest that the percentage of CCR5+CD4 T cells have no statistical contribution to progression to AIDS among elite and viremic controllers.

However, because CCR5 densities are independent of the percentage of CCR5+CD4 T cells and they have been associated with high viral loads (27-29), we carried out experiments to ascertain whether elite, viremic and non-controllers have differences in their CCR5 densities. Cells were stimulated and flow cytometry was carried out. The Mann Whitney test was used to compare

differences in MFI of CCR5-expressing $CD4^+$ among elite, viremic and non-controllers. *p* values < 0.05 were considered significant. We found significant variation in the Medium Fluorescent Intensity (MFI) of CCR5-expressing CD4+T cells between controllers (elite and viremic controllers) and the non-controllers; elite and non-controllers (P=0.0210), viremic and non-controllers (P=0.0312) (Fig 2C). However, there was no statistically significant difference in the MFI of CCR5-expressing $CD4^+$ T-lymphocytes between viremic and elite controllers (P=0.3048) (Fig 2C).

Fig 2. CCR5 expression on CD4+ T cells: A) A sequential gating strategy used to analyze CCR5 expression on CD4 + T cells. We first gated on the lymphocytes using a forward scatter-area (FSC-A) against side scatter-area (SSC-A) gate. (2) We excluded doublets with a singlet gate by gating on forward scatter-area (FSC-A) against forward scatter-height (FSC-H). (3) Live cells were selected by gating on scatter-height (FSC-H) against ARM Cyan (Live-dead marker). (4) Conventional T cells were selected by gating on $CD3^+$ cells from the total lymphocyte population, from which (**5**) $CD4^+$ T cells were selected. (6) From CD4+ T cells, CCR5+ T cells were selected. B) **Percentage of CCR5+CD4+ T cells** is higher among elite controllers (n=14), viremic controllers (n=9) compared to non-controllers (n=7) although the difference is not statistically significant; EC and VC (P=0.6010), EC and NC (P=0.9156), VC and NC (P=0.0702). C) **Differences in CCR5 densities** among EC, VC and NC is statistically significant (between EC and VC; P=0.3048, EC and NC; P=0.0210, VC and NC; P=0.0312).

3.4 SNPs identified within CCR5 promoter region in this cohort

The rare occurrence of delta 32 bp deletion within Africa (12), has led to a number of studies to explore additional CCR5 regions for possible causes of the phenotypes seen among African ART naïve individuals who have the capacity to control HIV (13). Studies have reported several CCR5 promoter polymorphisms associated with either reduced or increased CCR5 expression among different cohorts in Africa (13, 15). Controversies have arisen where some mutations are protective in some regions and detrimental in others, thus this study was set out to explore which CCR5 promoter variants are associated with the different phenotypes in this study. We used previously

stored PBMCs which were thawed and then DNA extracted using Qiagen Blood Genomic DNA Kit (QIAamp DNA kit; Qiagen, Inc., Valencia, California, USA). The DNA was PCR amplified and then sequenced.

We found that rs1799987 single nucleotide polymorphisms (SNPs) were predominant among elite controllers and viremic controllers (71% and 61% respectively) while rs41469351was more among non-controllers (68%). Furthermore, we also identified two Novel mutations; 1070 T>G (14.3%) and 785 A>G (14.3%) among elite controllers (Table 5).

Table 5: CCR5 promoter SNPs among elite, viremic and non-controllers

SNP	Chromosome position	dbNo.	Percentage of Occurrence (%)
Elite controllers (n=14)			
-2459A>G	chr3:46370444	rs1799987	71
1017C>T	chr3: 46370658	rs142710698	7
1070 T>G	Chr3:46370711	Novel	14.3
785 A>G	Chr3:46370426	Novel	14.3
Viremic controllers (n=9)			
-2459A>G	chr3:46370444	rs1799987	61
-2554G>T	chr3:46370349	rs2734648	56
Non controllers (n=7)			
-2132C>T	chr3:46370771	rs41469351	68
-1835C>T	chr3:46371068	rs1800024	53
-2733A>G	chr3:46370170	rs2856758	29

