REPUBLIQUE DU CAMEROUN
*Paix – Travail – Patrie*
********
UNIVERSITE DE YAOUNDE I
FACULTE DES SCIENCES
DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES
*********
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE, SANTE ET ENVIRONNEMENT
LABORATOIRE DE PARASITOLOGIE ET ECOLOGIE

REPUBLIC OF CAMEROUN
Peace – Work – Fatherland
*******
UNIVERSITY OF YAOUNDE I
FACULTY OF SCIENCE
DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY
*******
CENTRE OF RESEARCH AND DOCTORAL TRAINING IN LIFE SCIENCE, HEALTH AND ENVIRONMENT
LABORATORY OF PARASITOLOGY AND ECOLOGY

# Phenotypic and functional characterization of regulatory T cells in antiretroviral therapy naive HIV-1 infected people in Cameroon

THESIS
Submitted in partial fulfilment of the requirements for the award of the Ph.D in Animal Biology and Physiology

Par : **AMBADA NDZENGUE Georgia Elna**
DEA in Animal Biology

Sous la direction de
**TOA Francois-Xavier**
**Professor, University of Yaoundé I**
**COLIZZI Vittorio**
**Professor, University of Tor Vergata, Italy**

**Année Académique : 2020**

BIBLIOTHEQUE UNIVERSITAIRE UYI

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**FACULTE DES SCIENCES**
*FACULTY OF SCIENCE*

**DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES**
*DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY*

# ATTESTATION DE CORRECTION

Nous soussignés, membres du jury de soutenance de la Thèse de Doctorat/Ph.D en Biologie des Organismes Animaux (Option Parasitologie) de Madame AMBADA NDZENGUE Georgia Elna, matricule 97V254, soutenance autorisée par la correspondance N° 20-951/UYI/VREPDTIC/DAAC/DEPE du Recteur de l'Université de Yaoundé I en date du 10 Juin 2020, attestons que les corrections exigées à la candidate lors de l'évaluation faite le 17 Juin 2020 ont réellement été effectuées et que le présent document peut être déposé sous sa forme actuelle.

En foi de quoi la présente attestation lui est délivrée pour servir et valoir de ce que de droit.

Yaoundé, le 10 JUIL 2020

Président du Jury

Charles Félix Bilong Bilong
Professeur

Examinateur

Pr. Wilfred F. Mbacham
MS, DS, ScD (Harvard), FASI, FCAS
Public Health Biotechnologist

Chef de Département

UNIVERSITE DE YAOUNDE I – University of Yaounde I – DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE ANIMALES – Department of Animal Biology and Physiology – Le Chef de Département / The Head of Department – FACULTE DES SCIENCES – Faculty of Sciences

Charles Félix Bilong Bilong
Professeur

# FORMAL LIST OF THE PERMANENT TEACHING STAFF OF THE FACULTY OF SCIENCE OF THE UNIVERSITY OF YAOUNDE I

| UNIVERSITY OF YAOUNDE I<br>FACULTY OF SCIENCE<br>Division of Programming and follow-up of Academic Affairs | | UNIVERSITE DE YAOUNDE I<br>FACULTE DES SCIENCES<br>Division de la Programmation et du Suivi des Activités Académiques |
|---|---|---|
| **LIST OF PERMANENT TEACHING STAFF** | | **LISTE DES ENSEIGNANTS PERMANENTS** |

**ACADEMIC YEAR 2019/2020**

(Per Department and per rank)

**UPDATING DATE: February 19th 2019**

## ADMINISTRATION OF THE FACULTY OF SCIENCE

**DEAN:** TCHOUANKEU Jean- Claude, *Associate Professor*
**VICE-DEAN / DPSAA:** ATCHADE Alex de Théodore, *Associate Professor*
**VICE-DEAN / DSSE:** AJEAGAH Gideon AGHAINDUM, *Professor*
**VICE-DEAN/ DRC:** ABOSSOLO Monique, *Associate Professor*
**Head of service Academic Affairs Division, Education and Research:** MBAZE MEVA'A Luc Léonard, *Professor*
**Head of Administrative and Financial Division:** NDOYE FOE Marie C.F., *Associate Professor*

| **1- DEPARTMENT OF BIOCHEMISTRY (BC) (37)** | | | |
|---|---|---|---|
| **N°** | **NAMES AND SURNAMES** | **RANK** | **OBSERVATIONS** |
| 1 | BIGOGA DIAGA Jude | Professor | On duty |
| 2 | FEKAM BOYOM Fabrice | Professor | On duty |
| 3 | FOKOU Elie | Professor | On duty |
| 4 | KANSCI Germain | Professor | On duty |
| 5 | MBACHAM Wilfred | Professor | On duty |
| 6 | MOUNDIPA FEWOU Paul | Professor | **Head of Department** |
| 7 | NINTCHOM PENLAP V. spouse BENG | Professor | On duty |
| 8 | OBEN Julius ENYONG | Professor | On duty |
| 9 | ACHU Merci BIH | Associate Professor | On duty |
| 10 | ATOGHO Barbara Mma | Associate Professor | On duty |
| 11 | AZANTSA KINGUE Gabin Boris | Associate Professor | On duty |
| 12 | BELINGA Born NDOYE FOE M. C. F. | Associate Professor | *Head DFA / FS* |

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|---|---|---|---|
| 13 | BOUDJEKO Thaddée | Associate Professor | On duty |
| 14 | DJUIDJE NGOUNOUE Marcelline | Associate Professor | On duty |
| 15 | EFFA NNOMO Pierre | Associate Professor | On duty |
| 16 | NANA Louise spouse WAKAM | Associate Professor | On duty |
| 17 | NGONDI Judith Laure | Associate Professor | On duty |
| 18 | NGUEFACK Julienne | Associate Professor | On duty |
| 19 | NJAYOU Frédéric Nico | Associate Professor | On duty |
| 20 | MOFOR Born TEUGWA Clotilde | Associate Professor | *Service inspector/MINESUP* |
| 21 | TCHANA KOUATCHOUA Angèle | Associate Professor | On duty |
| 22 | AKINDEH MBUH NJI | Lecturer | On duty |
| 23 | BEBEE FADIMATOU | Lecturer | On duty |
| 24 | BEBOY EDJENGUELE Sara Nathalie | Lecturer | On duty |
| 25 | DAKOLE DABOY Charles | Lecturer | On duty |
| 26 | DJOKAM TAMO Rosine | Lecturer | On duty |
| 27 | DJUIKWO NKONGA Ruth Viviane | Lecturer | On duty |
| 28 | DONGMO LEKAGNE Joseph Blaise | Lecturer | On duty |
| 29 | EWANE Cécile Anne | Lecturer | On duty |
| 30 | FONKOUA Martin | Lecturer | On duty |
| 31 | KOTUE KAPTUE Charles | Lecturer | On duty |
| 32 | LUNGA Paul KEILAH | Lecturer | On duty |
| 33 | MANANGA Marlyse Joséphine | Lecturer | On duty |
| 34 | MBONG ANGIE MOUGANDE M. A. | Lecturer | On duty |
| 35 | PECHANGOU NSANGOU Sylvain | Lecturer | On duty |
| 36 | Palmer MASUMBE NETONGO | Lecturer | On duty |
| 37 | MBOUCHE FANMOE Marcelline Joelle | Assistant Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **2- DEPARTMENT OF ANIMAL BIOLOGY AND PHYSIOLOGY (B.P.A) (43)** | | | |
| 1 | AJEAGAH Gidéon AGHAINDUM | Professor | *VDean / DSSE* |
| 2 | BILONG BILONG Charles Félix | Professor | **Head of Department** |
| 3 | DIMO Théophile | Professor | On duty |
| 4 | DJIETO Lordon Champlain | Professor | On duty |
| 5 | ESSOMBA born NTSAMA MBALA | Professor | *VDean/FMSB/UYI* |
| 6 | FOMENA Abraham | Professor | On duty |
| 7 | KAMTCHOUING Pierre | Professor | On duty |
| 8 | NJAMEN Dieudonné | Professor | On duty |
| 9 | NJIOKOU Flobert | Professor | On duty |
| 10 | NOLA Moïse | Professor | On duty |
| 11 | TAN Paul VERNYUY | Professor | On duty |
| 12 | TCHUEM TCHUENTE Louis | Professor | *Coord. Progr. MINSANTE* |
| 13 | ZEBAZE TOGOUET Serge Hubert | Professor | On duty |
| 14 | BILANDA Danielle Claude | Associate Professor | On duty |
| 15 | DJIOGUE Séfirin | Associate Professor | On duty |
| 16 | DZEUFIET DJOMENI Paul Désiré | Associate Professor | On duty |
| 17 | JATSA H. spouse MEGAPTCHE | Associate Professor | On duty |

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|---|---|---|---|
| 18 | KEKEUNOU Sévilor | Associate Professor | On duty |
| 19 | MEGNEKOU Rosette | Associate Professor | On duty |
| 20 | MONY Ruth spouse NTONE | Associate Professor | On duty |
| 21 | NGUEGUIM TSOFACK Florence | Associate Professor | On duty |
| 22 | TOMBI Jeannette | Associate Professor | On duty |
| 23 | ALENE Désirée Chantal | Lecturer | On duty |
| 24 | ATSAMO Albert Donatien | Lecturer | On duty |
| 25 | BELLET EDIMO Oscar Roger | Lecturer | On duty |
| 26 | DONFACK Mireille | Lecturer | On duty |
| 27 | ETEME ENAMA Serge | Lecturer | On duty |
| 28 | GOUNOUE KAMKUMO Raceline | Lecturer | On duty |
| 29 | KANDEDA KAVAYE Antoine | Lecturer | On duty |
| 30 | LEKEUFACK FOLEFACK Guy Benoît | Lecturer | On duty |
| 31 | MAHOB Raymond Joseph | Lecturer | On duty |
| 32 | MBENOUN MASSE Paul Serge | Lecturer | On duty |
| 33 | MOUNGANG NGAMENI Luciane | Lecturer | On duty |
| 34 | MVEYO NDANKEU Yves Patrick | Lecturer | On duty |
| 35 | NGOUATEU KENFACK Omer BEBE | Lecturer | On duty |
| 36 | NGUEMBOCK | Lecturer | On duty |
| 37 | NJUA Clarisse Yafi | Lecturer | **CD/UBa** |
| 38 | NOAH EWOTI Olive Vivien | Lecturer | On duty |
| 39 | TADU Zéphirin | Lecturer | On duty |
| 40 | YEDE | Lecturer | On duty |
| 41 | KOGA MANG Dobara | Assistant Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **3- DEPARTMENT OF PLANT BIOLOGY AND PHYSIOLOGY (B. P. V) (25)** | | | |
| 1 | AMBANG Zachée | Professor | *CD / UYII* |
| 2 | BELL Joseph Martin | Professor | On duty |
| 3 | DJOCGOUE Pierre François | Professor | On duty |
| 4 | MOSSEBO Dominique Claude | Professor | On duty |
| 5 | YOUMBI Emmanuel | Professor | **Head of Department** |
| 6 | ZAPFACK Louis | Professor | On duty |
| 7 | ANGONI Hyacinthe | Associate Professor | On duty |
| 8 | BIYE Elvire Hortense | Associate Professor | On duty |
| 9 | KENGNE NOUMSI Ives Magloire | Associate Professor | On duty |
| 10 | MALA Armand William | Associate Professor | On duty |
| 11 | MBARGA BINDZI Marie Alain. | Associate Professor | *CT/MINESUP* |
| 12 | MBOLO Marie. | Associate Professor | On duty |
| 13 | NDONGO BEKOLO | Associate Professor | *CE / MINRESI* |
| 14 | NGONKEU MAGAPTCHE E. L. | Associate Professor | On duty |
| 15 | TSOATA Esaïe | Associate Professor | On duty |
| 16 | TONFACK Libert Brice | Associate Professor | On duty |

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|---|---|---|---|
| 17 | DJEUANI Astride Carole | Lecturer | On duty |
| 18 | GONMADGE Christelle | Lecturer | On duty |
| 19 | MAFFO MAFFO Nicole Liliane | Lecturer | On duty |
| 20 | MAHBOU SOMO TOUKAM Gabriel | Lecturer | On duty |
| 21 | NGALLE Hermine BILLE | Lecturer | On duty |
| 22 | NGOUO Lucas Vincent | Lecturer | On duty |
| 23 | NNANGA MEBENGA Ruth Laure | Lecturer | On duty |
| 24 | NOUKEU KOUAKAM Armelle | Lecturer | On duty |
| 25 | ONANA Jean Michel | Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **3- DEPARTMENT OF INORGANIC CHEMISTRY (C.I.) (32)** | | | |
| 1 | AGWARA ONDOH Moïse | Professor | *Vice-Rector Univ of Bamenda* |
| 2 | ELIMBI Antoine | Professor | On duty |
| 3 | Florence UFI CHINJE spouse MELO | Professor | *Rector, Univ.Ngaoundere* |
| 4 | GHOGOMU Paul MINGO | Professor | *Minister in charge of Miss.PR* |
| 5 | NANSEU NJIKI Charles Péguy | Professor | On duty |
| 6 | NDIFON Peter TEKE | Professor | *CT MINRESI/* **Head of Department** |
| 7 | NDIKONTAR Maurice KOR | Professor | *Vice Dean / Univ.Bamenda* |
| 8 | NGOMO Horace MANGA | Professor | *VC/UB* |
| 9 | NENWA Justin | Professor | On duty |
| 10 | NGAMENI Emmanuel | Professor | *Dean FS/Univ Dschang* |
| 11 | BABALE born DJAM DOUDOU | Associate Professor | *Charge Mission P.R.* |
| 12 | DJOUFAC WOUMFO Emmanuel | Associate Professor | On duty |
| 13 | KAMGANG YOUBI Georges | Associate Professor | On duty |
| 14 | KEMMEGNE MBOUGUEM Jean Claude | Associate Professor | On duty |
| 15 | KONG SAKEO | Associate Professor | *Charge Mission P.R.* |
| 16 | NDI NSAMI Julius | Associate Professor | On duty |
| 17 | NJIOMOU C. spouse DJANGANG | Associate Professor | On duty |
| 18 | NJOYA Dayirou | Associate Professor | On duty |
| 19 | YOUNANG Elie | Associate Professor | On duty |
| 20 | ACAYANKA Elie | Lecturer | On duty |
| 21 | BELIBI BELIBI Placide Désiré | Lecturer | CS/ENS Bertoua |
| 22 | CHEUMANI YONA Arnaud M. | Lecturer | On duty |
| 23 | EMADACK Alphonse | Lecturer | On duty |
| 24 | KENNE DEDZO GUSTAVE | Lecturer | On duty |
| 25 | KOUOTOU DAOUDA | Lecturer | On duty |
| 26 | MAKON Thomas Beauregard | Lecturer | On duty |
| 27 | MBEY Jean Aime | Lecturer | On duty |
| 28 | NCHIMI NONO KATIA | Lecturer | On duty |
| 29 | NEBA born NDOSIRI Bridget NDOYE | Lecturer | CT/MINFEM |

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|---|---|---|---|
| 30 | NYAMEN Linda Dyorisse | Lecturer | On duty |
| 31 | PABOUDAM GBAMBIE A. | Lecturer | On duty |
| 32 | TCHAKOUTE KOUAMO Hervé | Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **5- DEPARTMENT OF ORGANIC CHEMISTRY (C.O.) (32)** | | | |
| 1 | DONGO Etienne | Professor | On duty |
| 2 | GHOGOMU TIH Robert Ralph | Professor | *Dir. IBAF/UDA* |
| 3 | NGOUELA Silvère Augustin | Professor | On duty |
| 4 | NKENGFACK Augustin Ephraïm | Professor | **Head of Department** |
| 5 | NYASSE Barthélemy | Professor | On duty |
| 6 | PEGNYEMB Dieudonné Emmanuel | Professor | *Director/ MINESUP* |
| 7 | WANDJI Jean | Professor | On duty |
| 8 | Alex de Théodore ATCHADE | Associate Professor | *VICE-DEAN / DPSAA* |
| 9 | EYONG Kenneth OBEN | Associate Professor | *Chef Service DPER* |
| 10 | FOLEFOC Gabriel NGOSONG | Associate Professor | On duty |
| 11 | FOTSO WABO Ghislain | Associate Professor | On duty |
| 12 | KEUMEDJIO Félix | Associate Professor | On duty |
| 14 | KOUAM Jacques | Associate Professor | On duty |
| 15 | MBAZOA born DJAMA Céline | Associate Professor | On duty |
| 16 | MKOUNGA Pierre | Associate Professor | On duty |
| 17 | NOTE LOUGBOT Olivier Placide | Associate Professor | *Chef Service/MINESUP* |
| 18 | NGO MBING Joséphine | Associate Professor | *Sous/Direct. MINERESI* |
| 19 | NGONO BIKOBO Dominique Serge | Associate Professor | On duty |
| 20 | NOUNGOUE TCHAMO Diderot | Associate Professor | On duty |
| 21 | TABOPDA KUATE Turibio | Associate Professor | On duty |
| 22 | TCHOUANKEU Jean-Claude | Associate Professor | *Dean/FS/UYI* |
| 23 | TIH born NGO BILONG E. Anastasie | Associate Professor | On duty |
| 24 | YANKEP Emmanuel | Associate Professor | On duty |
| 18 | AMBASSA Pantaléon | Lecturer | On duty |
| 19 | KAMTO Eutrophe Le Doux | Lecturer | On duty |
| 20 | MVOT AKAK Carine | Lecturer | On duty |
| 21 | NGNINTEDO Dominique | Lecturer | On duty |
| 22 | NGOMO Orléans | Lecturer | On duty |
| 28 | OUAHOUO WACHE Blandine M. | Lecturer | On duty |
| 29 | TAGATSING FOTSING Maurice | Lecturer | On duty |
| 30 | ZONDENDEGOUMBA Ernestine | Lecturer | On duty |

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| + Teachers being recruted | | | |
| **6- DEPARTMENT OF COMPUTER (IN) (25)** | | | |
| 1 | ATSA ETOUNDI Roger | Professor | *Chef Div /MINESUP* |
| 2 | FOUDA NDJODO Marcel Laurent | Professor | *Chef Dpt ENS/Chef IGA MINESUP* |
| 3 | NDOUNDAM Réné | Associate Professor | On duty |
| 4 | ABESSOLO ALO'O Gislain | Lecturer | On duty |
| 5 | AMINATOU Halidou | Lecturer | On duty |
| 6 | DJAM Xaviera YOUH-KIMBI | Lecturer | On duty |
| 7 | EBELE Serge Alain | Lecturer | On duty |
| 8 | KAMGUEU Patrick Olivier | Lecturer | On duty |
| 9 | KOUOKAM KOUOKAM E. A. | Lecturer | On duty |
| 10 | MELATAGIA YONTA Paulin | Lecturer | On duty |
| 11 | MONTHE DJIADEU Valery M. | Lecturer | On duty |
| 12 | MOTO MPONG Serge Alain | Lecturer | On duty |
| 13 | OLLE OLLE Daniel Claude Delort | Lecturer | C/D Enset. Ebolowa |
| 14 | TAPAMO Hyppolite | Lecturer | On duty |
| 15 | TINDO Gilbert | Lecturer | On duty |
| 16 | TSOPZE Norbert | Lecturer | On duty |
| 17 | WAKU KOUAMOU Jules | Lecturer | On duty |
| 18 | BAYEM Jacques Narcisse | Assistant Lecturer | On duty |
| 19 | DOMGA KOMGUEM Rodrigue | Assistant Lecturer | On duty |
| 20 | HAMZA Adamou | Assistant Lecturer | On duty |
| 21 | JIOMEKONG AZANZI Fidel | Assistant Lecturer | On duty |
| 22 | KAMDEM KENGNE Christiane | Assistant Lecturer | On duty |
| 23 | MAKEMBE S. Oswald | Assistant Lecturer | On duty |
| 24 | MEYEMDOU Nadège Sylvianne | Assistant Lecturer | On duty |
| 25 | NKONDOCK MI. BAHANACK N. | Assistant Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **7- DEPARTMENT DE MATHEMATICS (MA) (26)** | | | |
| 1 | EMVUDU WONO Yves S. | Professor | *CD Info/Head of unit MINESUP* |
| 2 | AYISSI Raoult Domingo | Associate Professor | **Head of Department** |
| 3 | NKUIMI JUGNIA Célestin | Associate Professor | On duty |
| 4 | NOUNDJEU Pierre | Associate Professor | On duty |
| 5 | MBEHOU Mohamed | Associate Professor | On duty |

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| 6 | TCHAPNDA NJABO Sophonie B. | Associate Professor | *Director/AIMS Rwanda* |
| 7 | AGHOUKENG JIOFACK Jean Gérard | Lecturer | *Head of unit MINPLAMAT* |
| 8 | DJIADEU NGAHA Michel | Lecturer | On duty |
| 9 | DOUANLA YONTA Christophe | Lecturer | On duty |
| 10 | FOMEKONG Christophe | Lecturer | On duty |
| 11 | KIANPI Maurice | Lecturer | On duty |
| 12 | KIKI Maxime Armand | Lecturer | On duty |
| 13 | MBAKOP Guy Merlin | Lecturer | On duty |
| 14 | MBANG Joseph | Lecturer | On duty |
| 15 | MBELE BIDIMA Martin Ledoux | Lecturer | On duty |
| 16 | MENGUE MENGUE David Joe | Lecturer | On duty |
| 17 | NGUEFACK Bernard | Lecturer | On duty |
| 18 | NIMPA PEFOUNKEU Romain | Lecturer | On duty |
| 19 | POLA DOUNDOU Emmanuel | Lecturer | On duty |
| 20 | TAKAM SOH Patrice | Lecturer | On duty |
| 21 | TCHANGANG Roger Duclos | Lecturer | On duty |
| 22 | TCHOUNDJA Edgar Landry | Lecturer | On duty |
| 23 | TETSADJIO TCHILEPECK M. E. | Lecturer | On duty |
| 24 | TIAYA TSAGUE N. Anne-Marie | Lecturer | On duty |
| 25 | MBIAKOP Hilaire George | Assistant Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **8- DEPARTMENT OF MICROBIOLOGY (MB) (12)** | | | |
| 1 | ESSIA NGANG Jean Justin | Professor | *DRV/ IMPM* |
| 2 | BOYOMO ONANA | Associate Professor | On duty |
| 3 | NWAGA Dieudonné M. | Associate Professor | On duty |
| 4 | NYEGUE Maximilienne Ascension | Associate Professor | On duty |
| 5 | RIWOM Sara Honorine | Associate Professor | On duty |
| 6 | SADO KAMDEM Sylvain Leroy | Associate Professor | On duty |
| 7 | ASSAM ASSAM Jean Paul | Lecturer | On duty |
| 8 | BODA Maurice | Lecturer | On duty |
| 9 | BOUGNOM Blaise Pascal | Lecturer | On duty |
| 10 | ESSONO OBOUGOU Germain G. | Lecturer | On duty |
| 11 | NJIKI BIKOÏ Jacky | Lecturer | On duty |
| 12 | TCHIKOUA Roger | Lecturer | On duty |
| +Teachers being recruited | | | |

| 9-DEPARTMENT OF PHYSICS (PH) (40) | | | |
|---|---|---|---|
| 1 | BEN- BOLIE Germain Hubert | Professor | On duty |
| 2 | ESSIMBI ZOBO Bernard | Professor | On duty |
| 3 | KOFANE Timoléon Crépin | Professor | On duty |
| 4 | NANA ENGP Serge Guy | Professor | On duty |
| 5 | NDJAKA Jean Marie Bienvenu | Professor | ***Head of Department*** |
| 6 | NOUAYOU Robert | Professor | On duty |
| 7 | NJANDJOCK NOUCK Philippe | Professor | *SD/ MINRESI* |
| 8 | PEMHA Elkana | Professor | On duty |
| 9 | TABOD Charles TABOD | Professor | *Dean UBda* |
| 10 | TCHAWOUA Clément | Professor | On duty |
| 11 | WOAFO Paul | Professor | On duty |
| 12 | BIYA MOTTO Frédéric | Associate Professor | *DG/HYDRO Mekin* |
| 13 | BODO Bertrand | Associate Professor | On duty |
| 14 | DJUIDJE KENMOE spouse ALOYEM | Associate Professor | On duty |
| 15 | EKOBENA FOUDA Henri Paul | Associate Professor | *CD/ UN* |
| 16 | EYEBE FOUDA Jean sire | Associate Professor | On duty |
| 17 | FEWO Serge Ibraïd | Associate Professor | On duty |
| 18 | HONA Jacques | Associate Professor | On duty |
| 19 | MBANE BIOUELE César | Associate Professor | On duty |
| 20 | NANA NBENDJO Blaise | Associate Professor | On duty |
| 21 | NDOP Joseph | Associate Professor | On duty |
| 22 | SAIDOU | Associate Professor | *MINERESI* |
| 23 | SIMO Elie | Associate Professor | On duty |
| 24 | VONDOU Derbetini Appolinaire | Associate Professor | |
| 25 | WAKATA born BEYA Annie | Associate Professor | *SD/ MINESUP* |
| 26 | ZEKENG Serge Sylvain | Associate Professor | On duty |
| 27 | ABDOURAHIMI | Lecturer | On duty |
| 28 | EDONGUE HERVAIS | Lecturer | On duty |
| 29 | ENYEGUE A NYAM spouse BELINGA | Lecturer | On duty |
| 30 | FOUEDJIO David | Lecturer | *Head of unit MINADER* |
| 31 | MBINACK Clément | Lecturer | On duty |
| 32 | MBONO SAMBA Yves Christian U. | Lecturer | On duty |
| 33 | MELI'I JOELLE LARISSA | Lecturer | On duty |
| 34 | MVOGO ALAIN | Lecturer | On duty |