4. **DISCUSSION**

Expression of CCR5 on CD4+ T cells has been shown to be highly variable between individuals (30).*In vitro* studies have shown that this variability affects HIV Infectivity in cell lines (31), macrophages (32), and lymphocytes.(33). In our study, the number of CCR5+CD4 T cells and

CCR5 densities on the surface of CD4 T cells vary among elite, viremic and non-controllers and this could explain the different phenotypes among these populations. This is supported by Reynes et al. who demonstrated that CCR5 expression affects virus production and viral load, and individuals with a low viral load have reduced CCR5 densities on the CD4+ T cell surface. (27, 28)

In this study, we found no statistical difference in the reported percentage of CD4+CCR5+ T cells between elite, viremic and non-controllers. However, elite and viremic controllers had lower percentage of CD4+CCR5+ T cells compared to non-controllers. The reduction in CD4+CCR5+ T cells is in agreement with the findings by Potter et al who showed lower expression of CCR5 $CD4^+$ T lymphocytes in HIV controllers (28, 34). This reduction may contribute to the low levels of infection in elite and viremic controllers since CCR5 expressing CD4+ T cells are required during initial stages of HIV infection by the R5 tropic virus (35).

Of significance was the finding that showed that elite and viremic controllers had statistically significant reduction in CCR5 densities compared to non-controllers. This shows that even though there was no statistical significance in the percentage of the CCR5+CD4 T cells, there were differences in the number of CCR5 expressed on the CD4+ T cell surfaces. These results agree with findings by Reynes et al. who reported data supporting the hypothesis that the rate of evolution of HIV-1 disease in an individual is influenced by the median number of CCR5 co-receptors at the surface of the CD4 T cells of the individual. They also demonstrated that CCR5 expression affects virus production and viral load, and individuals with a low viral load have CCR5 densities below the threshold value (27). The low expression of CCR5 on CD4+ T cells could explain why the elite and viremic controllers have the capacity to control the R5 tropic HIV virus since they have reduced CCR5 expression on the surface of their CD4+ T cells.

In the present study we report a high frequency of rs1799987 A>G mutation among elite and viremic controllers. Joshi et al, in an *in vitro* study demonstrated that rs1799987 A>G was associated with reduced expression of CCR5 on the 293 T cell lines - which were transfected with plasmid DNA containing this specific CCR5 gene promoter variant ((36). These findings are in agreement with findings from studies by McDermott and Mehlotra who argued that this mutation was associated with reduced CCR5 expression (37, 38). Taken together, these findings could mean that rs1799987 SNP confers protection against HIV disease progression and may contribute to low HIV susceptibility, explaining its occurrence in the HIV controllers. Furthermore, -2459 A/A was noted to be enriched among HIV-1 non-controllers. Joshi et al in an individual SNP analysis *in vitro* study, also demonstrated that -2459 A/A was associated with increased CCR5 expression in individuals with the mutation compared to those without. These findings point to the potential role of -2459 A/A in CCR5 expression among Non-controllers. Still in the current study, we found rs41469351 C>T SNP in the promoter region of CCR5 gene which was highly concentrated among HIV-1 non-controllers (57%). Similar to our findings, Kostrikis LG et al who reported the -2132C/T SNP to be associated with higher viral loads and higher CCR5 densities on CD4 T cell in a cohort that was aimed at studying perinatal transmission (39). Our findings and those of previous researchers (40-42) could imply that -2132C/T increases HIV disease progression by facilitating increased CCR5 densities on CD4+ T cells among NCs thus increased viral replication. The roles of the other SNPs (1017C>T, -2554G>T, -1835C>T and -2733A>G) found in this present study in HIV disease progression have not been reported yet and as such require *in vitro* studies to elucidate their effect on CCR5 expression on the surface of CD4+ t cells.