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| 35 | OBOUNOU Marcel | Lecturer | DA/Univ Inter Etat/Sangmelima |
| 36 | WOULACHE Rosalie Laure | Lecturer | On duty |
| 37 | CHAMANI Roméo | Assistant Lecturer | On duty |
| + Teachers under recruitment process | | | |
| **10- DEPARTMENT OF EARTH SCIENCES (S.T.) (42)** | | | |
| 1 | BITOM Dieudonné | Professor | *Dean/FASA/UDs* |
| 2 | FOUATEU Rose epse YONGUE | Professor | On duty |
| 3 | KAMGANG Pierre | Professor | On duty |
| 4 | NDJIGUI Paul Désiré | Professor | **Head of department** |
| 5 | NDAM NGOUPAYOU Jules-Remy | Professor | On duty |
| 6 | NGOS III Simon | Professor | *DAAC/Uma* |
| 7 | NKOUMBOU Charles | Professor | On duty |
| 8 | NZENTI Jean-Paul | Professor | On duty |
| 9 | ABOSSOLO born ANGUE Monique | Associate Professor | *Vice-Dean/DRC* |
| 10 | GHOGOMU Richard TANWI | Associate Professor | CD/Uma |
| 11 | MOUNDI Amidou | Associate Professor | *Head of unit/MINIMDT* |
| 12 | NGUEUTCHOUA Gabriel | Associate Professor | *CEA/ MINRESI* |
| 13 | NJILAH Isaac KONFOR | Associate Professor | On duty |
| 14 | ONANA Vincent | Associate Professor | On duty |
| 15 | BISSO Dieudonne | Associate Professor | *Project Director/ Memve'ele barrage* |
| 16 | EKOMANE Emile | Associate Professor | On duty |
| 17 | GANNO Sylvestre | Associate Professor | On duty |
| 18 | NYECK Bruno | Associate Professor | On duty |
| 19 | TCHOUANKOUE Jean-Pierre | Associate Professor | On duty |
| 20 | TEMDJIM Robert | Associate Professor | On duty |
| 21 | YENE ATANGANA Joseph Q. | Associate Professor | *Head of unit/MINTP* |
| 22 | ZO'O ZAME Philémon | Associate Professor | *DG/ART* |
| 23 | ANABA ONANA Achille Basile | Lecturer | On duty |
| 24 | BEKOA Etienne | Lecturer | On duty |
| 25 | ELISE SABABA | Lecturer | On duty |
| 26 | ESSONO Jean | Lecturer | On duty |
| 27 | EYONG JOHN TAKEM | Lecturer | On duty |
| 28 | FUH Calistus Gentry | Lecturer | *Sec. D'Etat/MINIMIDT* |
| 29 | LAMILEN BILLA Daniel | Lecturer | On duty |
| 30 | MBESSE CECILE OLIVE | Lecturer | On duty |

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| 31 | MBIDA YEM | Lecturer | On duty |
| 32 | MBESSE CECILE OLIVE | Lecturer | On duty |
| 33 | MINYEM Dieudonné-Lucien | Lecturer | On duty |
| 34 | NGO BELNOUN Rose Noël | Lecturer | On duty |
| 35 | NGO BIDJECK Louise Marie | Lecturer | On duty |
| 36 | NOMO NEGUE Emmanuel | Lecturer | On duty |
| 37 | NTSAMA ATANGANA Jacqueline | Lecturer | On duty |
| 38 | TCHAKOUNTE J. Spouse NOUMBEM | Lecturer | *Chef cell/MINRESI* |
| 39 | TCHAPTCHET TCHATO De P. | Lecturer | On duty |
| 40 | TEHNA Nathanaël | Lecturer | On duty |
| 41 | TEMGA Jean Pierre | Lecturer | On duty |

**Distribution of permanent teachers per department and per rank (January 15th 2020)**

| | NUMBER OF TEACHERS | | | | |
|---|---|---|---|---|---|
| **Department** | **Professors** | **Associate Professors** | **Lecturers** | **Assistant lecturers** | **Total** |
| B.C. | 8 (1) | 13 (9) | 15 (7) | 1 (1) | **37 (18)** |
| B.P.A. | 13 (1) | 10 (4) | 19 (6) | 1 (0) | **43 (11)** |
| B.P.V. | 6 (0) | 10 (2) | 9 (6) | 0 | **25 (8)** |
| C.I. | 10(1) | 9(2) | 13 (2) | 0 | **32 (5)** |
| C.O. | 7 (0) | 16 (3) | 9 (3) | 0 | **32 (6)** |
| I.N. | 2 (0) | 1 (0) | 14 (0) | 8 (2) | **25 (2)** |
| M.A. | 1(0) | 5 (0) | 18 (1) | 1 (0) | **26 (1)** |
| M.B. | 1 (0) | 5 (2) | 6 (1) | 0 | **12 (3)** |
| P.H. | 11 (0) | 15 (2) | 13 (3) | 1(0) | **40 (5)** |
| S.T. | 8 (1) | 14 (1) | 20 (6) | 0 | **42 (8)** |
| **Total** | **67 (4)** | **98 (25)** | **136 (35)** | **12 (3)** | **314 (67)** |

**Giving a total of:** **314** (67), ie

- Professors : **67** (4)
- Associate professors : **98** (25)
- Lecturers : **136** (35)
- Assistant lecturers : **12** (3**)**

( ) = Number of women

**The Dean of the Faculty of Science**

# DEDICATION

I am dedicating this thesis to my daughter DJAMBANE ENYEGUE Léa Mélina.

## ACKNOWLEDGMENTS

At the end of this thesis, I would like to thank all the people without whom this project would never has been possible. I wish to express my sincere gratitude first and foremost to **All participants** who consented to participate in this study.

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- **Professor Claudine Mireille NTSAMA MBALA épouse ESSOMBA**

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A special acknowledgment goes to the members of jury for accepting to evaluate and edit this work.

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# LIST OF ABBREVIATIONS

**AIDS:** Acquired Immune Deficiency Syndrome

**ADP:** Adenosine DiPhosphate

**AMP:** Adenosine MonoPhosphate

**APC:** Antigen Presenting Cell

**APOBEC:** Apolipoprotein B mRNA-editing Enzyme, Catalytic polypeptide-like

**ART:** AntiRetroviral Therapy

**ATP:** Adenosine TriPhosphate

**Bcl:** B-cell lymphoma protein

**BSA:** Bovine Serum Albumin

**CCR5:** C-C chemokine Receptor type 5

**CCL:** Chemokine Ligand

**CD:** Cluster of Differentiation

**C/EBP-β:** CCAAT/Enhancer Binding Protein Beta

**CNLS :** Comité National de Lutte contre le SIDA

**CRF**: Circulating Recombinant Rorm

**CTIP 2:** Chicken ovalbumin upstream promoter Transcription factor-Interacting Protein 2

**CTL:** Cytotoxic T Lymphocyte

**CTLA-4:** Cytotoxic T Lymphocyte-associated Antigen 4

**CXCL:** CXC chemokine Ligand

**CXCR4:** Alpha-Chemokine Receptor type 4

**DCs:** Dendritic Cells

**DMSO:** Dimethyl Sulfoxide

**DNA:** DeoxyriboNucleic Acid

**dNTPs:** deoxyriboNucleotide TriPhosphates

**EDTA:** Ethylene-Diamine-Tetra-acetic Acid

**Env:** Envelope

**Fas:** First apoptosis signal

**FasL:** First apoptosis signal Ligand

**FBS:** Fetal Bovine Serum

**Foxp3:** Forkhead-box-P3

**FSC:** Forward Scatter

**Gag:** Group-specific antigen

**gp:** Glycoprotein

**HAART:** Highly Active Antiretroviral Therapy

**HIV:** Human Immunodeficiency Virus

**HLA:** Human Leukocyte Antigen

**HLA-DR**: Human Leukocyte Antigen-antigen D, isotype R

**ICAM:** IntraCellular Adhesion Molecule

**ICS**: Intracellular Cytokine Staining

**IDO:** Indoleamine 2,3-DiOxygenase

**IFN:** Interferon

**IL:** Interleukin

**IRF:** Interferon Regulatory Factor

**KIRs:** Killer-cell Immunoglobulin – like Receptors

**LAG:** Lymphocyte Activation Gene

**LFA-1**: Lymphocyte Function-associated Antigen-1

**LPS:** Lipopolysaccharide

**LTR:** Long -Terminal Repeat

**MFI:** Mean Fluorescence Intensity

**MHC:** Major Histocompatibility Complex

**MIP:** Macrophage Inflammatory Protein

**Nef:** Negative regulation factor

**NF-kB:** Nuclear Factor -kappa B

**NK:** Natural Killer

**PBMCs:** Peripheral Blood Mononuclear Cells

**PBS:** Phosphate Buffer Saline

**Pol:** Polymerase

**poly-ICLC:** Polyinosinic-polycytidylic stabilized with poly-lysine and carboxymethylcellulose

**PPAR-γ:** Peroxisome Proliferator-Activated Receptor gamma

**Rev:** Regulator of viral expression

**RNA:** Ribonucleic acid

**SamHD1:** Sterile alpha motif domain and HD domain containing protein 1

**SSC:** Side Scatter

**STAT:** Signal Transducer and Activator of Transcription

**Tat :** Transactivator

**T-bet :** T-box expressed in T cells

**T cell:** T lymphocyte

**TCR:** T-Cell Receptor

**TGF-β:** Transforming Growth Factor beta

**TLR:** Toll-Like Receptor

**TNF:** Tumor Necrosis Factor

**TRAIL:** Tumor necrosis factor-Related Apoptosis -Inducing Ligand

**TRAIL-R1:** Tumor necrosis factor-Related Apoptosis -Inducing Ligand-Receptor 1

**Treg:** Regulatory T cell

**TRIM22:** Tripartite motif containing protein 22

**TRIM5α :** Tripartite motif protein isoform 5 alpha

**URF:** Unique Recombinant Form

**UTR:** Untranslated Transcribed Region

**Vif:** Viral infectivity factor

**Vpr:** Viral protein R

**Vpu:** Viral protein U

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## ABSTRACT

Regulatory T (Treg) cells play a critical role in dampening excessive immune activation. However, in antiretroviral therapy (ART)-naive patients, HIV-1 infection maintains the immune system in a sustained state of activation that could alter both Treg cell surface markers and functions. The overall objective of this study was to assess the phenotypic and functional characteristics of Treg cells in ART naive HIV-1 infected adults. Treg cells were purified by magnetic sorting from Peripheral Blood Mononuclear Cells (PBMCs) obtained from 72 ART-naive HIV-1 infected and 38 HIV-negative participants. The phenotypic properties of the purified Treg cells were then determined by multiparametric flow cytometry. The correlations between Treg cell levels with either helper $CD4^+$ T cell count or plasmatic HIV-1 viral load were made with Spearman test. Treg cell functions were assessed by measuring the activation status and inflammatory cytokine formation by monocytes following co-culture with autologous Treg cells in the presence of Polyinosinic-polycytidylic stabilized with poly-lysine and carboxymethylcellulose (Poly-ICLC). Cytometric data were analyzed with FlowJo version 9.8.5. Graph Pad Prism version 5.0.was used for statistical analysis and P values less than 0.05 were considered to be statistically significant. Our data showed that in ART naive HIV-1 infected participants, Treg cells are dominated by effector and effector memory cells with. enhanced expression of CD39, CD73, HLA-DR and CD38 ($p<0.0001$). Overall Treg cell numbers decreased proportionally with helper $CD4^+$ T cells ($r = 0.4$, $P<0.0001$), whereas their frequencies within total $CD4^+$ T cells correlated positively with plasmatic HIV-1 viral load ($r= 0.5$; $P<0.0001$). When considering Treg cell subsets, effector and effector memory Treg cells were significantly depleted ($P<0.0001$) during the course of HIV-1 infection whereas naive and central memory Treg cells were not affected. With respect to Treg cell functions, we observed that Treg cells were able to down modulate autologous monocytes activation as well as IL-6 and TNF-α production after stimulation with poly-ICLC in immunocompetent participants. Compared to participants with CD4 count < 200 cells/$mm^3$, those with CD4 count ≥ 500 cells/$mm^3$ showed significantly higher levels of TGF-β and IL-10 ($p<0.001$ and $p<0.0001$, respectively), key cytokines used by Treg cells to exert their immunosuppressive functions. In conclusion, ART-naive HIV-1 infection negatively impacts Treg cell phenotypes, numbers and functions. Thus, immunotherapeutic strategies geared toward restoring Treg cell phenotypes, frequencies and function could be beneficial in the long-term management of HIV-1 infection.



**Keywords**: antiretroviral therapy-naive, HIV-1 infection, regulatory T cells, phenotype, inflammation.

# RESUME

Les cellules T régulatrices (Treg) jouent un rôle important dans le maintien de l'homéostasie immunitaire en supprimant l'excès d'activation des cellules effectrices. Cependant, au cours de l'infection chronique à VIH, le système immunitaire est maintenu dans un état d'activation permanent qui pourrait altérer le phénotype et la fonction des cellules Treg. L'objectif général de cette étude était de décrire le phénotype et la fonction des cellules Treg chez des personnes adultes infectées par le VIH-1, naïves au traitement antirétroviral. Pour cela, les cellules Treg ont été purifiées à partir des cellules mononucléées obtenues du sang périphérique des participants (72 VIH-$1^+$ et 38 $VIH^-$), grâce à un protocole optimisé de tri cellulaire magnétique. Les propriétés phénotypiques des cellules Treg purifiées ont été déterminées au moyen de la cytométrie en flux multiparamétrique. Les corrélations entre les nombres/pourcentages des cellules Treg et le nombre de lymphocytes T CD4 d'une part et la charge virale d'autre part ont été établies à l'aide du test de Spearman. L'activité suppressive des cellules Treg a été évaluée à la suite de leur mise en culture avec les monocytes autologues en présence du Poly-ICLC. Les niveaux d'activation et de production des cytokines pro-inflammatoires par les monocytes ont ainsi été mesurés. Les données cytométriques ont été analysées avec la version 9.8.5 de FlowJo. Le logiciel Graph Pad Prism version 5.0. a été utilisé pour l'analyse statistique et les valeurs de $P < 0$, indiquaient un résultat statistiquement significatif. Nos travaux ont montré que la population des cellules Treg est dominée par les cellules effectrices et effectrices mémoires chez les personnes infectées par le VIH-1. Par ailleurs, ces phénotypes expriment fortement les marqueurs CD39, CD73, HLA-DR et CD38 ($P<0,0001$). Une corrélation positive a été observée entre le nombre total des cellules Treg et celui des lymphocytes T CD4 ($r = 0,4$, $P <0.0001$) puis, entre le pourcentage des cellules Treg et la charge virale ($r= 0,5$ ; $P<0,0001$). Le nombre de cellules naïves et centrales mémoires n'est pas affecté au cours de l'infection. En revanche, le nombre de cellules effectrices et effectrices mémoires diminue significativement ($P<0,0001$). Sur le plan fonctionnel, les cellules Treg se sont révélées capables de réguler l'activation et la production de l'IL-6 et du TNF-α par les monocytes autologues stimulés chez les participants immunocompétents. Comparativement aux participants avec des CD4 < 200 cellules/ $mm^3$, les cellules Treg de ceux avec des CD4 ≥ 500 cellules/ $mm^3$ ont exprimé de grandes quantités de TGF-β ($P<0,001$) et d'IL-10 ($P<0,0001$), deux cytokines clés dans l'activité suppressive exercée par les cellules Treg. En conclusion, l'infection à VIH-1 a une incidence négative sur le phénotype, les quantités et la fonction des cellules Treg. Par conséquent, le développement des stratégies immuno-thérapeutiques qui visent à restaurer ces propriétés des cellules Treg pourrait être bénéfique pour la prise en charge de l'infection à VIH-1 à long terme.



**Mots clés** : Infection à VIH-1 naïve au traitement antirétroviral, cellules T régulatrices, phénotype, inflammation.

# INTRODUCTION

Thirty six years after the discovery of the Human Immunodeficiency Virus (HIV), the etiologic agent of the Acquired Immune Deficiency Syndrome (Barre-Sinoussi *et al.*, 1983), the global epidemic continues to expand. By the end of 2017, there were an estimated 36.9 [31.1–43.9] million worldwide people living with HIV, with 1.8 [1.4 –2.4] million new infections and 940,000 [670,000–1.3 million] deaths. Sub-Saharan Africa remains the most affected region with an estimated 25.7 million (70%) HIV cases among which about 6.1 [4.4 –8.1] million live in Western and central Africa (UNAIDS, 2018). Cameroon is the only country in Central Africa, where almost all HIV-1 major groups and subtypes co-circulate, with approximately 500,000 people living with HIV (CAMPHIA, 2018). Although the current therapeutic strategy against HIV-1 allows viral replication to be controlled (Volberding & Deeks, 2010, Deeks *et al.*, 2013), the disease remains incurable. This is due to an increased mutation rate of HIV-1 (Deforche *et al.*, 2006, Konings *et al.*, 2006b, McCutchan, 2006) and the inability of antiretroviral drugs to access latent reservoirs characterized by infected long-lived memory $CD4^+$ T lymphocytes and macrophages (Simon *et al.*, 2006); the latter being responsible for the persistent immune activation and inflammation observed during the course of HIV-1 infection (Tabb *et al.*, 2013). On the other hand, antiretroviral drugs have several long-term side effects, all mainly related to cellular mitochondrial disturbances including kidney, liver and pancreatic diseases. Changes in fat metabolism resulting in elevated cholesterol and triglyceride levels as well as an increased risk for heart attacks were also reported (Volberding & Deeks, 2010, Deeks *et al.*, 2013). Thus, understanding the mechanisms by which HIV-1 infected people can spontaneously control viral replication in the absence of antiretroviral therapy is of paramount importance.

HIV-1 infection is hallmarked by a chronic activation of the immune system, which is considered as the driving force of $CD4^+$ T-cell depletion, increase rate of viral replication and AIDS (Tabb *et al.*, 2013). An increase suppression of $CD4^+CCR5^+$ T-cells, primarily in mucosal tissue is invariably associated with a loss of the integrity of mucosal barrier leading to sustained exposure to gut microbial products like bacterial endotoxin lipopolysaccharide (LPS), a strong stimulator of the immune system (Mogensen *et al.*, 2010). In response to LPS, monocytes produce cuprous amounts of pro-inflammatory cytokines including interleukin (IL)-1 β, IL-6, tumor necrosis factor-alpha (TNF-α) and type 1 interferons (Deeks *et al.*, 2013, Doitsh & Greene, 2016). In addition, a wide range of chemokines, such as CCL2, CCL3, CCL4 are also produced by

activated monocytes and macrophages further facilitating the recruitment of additional leukocytes to the inflammation site (Sassé *et al.*, 2012, Roberts *et al.*, 2015). This as a consequence escalates inflammation, activating naive and central memory $CD4^+$ T cells creating new targets for HIV-1 infection (Tabb *et al.*, 2013).

An effective immune response against HIV must strike a balance between maximizing recognition and control of viral replication and minimizing collateral damage to tissues or immune dysfunctions. One mechanism by which the immune system finds this balance is through the action of regulatory T (Treg) cells.

Treg cells represent a subset of $CD4^+$ T cells characterized by the constitutive expression of IL-2 receptor alpha chain component CD25, (Sakaguchi, 2005, Zorn *et al.*, 2006, Duhen *et al.*, 2012), and the transcription factor forkhead box P3 (FoxP3), which acts as a master regulator for their development and suppressive function (Fontenot *et al.*, 2003, Sakaguchi, 2003, Chatila, 2005, Yang *et al.*, 2015). Unlike the majority of activated, and memory $CD4^+$ T cells that express high levels of the IL-7 receptor alpha chain CD127, the Treg cell population shows low or no expression of CD127 (Liu *et al.*, 2006, Presicce *et al.*, 2011, Yang *et al.*, 2015).

Treg cells are expected in steady states to dampen excessive immune activation, thereby ensuring homeostasis following immune activation(Keynan & Rubinstein, 2008). However, in the context of a challenging persistent infection such as HIV, Treg cell function *in vivo* is probably limited as a result of either direct infection of Treg cells by HIV (Pion *et al.*, 2013) or poor interaction of Treg cells with other immune cells like dendritic cells in the destroyed tissue micro-environment (O'Brien *et al.*, 2013). Nevertheless, previous studies have demonstrated the beneficial effect of Treg cells in reducing HIV-1-associated immune activation and inflammation (Weiss *et al.*, 2004, Velavan & Ojurongbe, 2011, Angin *et al.*, 2012). Treg cells have also been shown to suppress both HIV-1 specific T cell proliferation and cytokines production (Kinter *et al.*, 2007, Kared *et al.*, 2008, Macatangay & Rinaldo, 2010). This on the one hand can result to a reduction of the available target cells for HIV replication, thereby limiting disease progression (Moreno-Fernandez *et al.*, 2009, Owen *et al.*, 2010, Velavan & Ojurongbe, 2011). On the other hand, the suppression of critical virus-specific immune responses could be deleterious to the individual especially with respect to unchecked viral expansion and inflammation (Macatangay & Rinaldo, 2010, Brezar *et al.*, 2016). So far,

no studies have been able to establish definitely if Treg cells can hasten or delay HIV infection (Allan *et al.*, 2008).

Chronic HIV-1 infection exacerbates immune activation which could accelerate disease progression and death. However, Treg cells in dampening excessive activation could modulate the outcome of the interaction between the virus and the immune system. Our hypothesis is that such an interaction could drive changes in the phenotype and functions of Treg cells which can be exploited to harness immune functions in the long-term management of HIV-1 infection.

The overall objective of this study was to assess Treg cell phenotype and functions during ART- naïve HIV-1 infection in view of optimizing immunotherapeutic strategies for the long-term management of HIV/AIDS. Indeed, deeper knowledge of the immune system has in the past allowed the development of novel immunotherapeutic strategies including tumor therapy, allergies, vaccinations and organ transplantation.

Our specific objectives were:

1. to purify Treg cells from peripheral blood mononuclear cells (PBMCs) of ART-naive HIV-1 infected participants;
2. to describe the phenotypes of Treg cells in the context of ART-naive HIV-1 infection;
3. to assess the relationship between Treg cell levels and the virologic or immunologic parameters;
4. to define Treg cells cytokine profile in the context of ART-naive HIV-1 infection;
5. to assess the capacity of Treg cells to modulate autologous' monocytes activation as well as their production of pro-inflammatory cytokines during *in vitro* stimulation with poly-ICLC.

This document includes three parts presented in chapter form: the first focuses on the literature review, the second presents the methods and techniques used to reach our objectives, and the third is focused on the results obtained and the discussion of the relevance and implication of our results. The document is terminated with a conclusion.

# CHAPTER I. LITERATURE REVIEW

## I.1 VIRUS DEFINITION

Viruses are infectious agents that are obligate intracellular parasites because they must replicate inside a host cell, utilizing its macromolecular machinery and energy supplies for their replication process. To accomplish this, viruses use their genomes that may be encoded as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)(Gelderblom, 1996). Viral genomes represent essential elements of the phylogeny of life and have played important parts in evolution. They are able to produce progeny genomes, possess internal regulation, adapt and respond to changing environmental conditions, and maintain structural organization (Ludmir & Enquist, 2009). André Wolff (French Nobel Prize) is credited with giving in 1953 and refining in 1962 the definition of the virus. Among the viruses, HIV is gouped to the genus *Lentivirus* within the family of *Retroviridae,* subfamily *Orthoretrovirinae*. This virus uses a reverse transcriptase enzyme to transcribe its RNA into DNA which can then allow viral multiplication (German Advisory Committee Blood, 2016).

## I.2 HIV GENOME STRUCTURE

The HIV-1 RNA genome encodes three major genes including *Env* (envelope), *Gag* (group-specific antigen) and *Pol* (polymerase) (Simon *et al.*, 2006). *Env* codes for the glycoprotein gp160, the precursor of gp120 and gp41. The outer envelope glycoprotein gp120 interacts with the CD4 receptor, while the trans membrane glycoprotein gp41 fuses with the host cell membrane and helps the virus enter the cell (Kwong *et al.*, 1998). The major components coded by the *Gag* gene include P24 (capsid protein), P6 and P7 (nucleocapsid proteins) and P17 (matrix protein) as shown in Figure 1a & b. The nucleocapsid proteins protect the RNA from digestion by nucleases, while the matrix protein ensures the integrity of the virion particle. The important proteins coded by *Pol* gene are enzyme proteins P66 and P51 (Reverse Transcriptase), P32 (integrase) and P11 (Protease) that participate in the reverse transcription, integration and maturation of HIV, respectively (Nagata *et al.*, 2017).

In addition to the proteins derived from the *gag*, *pol* and *env*, there are six other proteins synthetised by HIV. Three of them, *Vif* (viral infectivity), *Vpr* (viral protein R) and *Nef* (negative regulation factor), are incorporated into the virus, while the others *Tat* (transactivator), *Rev* (regulator of viral expression) and *Vpu* (viral protein U) are not found in the mature virus. *Vif* overcomes inhibition by the host factor, apolipoprotein B

mRNA-editing enzyme, catalytic polypeptide-like (APOBEC)3G and promotes more stable reverse transcriptase complexes. *Vpr* promotes cell-cycle arrest and facilitates infection of macrophages. *Nef* promotes CD4 lymphocyte activation, blocks cell suicide, enhances infectivity and is associated with disease progression. *Tat* enhances viral RNA transcription, *Rev* regulates viral RNA nuclear export proteins and *Vpu* indirectly assists in assembly and enhances release of virus from cell membrane (Odigie & Bhattacharya, 2009). At each end of the genome are long-terminal repeat (LTR) sequences that contain promoters, enhancers, and other gene sequences required for transcription of the viral genome and for proviral integration into host cell chromosomes (Fig. 1b).