This study's limitations included a limited sample size due to the low occurrence of elite and viremic controllers in the general population. Furthermore, the Test and treat policy rolled out by

the World Health Organization in 2016, where all individuals that test positive for HIV are enrolled on HAART, made it impossible to identify and recruit more controllers.

Additionally, this study didn't consider other factors for example the Human Leukocyte Antigen which might have a contributory protection role against HIV disease progression to AIDS among elite and viremic controllers (43, 44).

5. Conclusion

In summary, our study has confirmed the presence of SNPs which have been previously associated with either delaying or increasing HIV progression to AIDS. Rs1799987 A>G mutation identified among HIV-1 elite and viremic controllers was associated with reduced CCR5 density on CD4+ T cells as compared to non-controllers, while -2459 A/A and rs41469351 C>T SNP identified among non-controllers were associated with increased CCR5 density on CD4+ T cells as compared to controllers. This suggests that the high frequency of Rs1799987 A>G mutation among elite and viremic controllers may be associated with delayed HIV progression, while the high frequency of -2459 A/A and rs41469351 C>T SNP among non-controllers may facilitate HIV progression. Additionally, the study identified two novel mutations among elite controllers - 1070 T>G and 785 A>G, however, *in vitro* studies are needed to study their effect on CCR5 expression.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Abbreviations

ART: Antiretroviral Treatment; ORF: Open reading frame; *CCR5*: C-C chemokine receptor type 5; HIV: Human Immunodeficiency Virus; AIDS: Acquired Immunodeficiency Virus; SNPS: Single Nucleotide polymorphisms; MJAP: Makerere University Joint Aids Program; MFI: mean fluorescence intensity; DNA: Deoxyribonucleic acid; PCR: Polymerase Chain reaction; PBMCS:

Peripheral Blood Mononuclear cells; UNCST: Uganda National Council of Science and Technology; LTNPs : Long Term Non Progressors.

Acknowledgements

We would like to thank Ms. Geraldine Nalwadda, Department of Immunology and Molecular Biology for her administrative support. We thank the staff of Immunology and molecular laboratories at Makerere University as well as the Center for AIDS Research laboratories at the Joint Clinical Research Center, Kampala Uganda for their technical input during the research.

Funding

This work was done as partial fulfillment for the award of a degree of Master of Science in Immunology and Clinical Microbiology to BN. BN is a MITHU fellow and this research work was funded by NIH Fogarty, Grant No. D43TW010319 (PI: Henry Boom). Part of the work was funded by the African Centre of Excellence in Materials, Product development, and Nanotechnology (ACE_ MAPRONANO) Project ID: P151847/IDA 5797-UG. OJS is a NURTURE fellow under NIH grant D43TW010132 (PI: Nelson Sewankambo).

Confidentiality

No patient identifiers were attached to patient data. All data was password protected, stored in fully encrypted databases and accessible only to research assistants and investigators responsible for analysis.

Contributions

Conceptualization, NB and OJS; Methodology, NB, SBA, AB, DK, JK, EN, JM, FW and OJS; Investigation, Data curation, Visualization and Project management were performed by NB; Writing—Original Draft, NB; Writing—Review and Editing, NB, SBA, OJS, DPK, RN, AK and

IN; Funding Acquisition, DPK, and OJS; Resources, IN, RN, OJS and SBA; Supervision, DPK, and OJS. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

The parent study was approved by the Makerere University School of Biomedical Sciences Higher Degree Research and Ethics Committee (SBS-HDREC, study no. SBS-372) and the Uganda National Council of Science and Technology (UNCST, study no. HS 2169).

For this study, we obtained a waiver of consent from the Institutional Review Board of the School of Biomedical Sciences, Makerere University to use the stored samples collected from the approved parent study (SBS-605).

Consent for publication

Not applicable.

Conflict of interest:

The authors declare no conflict of interest.