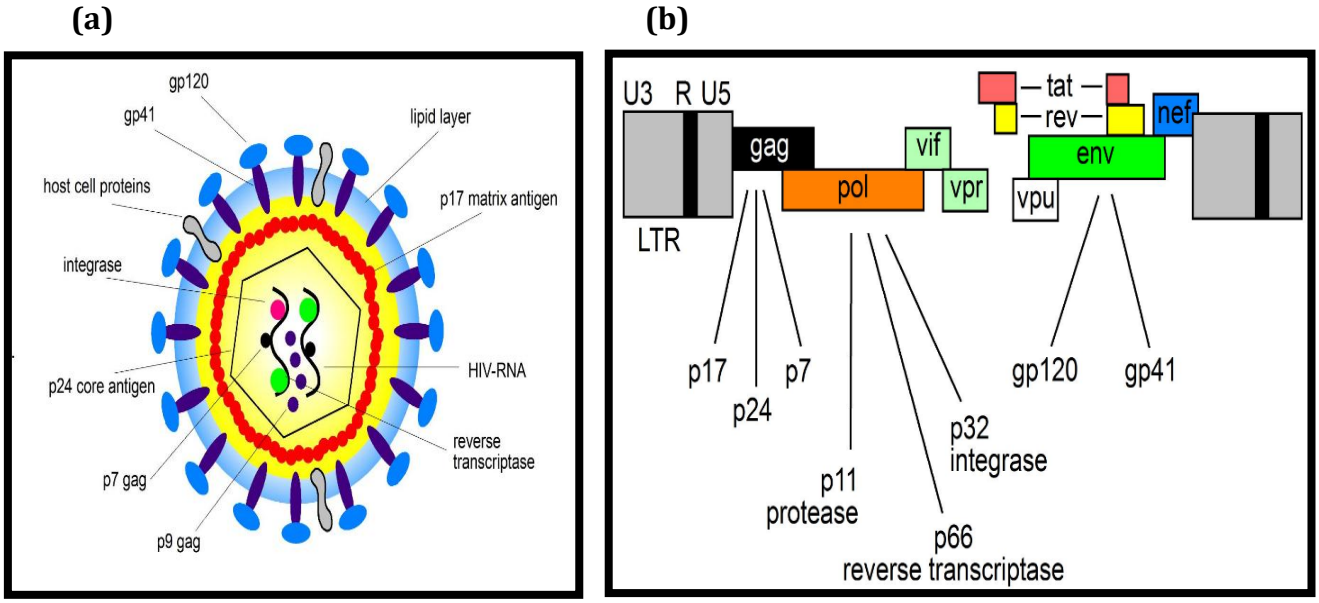



**Figure 1**:The structure of the HIV virion (a) and the HIV genome structure (b) (Rubbert *et al.*, 2011). *The HIV genome is composed of two copies of single-stranded, positive-sense RNA molecules, encoding nine different genes. The gag gene contains the sequence information for structural proteins matrix (p17), capsid (p24) and nucleocapsid (p6, p7, p9). The pol gene carries the information for the viral enzymes reverse transcriptase (p66), protease (p11) and integrase (p32). The env gene encodes surface glycoprotein gp160, a precursor of glycoproteins gp120 and gp41. The six remaining auxiliary genes, tat, rev, nef, vif, vpr, and vpu are regulatory genes for proteins involved in viral maturation, replication, and infectivity.*

## I.3 HIV VARIANTS AND DISTRIBUTION

HIV includes extremely various viruses which are currently classified in two types: HIV type 1 and HIV type 2 (Jacobs *et al.*, 2006). While HIV type 2 is less diversified, because formed only by six genetic subtypes named A through F and remains essentially confined to West Africa (Esteves *et al.*, 2000), HIV type 1 shows a large number of genetic variants and spreads around the world (Fischetti *et al.*, 2004).

HIV-1 is divided into four main groups including groups M (Major), O (Outlier), N (non-M/non-O, or New) and P (putative) (Vallari *et al.*, 2011, Abecasis *et al.*, 2013). Contrary to groups O, N and P that are mainly restricted to Central Africa, group M has caused the HIV pandemic. This latter has been further classified into 9 distinct subtypes including subtypes A, B, C, D, F, G, H, J and K. HIV-1 has a high capacity for replication and genomic malleability that allow it to evolve and adapt quickly to its environment, leading to numerous viral variants. This can be due to gene mutations and recombination. There are more than 90 inter-subtypes recombinant forms (www.hivlanl.org) called Circulating Recombinant Forms (CRFs) with significant epidemic spread (Fischetti *et al.*, 2004, Deforche *et al.*, 2006) and Unique Recombinant Forms (URFs) identified from a single individual (McCutchan, 2006).

The distribution of HIV-1 subtypes and recombinants is usually specific to one locality, one region, or one continent. Thus, subtype B is mainly found in Western and Eastern Europe, Latin America and Central Asia (Abecasis *et al.*, 2013). The subtype F is encountered in Brazil (Louwagie *et al.*, 1995) whereas subtypes A, D, and G are frequently found in West Africa (Esteves *et al.*, 2000, Fischetti *et al.*, 2004). The subtype C is responsible for the majority of infections in South Africa (Jacobs *et al.*, 2006). Concerning the recombinant variants, CRF 02_AG is the predominant subtype in West Africa particularly in Ghana and Guinea Bissau (Fischetti *et al.*, 2004) but is also present in Central Africa (Konings *et al.*, 2006a). The CRF07_BC and CRF08_BC are found in China (Su *et al.*, 2000), while CRF 01_AE is the major strain throughout South and Southeast-Asia (McCutchan, 2006, Abecasis *et al.*, 2013). CRF12_BF and CRF04_cpx are found in South America (Thomson *et al.*, 2004), in Ciprus and Greece (Paraskevis *et al.*, 2001) respectively. Unlike the case in other countries, where one or two HIV-1 subtypes dominate, Cameroon is the only country where diverse strains of HIV-1, M, N, O cocirculate, including CRFs (CRF02_AG, CRF01_AE, CRF06_cpx, CRF09_cpx, CRF11_cpx, CRF13-cpx, CRF18-cpx, CRF22-cpx, CRF25_cpx, and CRF37_cpx), URFs and inter-subtype recombinants (CRF02_AG/F2) (Konings *et al.*, 2006a, Ndongmo *et al.*, 2006, Ndembi *et al.*, 2008, Torimiro *et al.*, 2009, Veras *et al.*, 2011).

## I.4 HIV INFECTION

HIV infection causes a chronic infection that is characterized by a progressive loss and dysfunctions of CD4+ T cells (Bi *et al.*, 2009). Although the manifestations of the

disease vary in individuals, the infection progresses through a clinical course that begins with acute infection, followed by a period of clinical latency and eventually culminating in AIDS in the absence of antiretroviral therapy.

### I.4.1 Acute infection

Acute or primary HIV infection is the earliest stage of HIV infection, and it generally develops within 2 to 4 weeks after a person is infected with HIV (Mogensen *et al.*, 2010). This stage is characterized by a massive depletion of $CCR5^{+}CD4^{+}$ effector memory T cells (Fig.2), associated with a high increase of plasma viraemia which reaches a peak after 21-28 days of infection (Appay & Sauce, 2008, Streeck & Nixon, 2010b). At the time of peak viraemia, HIV-1 infected people may develop symptoms of the acute retroviral syndrome, including fever, malaise, fatigue, sore throat, persistent generalized lymphadenopathy, hematologic disturbances, neurologic and dermatologic disorders (Zetola & Pilcher, 2007). The establishment of the adaptive immune response, in particular HIV- specific $CD8^{+}$ T cells and anti-HIV antibodies leads to increase numbers of circulating virus-specific $CD4^{+}$ T cells and the gradual decline of the viral load to its lowest point, also known as the set point (Mogensen *et al.*, 2010). This initiates the chronic phase of HIV infection (Zetola & Pilcher, 2007).

### I.4.2 Chronic infection

Chronic HIV infection also called clinical latency is an asymptomatic phase that is characterized by the permanent viral replication in peripheral lymphatic tissue and the gradual loss of the $CD4^{+}$T cells, at an average rate of approximately 50 cells/µL/year (Stilianakis & Schenzle, 2006). During chronic infection, there is a sustained immune activation and an accelerated cell turnover. In the gut, naive and central memory T cells are supplied, but these cells are short-lived and only partially substitute for the $CD4^{+}$ effector memory T cells depleted during the acute phase of infection (Fig.2). Another important factor is the accelerated viral evolution at this stage, provided by an excessively high viral mutation rate and alteration in cellular tropism, resulting in progression from a pool of CCR5-trophic to dual trophic or dominantly CXCR4 trophic strains able to infect activated naive and central memory $CD4^{+}$ T cells. The rate of disease progression correlates directly with HIV RNA levels (Mogensen *et al.*, 2010). For instance, HIV-infected individuals with high levels of HIV RNA progress to symptomatic

HIV disease faster than those with low levels of HIV RNA. Based on the degree of immunodeficiency, chronic HIV disease can be divided into 4 stages (WHO, 2005) including not significant immunodeficiency ($CD4^+$ T-cell count above 500 cells/$mm^3$), mild immunodeficiency ($CD4^+$ T-cell count between 350-499 cells/ $mm^3$), advanced immunodefciency ($CD4^+$ T-cell count between 200-349 cells/ $mm^3$) and severe immunodeficiency ($CD4^+$ T-cell count less than 200 cells/ $mm^3$). Without anti-retroviral treatment, chronic HIV infection usually advances to Acquired Immune Deficiency Syndrome (AIDS) in roughly 10 years (Deeks & Walker, 2007).

### I.4.3 Acquired Immune Deficiency Syndrome

Acquired Immune Deficiency Syndrome is the final stage of HIV infection. The interval between acute HIV infection and AIDS is highly variable, with a median time of 8-11 years. This stage is defined by an absolute CD4 cell count of less than 200 cells/ $mm^3$ and specific opportunistic infections or malignancies (Stilianakis & Schenzle, 2006).

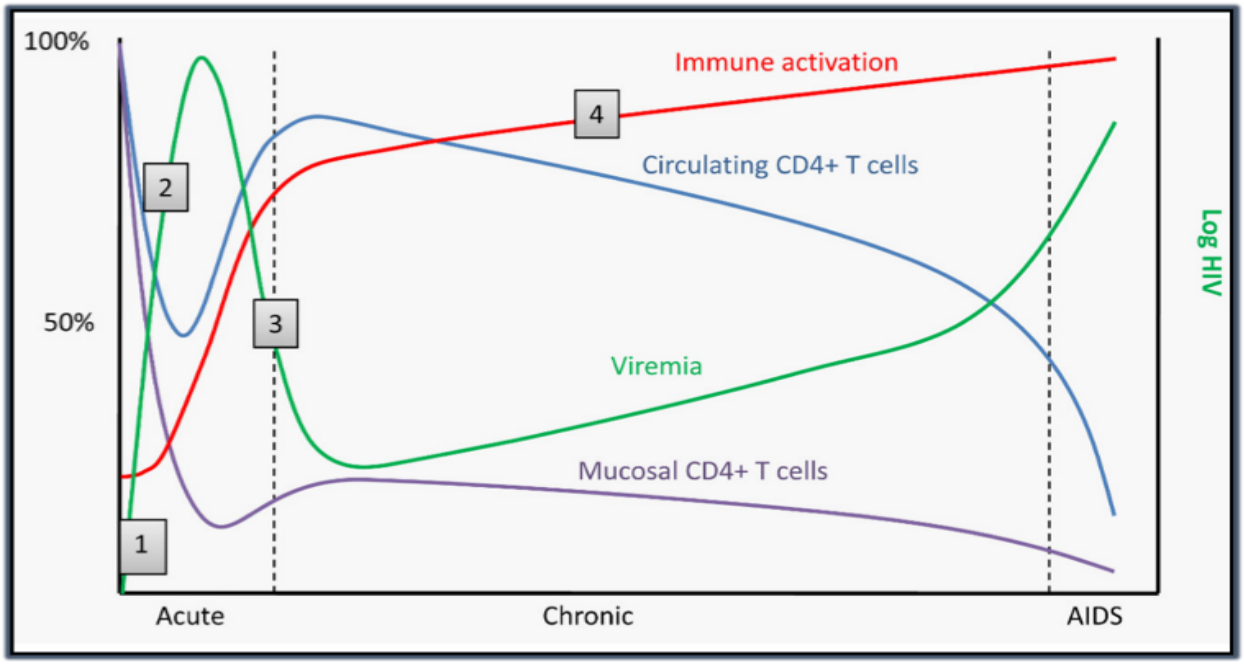



**Figure 2**: The clinical course of HIV infection (Mogensen *et al.*, 2010). *(1) stage before detectable viral RNA in plasma, referred as eclipse phase (2) Immune -mediated T cell death and progression of infection (3) Defense against established infection. (4) Chronic immune activation.*

## I.5 INTERACTION BETWEEN HIV AND THE IMMUNE SYSTEM

### I.5.1 HIV and the innate immune system

The innate immune system represents the first line of defense against infectious organisms; it plays a major role in initial containment of infection and hence may be crucial in acute HIV infection (Mogensen *et al.*, 2010). Among the innate immune cells, we pay more attention to monocytes, macrophages, dendritic cells and NK cells that act in concert with humoral players to control the virus's replication (Paranjape, 2005); without forgetting the complement system that will be developed later.