436 **References**

437 1. WHO. HIV/AIDS: World Health Organization; 2017 [Available from:
438 http://www.who.int/mediacentre/factsheets/fs360/en/.

439 2. Laeyendecker O, Redd AD, Lutalo T, Gray RH, Wawer M, Ssempijja V, et al. Frequency
440 of long-term nonprogressors in HIV-1 seroconverters from Rakai Uganda. Journal of acquired
441 immune deficiency syndromes (1999). 2009;52(3):316.

442 3. Pereyra F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A, et al. Genetic and
443 immunologic heterogeneity among persons who control HIV infection in the absence of therapy.
444 The Journal of infectious diseases. 2008;197(4):563-71.

445 4. Kayongo A, Gonzalo-Gil E, Gümüşgöz E, Niwaha AJ, Semitala F, Kalyesubula R, et al.
446 Brief Report: Identification of Elite and Viremic Controllers From a Large Urban HIV Ambulatory
447 Center in Kampala, Uganda. Journal of acquired immune deficiency syndromes (1999).
448 2018;79(3):394.

449 5. Barmania F, Pepper MS. CC chemokine receptor type five (CCR5): an emerging target for
450 the control of HIV infection. Applied & translational genomics. 2013;2:3-16.

451 6. Singh KK, Hughes MD, Chen J, Phiri K, Rousseau C, Kuhn L, et al. Associations of
452 chemokine receptor polymorphisms With HIV-1 mother-to-child transmission in sub-Saharan
453 Africa: possible modulation of genetic effects by antiretrovirals. Journal of acquired immune
454 deficiency syndromes (1999). 2008;49(3):259.

455 7. An P, Martin MP, Nelson GW, Carrington M, Smith MW, Gong K, et al. Influence of
456 CCR5 promoter haplotypes on AIDS progression in African–Americans. Aids. 2000;14(14):2117-
457 22.

458 8. John GC, Bird T, Overbaugh J, Nduati R, Mbori-Ngacha D, Rostron T, et al. CCR5
459 promoter polymorphisms in a Kenyan perinatal human immunodeficiency virus type 1 cohort:

460 association with increased 2-year maternal mortality. The Journal of infectious diseases.
461 2001;184(1):89-92.
462 9. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber C-M, et al. Resistance to
463 HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor
464 gene. Nature. 1996;382(6593):722.
465 10. Novembre J, Galvani AP, Slatkin M. The geographic spread of the CCR5 Δ32 HIV-
466 resistance allele. PLoS biology. 2005;3(11):e339.
467 11. Martinson JJ, Chapman NH, Rees DC, Liu Y-T, Clegg JB. Global distribution of the CCR5
468 gene 32-basepair deletion. Nature genetics. 1997;16(1):100.
469 12. Salem A-H, Batzer MA. Distribution of the HIV resistance CCR5-Δ32 allele among
470 Egyptians and Syrians. Mutation Research/Fundamental and Molecular Mechanisms of
471 Mutagenesis. 2007;616(1-2):175-80.
472 13. Barmania F, Potgieter M, Pepper MS. Mutations in CC chemokine receptor type 5 (CCR5)
473 in South African individuals. International Journal of Infectious Diseases. 2013;17(12):e1148-e53.
474 14. Chang HY, Ahn SH, Kim DY, Shin JS, Kim YS, Hong SP, et al. Association between
475 CCR5 promoter polymorphisms and hepatitis B virus infection. The Korean journal of hepatology.
476 2005;11(2):116-24.
477 15. Picton AC, Paximadis M, Tiemessen CT. CCR5 promoter haplotypes differentially
478 influence CCR5 expression on natural killer and T cell subsets in ethnically divergent HIV-1
479 uninfected South African populations. Immunogenetics. 2012;64(11):795-806.
480 16. Gonzalez E, Bamshad M, Sato N, Mummidi S, Dhanda R, Catano G, et al. Race-specific
481 HIV-1 disease-modifying effects associated with CCR5 haplotypes. Proceedings of the National
482 Academy of Sciences. 1999;96(21):12004-9.

17. Tang J, Shelton B, Makhatadze NJ, Zhang Y, Schaen M, Louie LG, et al. Distribution of chemokine receptor CCR2 and CCR5 genotypes and their relative contribution to human immunodeficiency virus type 1 (HIV-1) seroconversion, early HIV-1 RNA concentration in plasma, and later disease progression. Journal of virology. 2002;76(2):662-72.

18. Li M, Song R, Masciotra S, Soriano V, Spira TJ, Lal RB, et al. Association of CCR5 human haplogroup E with rapid HIV type 1 disease progression. AIDS Research & Human Retroviruses. 2005;21(2):111-5.

19. Nguyen L, Li M, Chaowanachan T, Hu DJ, Vanichseni S, Mock PA, et al. CCR5 promoter human haplogroups associated with HIV-1 disease progression in Thai injection drug users. Aids. 2004;18(9):1327-33.

20. Williamson C, Loubser SA, Brice B, Joubert G, Smit T, Thomas R, et al. Allelic frequencies of host genetic variants influencing susceptibility to HIV-1 infection and disease in South African populations. Aids. 2000;14(4):449-51.

21. Picton AC, Paximadis M, Tiemessen CT. Genetic variation within the gene encoding the HIV-1 CCR5 coreceptor in two South African populations. Infection, Genetics and Evolution. 2010;10(4):487-94.

22. Mummidi S, Bamshad M, Ahuja SS, Gonzalez E, Feuillet PM, Begum K, et al. Evolution of human and non-human primate CC chemokine receptor 5 gene and mRNA Potential roles for haplotype and mRNA diversity, differential haplotype-specific transcriptional activity, and altered transcription factor binding to polymorphic nucleotides in the pathogenesis of HIV-1 and simian immunodeficiency virus. Journal of Biological Chemistry. 2000;275(25):18946-61.

23. Meijerink H, Indrati AR, van Crevel R, Joosten I, Koenen H, van der Ven AJ. The number of CCR5 expressing CD4+ T lymphocytes is lower in HIV-infected long-term non-progressors

506 with viral control compared to normal progressors: a cross-sectional study. BMC infectious
507 diseases. 2014;14(1):683.
508 24. Veazey RS, Mansfield KG, Tham IC, Carville AC, Shvetz DE, Forand AE, et al. Dynamics
509 of CCR5 expression by CD4+ T cells in lymphoid tissues during simian immunodeficiency virus
510 infection. Journal of virology. 2000;74(23):11001-7.
511 25. Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors—central
512 to understanding the transmission and pathogenesis of human immunodeficiency virus type 1
513 infection. AIDS research and human retroviruses. 2004;20(1):111-26.
514 26. Grivel J-C, Shattock RJ, Margolis LB. Selective transmission of R5 HIV-1 variants: where
515 is the gatekeeper? Journal of translational medicine. 2011;9(1):S6.
516 27. Reynes J, Portales P, Segondy M, Baillat V, André P, Réant B, et al. CD4+ T cell surface
517 CCR5 density as a determining factor of virus load in persons infected with human
518 immunodeficiency virus type 1. The Journal of infectious diseases. 2000;181(3):927-32.
519 28. Reynes J, Portales P, Segondy M, Baillat V, André P, Avinens O, et al. CD4 T cell surface
520 CCR5 density as a host factor in HIV-1 disease progression. Aids. 2001;15(13):1627-34.
521 29. Yang X, Jiao Y-m, Wang R, Ji Y-x, Zhang H-w, Zhang Y-h, et al. High CCR5 density on
522 central memory CD4+ T cells in acute HIV-1 infection is mostly associated with rapid disease
523 progression. PloS one. 2012;7(11):e49526.
524 30. Moore JP. Coreceptors--Implications for HIV Pathogenesis and Therapy. Science.
525 1997;276(5309):51-2.
526 31. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D. Effects of CCR5 and CD4 cell
527 surface concentrations on infections by macrophagetropic isolates of human immunodeficiency
528 virus type 1. Journal of virology. 1998;72(4):2855-64.