#### I.5.1.1 Interaction between HIV and monocytes

**a. Mechanisms of monocytes action**

Monocytes are vitally important cells in the immune system, as they are the precursor cells to professional antigen presenting cells (APCs), such as macrophages and Dendritic Cells (Coleman & Wu, 2009). On the other hand, monocytes express both CD4 receptor and CCR5/CXCR4 co-receptors required for the virus entry, and are therefore susceptible to HIV-1 infection (Coleman & Wu, 2009, Sassé *et al.*, 2012). However, several studies showed that monocytes are resistant to the cytopathic effects of HIV. Low levels of both CD4 receptor as well as CCR5 co-receptor (Fig.3) are related to monocyte resistance to infection (Sassé *et al.*, 2012). This resistance has been also explained by the activity of several restriction factors. For instance, Sterile alpha motif domain and HD domain containing protein 1 (SAMHD1) functions as a deoxynucleoside triphosphate triphosphohydrolase that depletes the intracellular pool of deoxynucleoside triphosphates (dNTPs) needed for further cDNA synthesis in monocytes, thus impairing reverse transcription (Iwasaki, 2012, Sattentau & Stevenson, 2016). In addition, restriction factors like, APOBEC 3A and 3G have been shown to inhibit HIV replication in monocytes (Fig. 3). It has been proposed that anti-HIV micro-RNA (miR-198) also contributes to HIV-1 restriction in monocytes by repressing the HIV-1 *Tat* activity (Coleman & Wu, 2009, Bergamaschi & Pancino, 2010). Moreover, monocytes, when compared with activated $CD4^+$ T cells and macrophages, are known to have much lower levels of cyclin T1 protein (CycT1) and active, phosphorylated cyclin-dependent kinase 9 (CDK9) form, which interact with *Tat* to stimulate the elongation of viral RNA (Coleman & Wu, 2009, Bergamaschi & Pancino, 2010). Besides antiviral mechanisms developed by monocytes, these cells can also release numerous pro-

inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and chemokines (CCL2, CCL3, CCL4, CCL22, CXCL8, and MIP-1a) that can stimulate other immune cells to clear HIV infection (Tiemessen *et al.*, 2007, Sassé *et al.*, 2012).

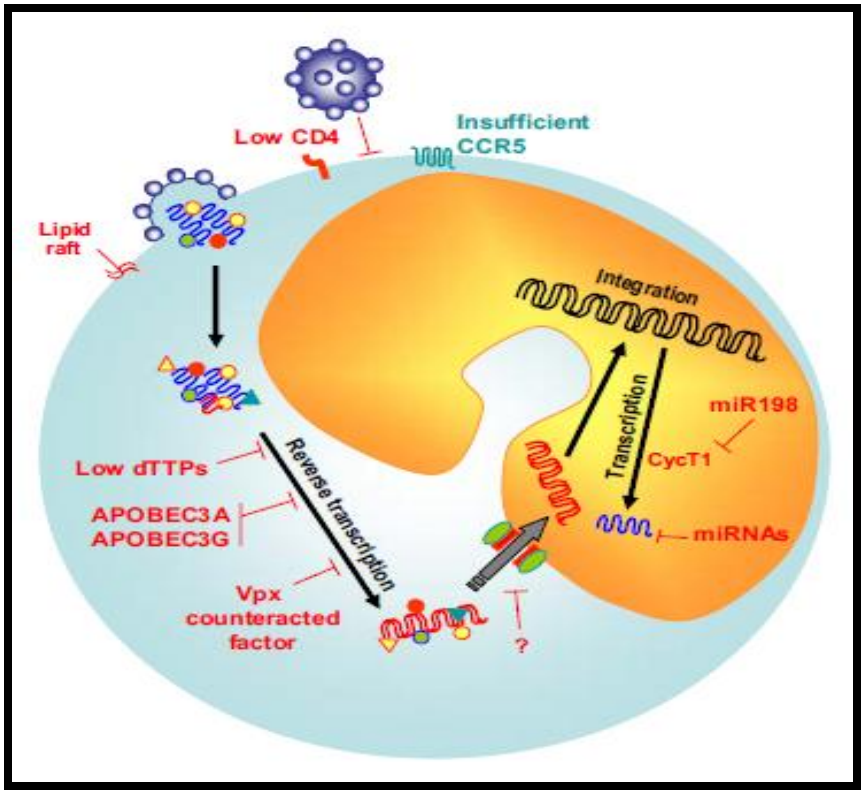



**Figure 3**:Representation of host restriction factors in human Monocytes (Bergamaschi & Pancino, 2010). *Low levels of CD4 and CCR5 may limit viral entry in monocytes. Low expression of thymidine phosphorylase associated with a limited stock of dTTP reduces reverse transcription (RT) rate. APOBEC3A and 3G may interfere with HIV-1 RT in Monocytes. Vpx from HIV-2 antagonizes the restriction of HIV-1 by counteracting an unidentified host factor. Cellular miRNAs have been proposed to target the 3'UTR of HIV-1 transcripts. miR-198 may repress CycT1 expression that contributes to Tat transactivation.*

### b. HIV mechanisms to escape monocytes mediated action

HIV has developed several strategies to avoid specific restriction factors that pose a barrier to replication. For instance, HIV-Vif protein can mark APOBEC3G for degradation through ubiquitination and degradation by the proteasome pathway and thus prevents its incorporation into virions (Odigie & Bhattacharya, 2009, Bergamaschi & Pancino, 2010, Guha & Ayyavoo, 2013, Manches *et al.*, 2014). On the other hand, exogenous recombinant HIV-1 Tat protein has been shown to cause increased production of the IL-10 from monocytes *in vitro*. This production positively correlated with increased levels of soluble CD14, a marker for monocyte activation and a mediator of gram negative bacterial LPS action (Coleman & Wu, 2009, Sassé *et al.*, 2012).

Exogenous HIV-1 Tat protein has been shown also to increase monocyte survival, through increased expression of the anti-apoptotic protein Bcl-2 (Coleman & Wu, 2009). Under the HIV-1 gp-120 antigen pressure, monocytes upregulate the expression of chemokines, such as chemotactic protein-1 (MCP-1), which increases monocyte recruitment to the sites of HIV-1 infection, increasing the available monocyte pool for infection by HIV-1. HIV-1 infected monocytes will then migrate to peripheral tissues such as brain, lungs and gastrointestinal tract where they differentiate into macrophages and DCs, then the virus can start replicating and infecting the tissue (Coleman & Wu, 2009, Bergamaschi & Pancino, 2010).

### I.5.1.2 Interaction between HIV and macrophages

#### a. Mechanisms of macrophages action

Macrophages are terminally differentiated immune cells which play an important role in the clearing of pathogens and cellular debris by phagocytosis. They also act as the antigen presenting cells, thus playing an essential role in the adaptive immunity (Kumar & Herbein, 2014). Macrophages in the mucosa contribute to the humoral and cellular immune responses which suppress HIV-1 viral loads at the onset of the chronic phase of HIV-1 infection (Koppensteiner *et al.*, 2012). They help to optimize the anti-HIV Cytotoxic T Lymphocyte (CTL) response due to cross presentation of HIV-1 derived peptides via histocompatibility leukocyte antigen (HLA) class I, and they are involved in the orchestration of the primary antibody response (Koppensteiner *et al.*, 2012, Kumar & Herbein, 2014). However, as monocytes, macrophages express both CD4 receptor and CCR5 and CXCR4 coreceptors required for HIV-1 infection, but they are able to resist to HIV-mediated cytopathic effects (Sassé *et al.*, 2012). A decreased expression of the CD4 and CCR5 molecules at the cell surface, a homozygous CCR5Δ32 mutation or the heterozygous CCR5Δ32 (Fig. 4) have been associated with a decreased susceptibility of macrophage to R5 HIV-1 infection (Bergamaschi & Pancino, 2010). In addition to the CD4/CCR5 mediated entry of HIV-1 into the cell by membrane fusion, an alternative route of infection has been described in macrophage that involves the uptake of the virus via micropinocytosis. This process requires an intact lipid raft, and notably the correct amount and distribution of cholesterol molecules. Depletion of cholesterol, has been shown to disrupt HIV-1 entry into macrophages, possibly by reducing the fusion capacity with the HIV-1 envelope and CCR5-mediated CCR5 signaling

(Bergamaschi & Pancino, 2010). The presence of host transcriptional repressor, C/EBP-β is known to block HIV-1 transcription in macrophages (Fig. 4) which may contribute to HIV latency (Kumar & Herbein, 2014). On the other hand, the human urokinase protein also referred as Urokinase-type plasminogen activator (uPA) blocks the release of viral particles from intracellular vacuoles (Bergamaschi & Pancino, 2010). Whereas APOBEC3G and APOBEC3A are down-regulated in macrophages, high amounts of SamHD1, TRIM22 and Tetherin have been reported. These restriction factors are known able to inhibit HIV replication and viral assembly as well as HIV-1 release from the infected cell. Moreover, Cyclin dependent kinase inhibitor p21 inhibits HIV-1 replication in macrophages by interfering with reverse transcription of the viral genome by a mechanism independent of SAMHD1 (Koppensteiner *et al.*, 2012, Kumar & Herbein, 2014).

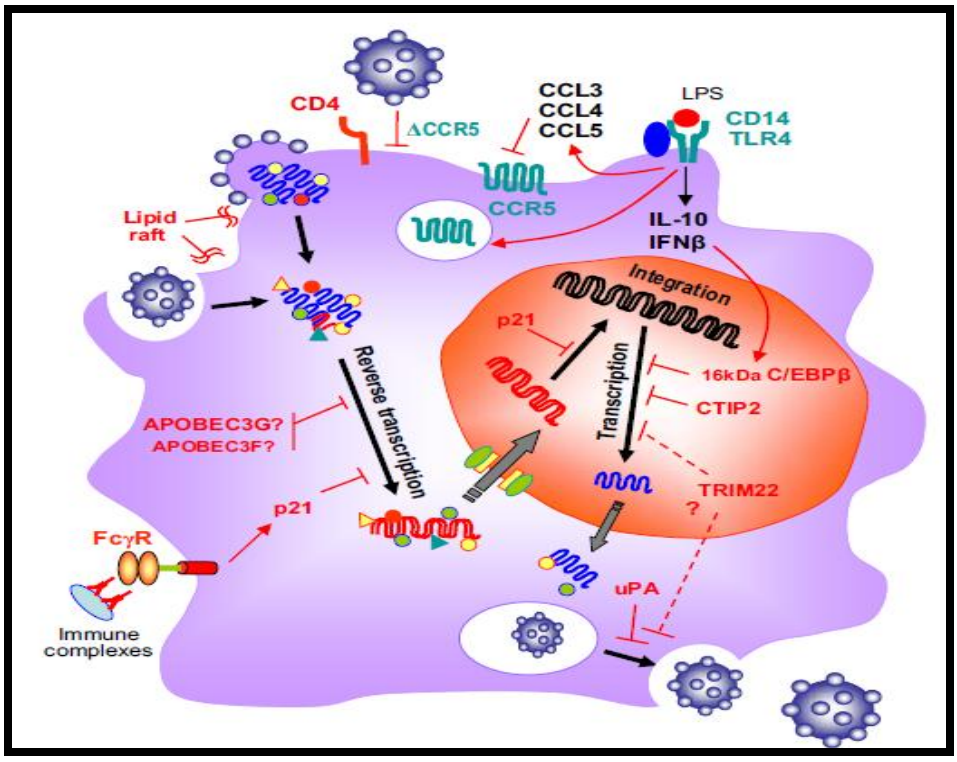



**Figure 4**: Representation of host restriction factors in human macrophages (Bergamaschi & Pancino, 2010). *The CCR5Δ32 mutation restricts viral entry of R5 HIV-1 in Macrophages. LPS targets the early phases of the HIV-1 cycle in macrophages, through the down-regulation of CCR5 expression and the LTR-driven transcription by IL-10/IFN-β-induced expression of 16 kDa C/EBPβ. p21Waf1 interferes with both RT and integration and is induced by FcγR engagement. CTIP2 and TRIM22 have been implicated in the inhibition of HIV-1 transcription. Urokinase-type plasminogen activator (uPA) blocks the release of viral particles from intracellular vacuoles.*

**b. HIV mechanisms to escape macrophages mediated action**

HIV-1 has developed sophisticated mechanisms to counteract host restriction factors, and thus to prolong the life span of infected macrophage. This enables HIV-1 infected macrophages to contribute to disease progression by serving as a viral reservoir, as a mobile source of virus, and as an amplifier of immune dysfunction (Koppensteiner *et al.*, 2012, Wang *et al.*, 2015). Among HIV-1 proteins, the viral proteins *Tat*, *Vpr* and *Nef* interfere with signaling pathways in macrophages. HIV-1 *Tat* is known to trigger the expression of HIV coreceptors (CXCR4, CCR5 and CCR3) in macrophages in a dose-dependent manner which might positively influence HIV-1 infection. In addition, *Tat* enhances the endogenous levels of $Ca2^{+}$ in macrophages which may subsequently lead to the production of chemokines and pro-inflammatory cytokines by macrophages. This pro-inflammatory cytokine profile is suggested not only to enhance virus replication and persistence of chronically activated macrophages in vivo, but also to promote viral infection by enlarging the number of primary target cells (Koppensteiner *et al.*, 2012, Kumar & Herbein, 2014). *Vpr* stimulates the viral replication in acutely and latently infected macrophages by enhancing the expression of p21 in macrophages (Kumar & Herbein, 2014). HIV-1 *Nef* induces the production of macrophage colony stimulating factor (M-CSF) which inhibits the expression of TRAILR1 on macrophages and upregulates the expression of anti-apoptotic proteins. In addition, *Nef* down-regulates the CD4 expression in macrophages, therefore avoiding superinfection which otherwise could lead to premature cell death. CD4 down-regulation in infected cells may also promote the release of viral progeny by avoiding sequestration of viral envelope by CD4 (Kumar & Herbein, 2014).