32. Tuttle DL, Coberley CR, Xie X, Kou ZC, Sleasman JW, Goodenow MM. Effects of human immunodeficiency virus type 1 infection on CCR5 and CXCR4 coreceptor expression on CD4 T lymphocyte subsets in infants and adolescents. AIDS research and human retroviruses. 2004;20(3):305-13.

33. Wu L, Paxton WA, Kassam N, Ruffing N, Rottman JB, Sullivan N, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. The Journal of experimental medicine. 1997;185(9):1681-92.

34. Potter SJ, Lacabaratz C, Lambotte O, Perez-Patrigeon S, Vingert B, Sinet M, et al. Preserved central memory and activated effector memory CD4+ T-cell subsets in human immunodeficiency virus controllers: an ANRS EP36 study. Journal of virology. 2007;81(24):13904-15.

35. Blaak H, Ran LJ, Rientsma R, Schuitemaker H. Susceptibility of in vitro stimulated PBMC to infection with NSI HIV-1 is associated with levels of CCR5 expression and β-chemokine production. Virology. 2000;267(2):237-46.

36. Joshi A, Punke EB, Sedano M, Beauchamp B, Patel R, Hossenlopp C, et al. CCR5 promoter activity correlates with HIV disease progression by regulating CCR5 cell surface expression and CD4 T cell apoptosis. Scientific reports. 2017;7(1):1-11.

37. McDermott DH, Zimmerman PA, Guignard F, Kleeberger CA, Leitman SF, Murphy PM, et al. CCR5 promoter polymorphism and HIV-1 disease progression. The Lancet. 1998;352(9131):866-70.

38. Mehlotra RK. CCR5 Promoter Polymorphism− 2459G> A: Forgotten or Ignored? Cells. 2019;8(7):651.

39. Kostrikis LG, Neumann AU, Thomson B, Korber BT, McHardy P, Karanicolas R, et al. A polymorphism in the regulatory region of the CC-chemokine receptor 5 gene influences perinatal

553 transmission of human immunodeficiency virus type 1 to African-American infants. Journal of
554 virology. 1999;73(12):10264-71.
555 40. Madlala P, Singh R, An P, Werner L, Mlisana K, Karim SSA, et al. Association of
556 polymorphisms in the regulatory region of the cyclophilin A gene (PPIA) with gene expression
557 and HIV/AIDS disease progression. Journal of acquired immune deficiency syndromes (1999).
558 2016;72(5):465.
559 41. Bleiber G, May M, Martinez R, Meylan P, Ott J, Beckmann JS, et al. Use of a combined
560 ex vivo/in vivo population approach for screening of human genes involved in the human
561 immunodeficiency virus type 1 life cycle for variants influencing disease progression. Journal of
562 virology. 2005;79(20):12674-80.
563 42. An P, Wang LH, Hutcheson-Dilks H, Nelson G, Donfield S, Goedert JJ, et al. Regulatory
564 polymorphisms in the cyclophilin A gene, PPIA, accelerate progression to AIDS. PLoS pathogens.
565 2007;3(6):e88.
566 43. Gaudieri S, DeSantis D, McKinnon E, Moore C, Nolan D, Witt C, et al. Killer
567 immunoglobulin-like receptors and HLA act both independently and synergistically to modify
568 HIV disease progression. Genes & Immunity. 2005;6(8):683-90.
569 44. den Uyl D, van der Horst-Bruinsma IE, van Agtmael M. Progression of HIV to AIDS: a
570 protective role for HLA-B27. AIDS Rev. 2004;6(2):89-96.
571 WHO and UNAIDS announce recommendations from expert consultation on male circumcision
572 for HIV prevention. March 2007. Available
573 athttp://www.who.int/hiv/topics/malecircumcision/en/index.html. Accessed May 28,08
574 http://www.unaids.org/en/resources/fact-sheet
575 http://www.unaids.org/en/resources/documents/2017/20170720_Global_AIDS_update_2017

576 UNAIDS. 2015

577 http://www.unaids.org/sites/default/files/media_asset/AIDS_by_the_numbers_2015_en.pdf/

Figures

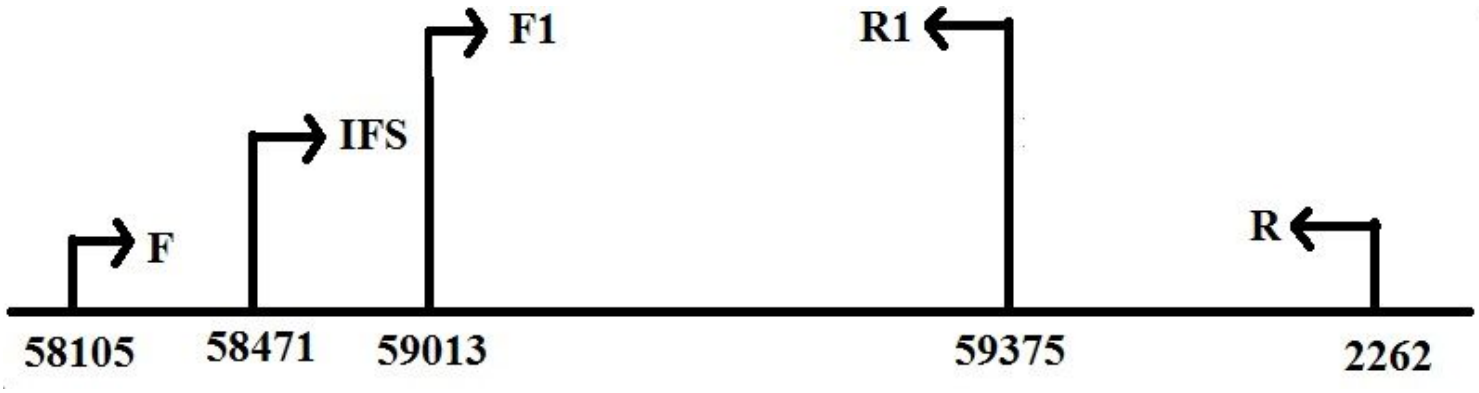

Figure 1

The CCR5 promoter primer map for the primers used in sanger sequencing.