### I.5.1.3 Interaction between HIV and Dendritic Cells

**a. Mechanisms of Dendritic Cells action**

Also called antigen-presenting cells, Dendritic cells (DCs) are highly specialized in the function of capturing / transforming foreign molecules in order to obtain maximum stimulation of the immune system. In fact, their action lies between innate immunity and acquired immunity as if it were a conductor of the overall immune reaction. DCs are among the first cell types to encounter HIV because of their location at all mucosal surfaces (Manches *et al.*, 2014) and therefore, play an important role in HIV infection.

Conventional DCs (cDCs) show a high phagocytic activity and favor the transmission of HIV to $CD4^+$ T cells in draining lymph nodes to initiate acquired immune response (Barroca *et al.*, 2014, Manches *et al.*, 2014). Indeed, this subset express on its surface, the DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN), which binds to the HIV gp 120 envelope protein. This binding results in HIV internalization, followed by its partial degradation in early endosomal compartments and its delivery to $CD4^+$ T cells through virologic synapses (Barroca *et al.*, 2014, Manches *et al.*, 2014). In contrast, Langerin–mediated uptake by epidermal Langerhans cells (LCs) directs virions to acidic compartments containing Birbeck granules where virus is rapidly degraded, thus preventing the productive infection of LCs and also the HIV-1 transfer to $CD4^+$ T cells. In plasmacytoid DCs (pDCs), the interaction of viral RNA with toll-like receptor (TLR)-7 in the endosome triggers interferon regulatory factor (IRF)-7 activation and interferon (IFN)- α induction. This cytokine is involved in shutting down viral replication in infected cells while also promoting the recruitment and activation of other immune cells to the sites of infection as well as to the lymph nodes (Gerosa *et al.*, 2002, Megjugorac *et al.*, 2004, Borrow, 2011, Carrington & Alter, 2012). Both IRF-1 and 7 also induce the expression of hundreds of interferon-stimulated genes (ISGs), resulting in a number of type I IFN-inducible restriction factors which have been shown to block HIV replication at different stages of infection (Fig. 5). For instance, APOBEC3G has been shown to inhibit HIV replication in DCs (Odigie & Bhattacharya, 2009). TRIM5α binds to the incoming HIV capsid protein and promotes disassembly before completion of reverse transcription, even though its activity is limited in human (Towers & Noursadeghi, 2014). Tetherin blocks HIV-1 release from the infected cell (Tabb *et al.*, 2013). SAMHD1 prevents reverse transcription and productive cell-to-cell transmission through depletion of deoxynucleotides (Iwasaki, 2012, Sattentau & Stevenson, 2016).

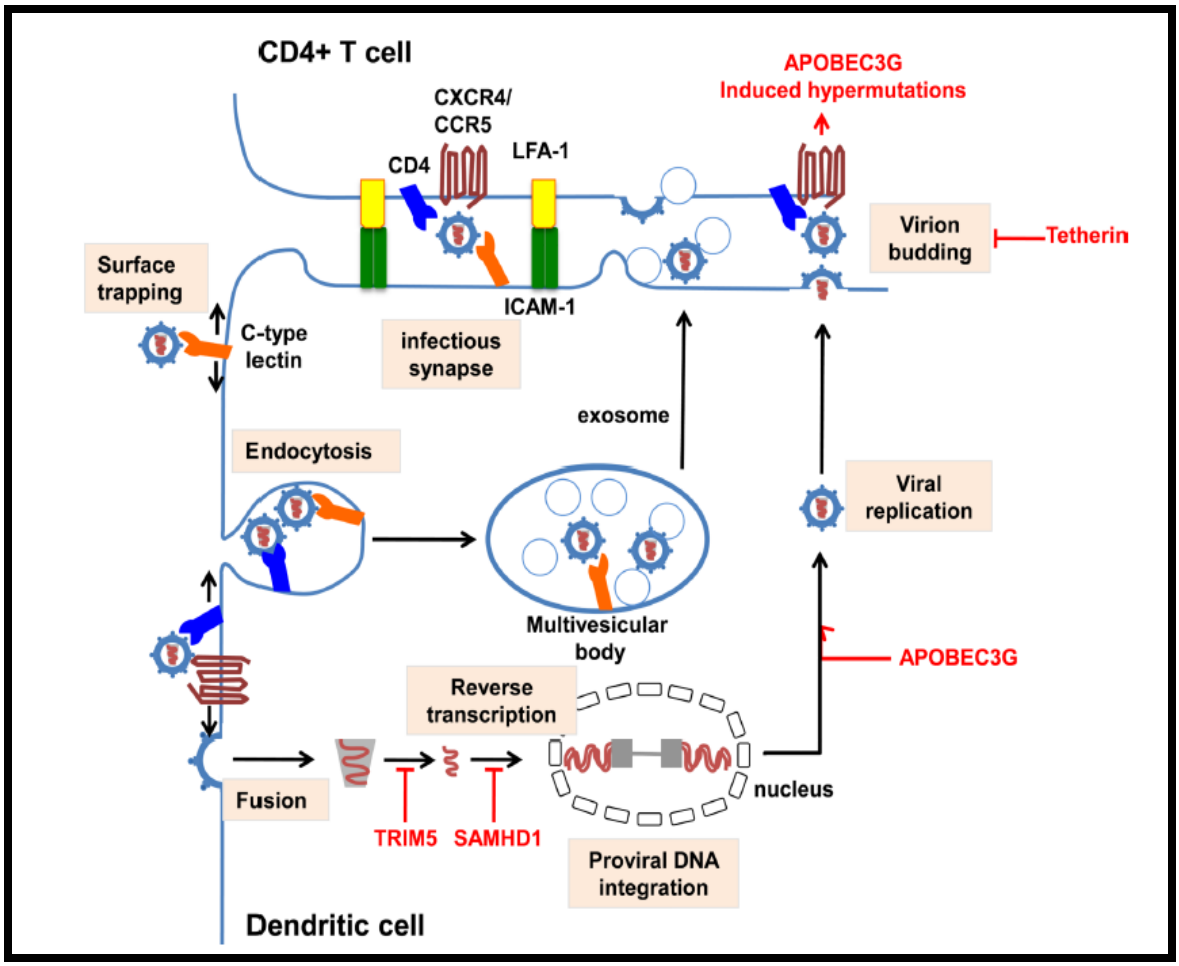



**Figure 5**: Interaction between HIV and dendritic cells (Manches *et al.*, 2014). *HIV binding to CD4 and CXCR4 or CCR5 allows infection of DC through fusion with the cell membrane. However, several restrictions factors (red) prevent efficient infection of DC, acting at different stages. Despite low infectivity, DC can participate to CD4⁺ T cells trans-infection. HIV attachment to DC through C-type lectins, such as DC-SIGN, can lead to endocytosis and storage of intact virions, or to its retention on the cell surface. HIV into multivesicular bodies can be delivered through an exocytic route that converges with the exosome dissemination pathway. Alternatively, virions trapped on the surface of DC can be directly transmitted to target T cells. The efficiency of trans-infection is highly augmented by the formation of an 'infectious synapse', which concentrate virions and HIV receptors in a limited intercellular region. The interaction between the adhesion molecules (LFA-1, ICAM-1) help stabilize the infectious synapse. Newly formed virions from infected DC can also bud to be released in the infectious synapse and binding to HIV receptors on CD4⁺ T cells.*

### b. HIV mechanisms to escape Dendritic Cells mediated action

Following chronic exposure to HIV-1 antigen, pDCs produce increased levels of type I IFNs, which induce the expression of TNF-related apoptosis inducing ligand (TRAIL), involved in triggering apoptosis of uninfected CD4⁺ T cells (Chang & Altfeld, 2010, Guha & Ayyavoo, 2013). IFN secretion can also augment central memory CD4⁺ T cell proliferation and differentiation, leading to exhaustion of the CD4⁺ T cell pool, while

at the same time generating increased target cell numbers for HIV infection (Barroca *et al.*, 2014). On the other hand, HIV-exposed pDCs prime Treg cells due to upregulated expression of indoleamine 2,3 dioxygenase (IDO). In turn, IL-10 produced by Treg cells will impair cDC function, further blunting adaptive immune response (Coleman & Wu, 2009, Barroca *et al.*, 2014). HIV-1 has been shown also to counteract the activity of certain restriction factors. For example, HIV-1 *Vif* protein can induce the degradation of APOBEC3G (Odigie & Bhattacharya, 2009, Guha & Ayyavoo, 2013, Manches *et al.*, 2014). Additionally, HIV-1 *Vpu* reduces tetherin expression at the surface, by targeting it for degradation and sequestering it in a perinuclear compartment leading to the detachment of virus particles from cell membrane (Odigie & Bhattacharya, 2009, Borrow, 2011). The HIV-1 capsid protein has evolved to be invisible to human TRIM5a and is therefore not restricted by this protein, even when expressed at high levels (Towers & Noursadeghi, 2014).

### I.5.1.4 Interaction between HIV and Natural Killer cells

#### a. Mechanisms of Natural Killer cells action

Identified in the 1970s, Natural killer (NK) cells differentiate in the bone marrow and are effectors of natural immunity involved in the early stages of viral infections and may be active depending on the cytokine affinity. NK cells play a crucial role during the earliest stages of HIV-1 infection (Hens *et al.*, 2016). NK cells are expanded following activation by IFN- α, IL-12 and IL-15 produced by DCs (Chang & Altfeld, 2010, Altfeld & Gale, 2015). These stimulated NK cells proliferate and release numerous cytokines and β-chemokines such as IFNγ, Tumor necrosis factor alpha (TNF-α), MIP-1A, MIP-1B, RANTES and GM-CSF to activate T-cell proliferation (Carrington & Alter, 2012, Altfeld & Gale, 2015). On the other hand, NK cells express different activating and inhibitory killer cell immunoglogulin-like receptors (KIRs), which in conjunction with their HLA class I ligands have been associated with better control of HIV-1 replication and slower HIV-1 disease progression (Chiesa *et al.*, 2015). Several data show that KIR3DS1$^+$ NK cells, and to a lesser extent KIR3DL1$^+$ NK cells have a protective role during acute and chronic HIV-1 infection by killing HIV-1 infected cells and thus inhibiting viral replication (Fig. 6). However, this occurs only in individuals carrying HLA-Bw4 alleles with an isoleucine at position 80 (Bw4-80I) of the peptide-binding groove (Chang & Altfeld, 2010, Borrow, 2011, Carrington & Alter, 2012).

However, after seroconversion, NK cells can combat HIV-1-infected cells via antibody-dependent cell mediated cytotoxicity (Paranjape, 2005). Apart from their direct antiviral activity, NK cells play an important role in regulating the function of DCs, by rapid elimination of immature DCs, in order to avoid deficient T cell priming (Chang & Altfeld, 2010, Hens *et al.*, 2016).

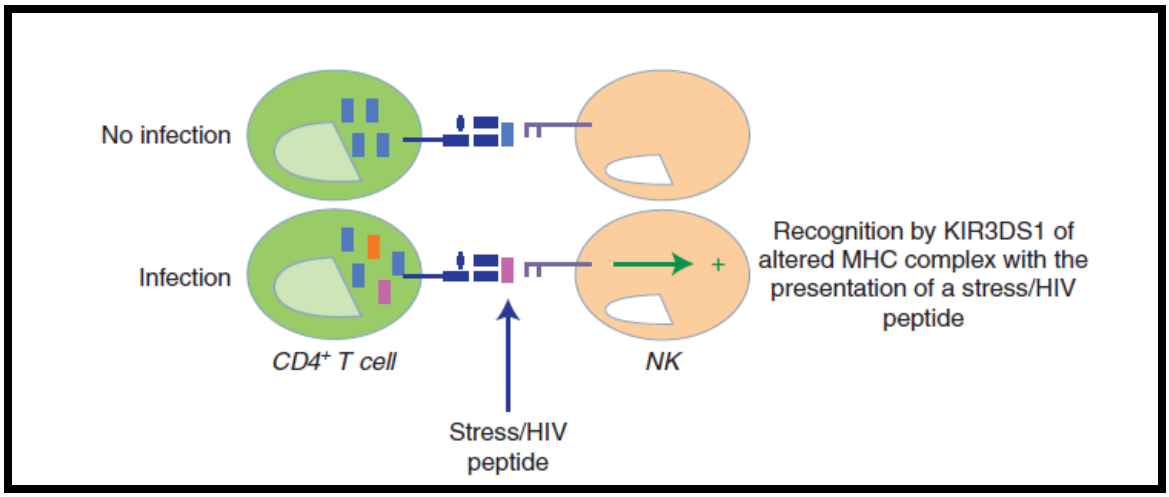



**Figure 6**: A model of KIR3DS1$^+$ NK-cell recognition of an HIV-infected target cell (Carrington & Alter, 2012). *A viral or stress peptide generated during infection presented by Bw4-80I may alter the affinity of the activating KIR3DS1 receptor expressed on NK cells for its putative ligand, resulting in the potent activation of NK cells and rapid elimination of virally infected cells.*

### b. HIV mechanisms to escape natural killer cells mediated action

HIV-1 has evolved multiple strategies for evading NK-cell-mediated control. HIV-1 Nef functions as a potential regulator of NK cell cytotoxicity due to its involvement in HLA- class I downregulation on HIV-1 infected CD4$^+$ T cells. HIV-1 Nef selectively downregulates HLA- A/B molecules but not HLA- C/E molecules, which have a greater relative role in inhibiting NK activation leading to inhibition of NK cell cytotoxicity (Guha & Ayyavoo, 2013, Hens *et al.*, 2016). Nef also down-regulates expression of the gp41-induced NKp44 ligand on HIV-1 infected cells (Borrow, 2011). HIV-1 *Tat* inhibits lymphocyte function-associated antigen (LFA)- 1-mediated $Ca^{2+}$ influx through the binding of L-type $Ca^{2+}$ channel and thereby impairs NK cell cytotoxicity (Guha & Ayyavoo, 2013). Chronic viral stimulation may also lead to inappropriate activation of peripheral NK cells, thus resulting in NK cell exhaustion or anergy (Alter *et al.*, 2005, Mavilio *et al.*, 2005).