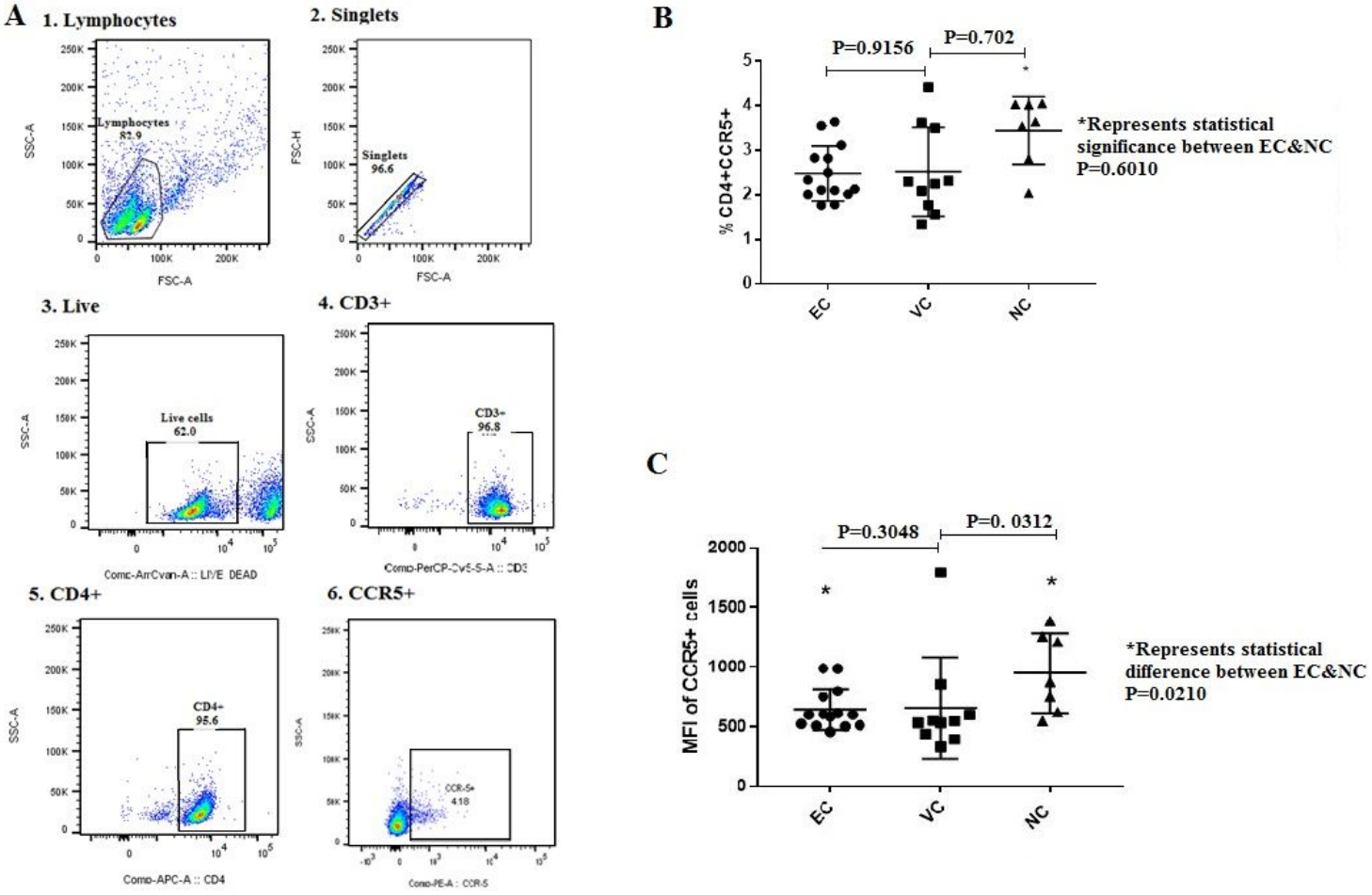

Figure 2

CCR5 expression on CD4+ T cells: A) A sequential gating strategy used to analyze 289 CCR5 expression on CD4 + T cells. We first gated on the lymphocytes using a forward scatter-290 area (FSC-A) against side scatter-area (SSC-A) gate. (2) We excluded doublets with a singlet gate 291 by gating on forward

scatter-area (FSC-A) against forward scatter-height (FSC-H). (3) Live cells 292 were selected by gating on scatter-height (FSC-H) against ARM Cyan (Live-dead marker). (4) 293 Conventional T cells were selected by gating on CD3+ cells from the total lymphocyte population, 294 from which (5) CD4+ T cells were selected. (6) From CD4+ T cells, CCR5+ T cells were selected. 295 B) Percentage of CCR5+CD4+ T cells is higher among elite controllers (n=14), viremic 296 controllers (n=9) compared to non-controllers (n=7) although the difference is not statistically 297 significant; EC and VC (P=0.6010), EC and NC (P=0.9156), VC and NC (P=0.0702). C) 298 Differences in CCR5 densities among EC, VC and NC is statistically significant (between EC 299 and VC; P=0.3048, EC and NC; P=0.0210, VC and NC; P=0.0312).