### I.5.1.5 Interaction between HIV and the complement system

#### a. Mechanisms of the complement action

The complement system is a well-regulated complex system made of several circulating proteins synthesized by various organs (hepatocytes, tissue macrophages) and cellular receptors. It lyses viruses by a complementary activity of antibodies through a triggered-enzyme cascade as shown in figure 7.

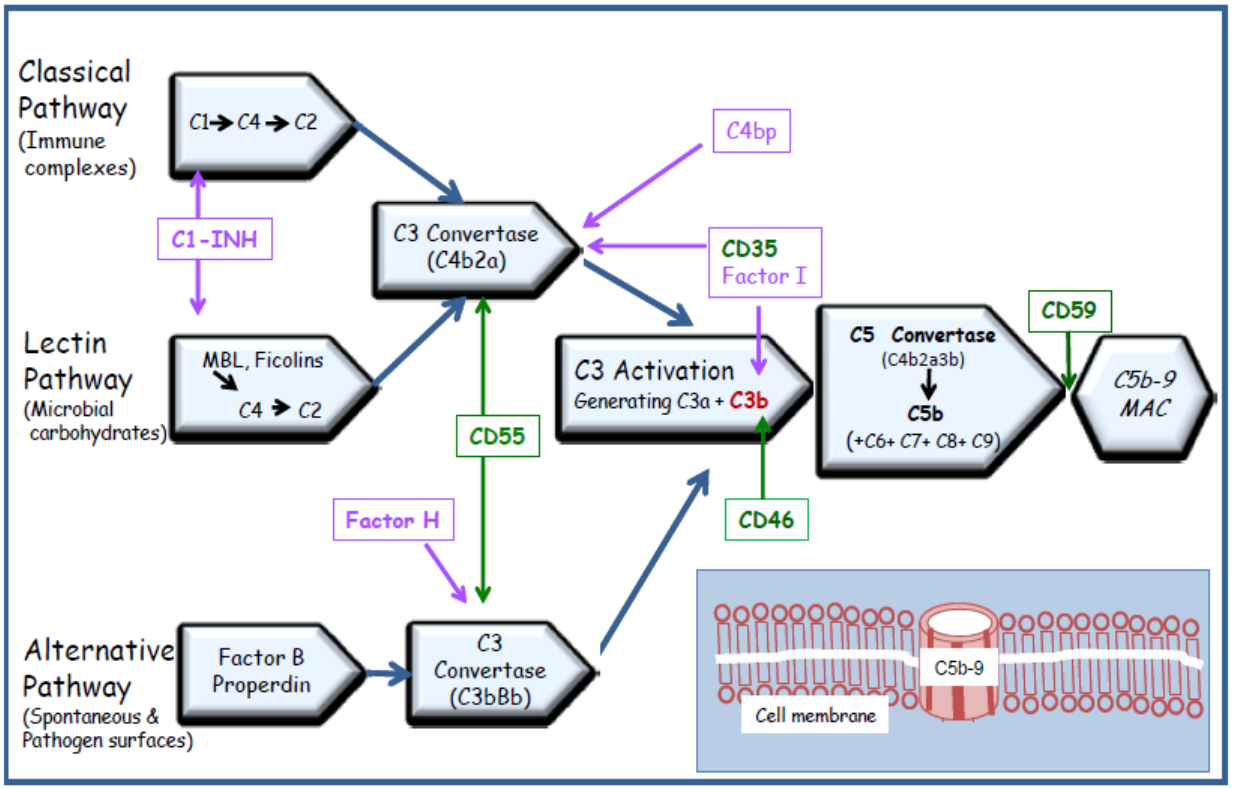



**Figure 7**: activation events of the complement cascade (Nesargikar *et al.*, 2012). *IgM or IgG antigen/antibody complexes binding to C1q, the first protein of the cascade, initiates the classical pathway. The alternative pathway is not so much an activation pathway, as it is a failure to regulate the low level continuous formation of a soluble C3 convertase. The third pathway is known as MBL (Mannosebinding lectin)/MASP (MBL associated Serine Protease) pathway. The initiating molecules for the MBL pathway are multimeric lectin complexes that bind to specific carbohydrate patterns uncommon in the host, leading to activation of the pathway through enzymatic activity of MASP. The sites of action of the membrane bound complement regulators– CD35, CD46, CD55 & CD59 (green boxes) and the fluid phase regulators – C1-INH, Factor H, Factor I and C4bp (violet boxes) are represented with arrows.*

The complement system is central to innate humoral immunity and plays an important role in the protection against HIV infection. It fights against the infection through antibody-mediated complement-dependent lysis of HIV-1 virions and HIV-1-infected cells and neutralizes HIV-1 particles (Liu *et al.*, 2014). Complement activation occurs through three different pathways including the classical, the alternative and the

lectin pathway (Stoermer & Morrison, 2011, Shishido *et al.*, 2012). All three pathways converge in the cleavage of C3, the main complement component, to the anaphylatoxin C3a and the opsonin C3b. Upon entering the host, HIV-1 spontaneously activates the complement system via gp120 and gp41, and is hence already coated with the opsonin C3b, which associates with the C3 convertase to generate the C5 convertase. This convertase cleaves C5 into C5a and C5b. C5b triggers the formation of the membrane attack complex (MAC), that consists of C5b, C6, C7, C8 and polymeric C9 molecules (Fig.7). The formation of the MAC disrupts the viral membrane, resulting in lysis of viral particle (Nesargikar *et al.*, 2012).

**b. HIV mechanisms to escape the complement mediated action**

During the budding process, HIV acquires membrane-anchored regulators of complement activation (RCAs) such as decay accelerating factor (DAF/CD55), CD59 and Factor H, which protect virions from complement-mediated lysis (Yu *et al.*, 2010, Stoermer & Morrison, 2011). HIV not killed by complement-mediated lysis, persists covered with C3 and C5a fragments which are upregulated by HIV-1 viral proteins Nef and gp41. Thus, opsonized HIV is able to interact with cells, such as DCs, monocytes and macrophages expressing complement receptor CR3 and CR4 leading to viral entry and intracellular viral spread (Shishido *et al.*, 2012, Liu *et al.*, 2014). In addition, C5a product may induce the production of TNF-α and IL-6 by monocytes and monocyte-derived macrophages, leading to the recruitment of DCs, which in turn promote the productive infection of $CD4^+$ T cells. The binding of anti-HIV antibodies to complement opsonized HIV-1 virions also facilitates HIV-1 interaction with non-infected cells like erythrocytes and B cells. Therefore, immune-complexed HIV-1, but also free HIV are delivered to organs susceptible to infection (Yu *et al.*, 2010).

## I.5.2 HIV and the adaptive immune system

The Adaptive immune response involves three populations of lymphocytes including $CD4^+$ helper T cells, $CD8^+$ cytotoxic T Lymphocytes and B cells that play a key role in controlling HIV infection.

### I.5.2.1 Interaction between HIV and CD4+ T cells

#### a. Mechanisms of CD4+ T cells action

CD4+ T cells are a heterogeneous population that can be phenotypically classified into naive, central memory, transitional memory and effector memory cells based on their differentiation status. These cells can also be categorized into CD4+ Type 1 helper (Th1), Th2, Th9, Th17, Th22, CD4+ T follicular helper (Tfh) and Treg cell subsets based on their cytokine secretion patterns and their tissue locations (Fevrier *et al.*, 2011, Tabb *et al.*, 2013) as shown in Fig. 8.

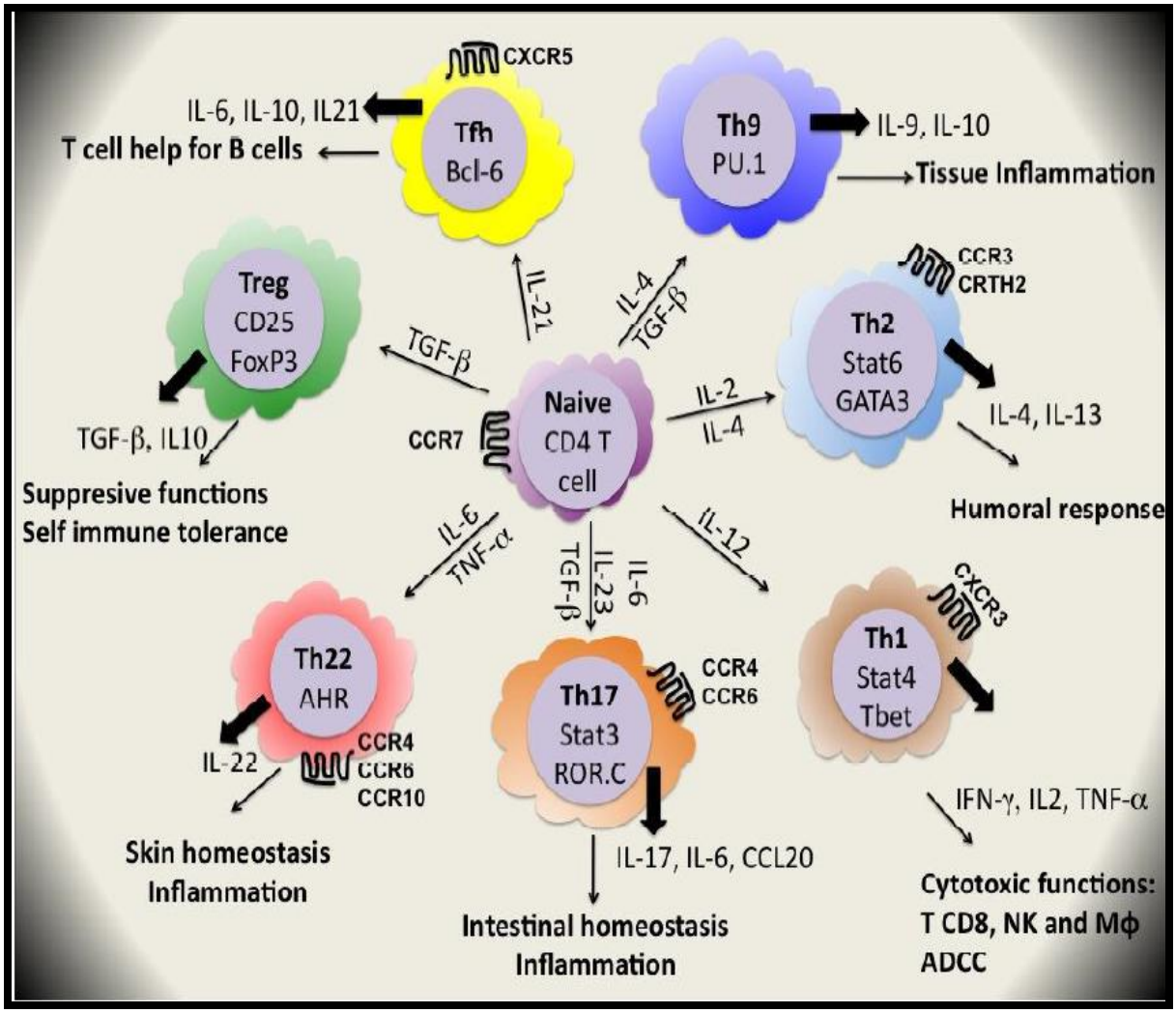



**Figure 8**: Differentiation of effector CD4+ T cells following activation by a given antigen (Fevrier *et al.*, 2011).*cytokines secreted in the microenvironment dictate the type of effector cells subsequently induced from naive T cells. Th1, Th2, Th9, Th17, TH22, TFH and Treg lineages are defined depending on the expression of transcription factors, effector cytokines, and chemokine receptors.*

Some of these subsets play an important role in the non-progressive outcome of HIV-1 infection.

Th1 lymphocytes secrete IL-2 and IFNγ, which activate macrophages, NK cells and cytotoxic T cells to kill HIV-infected cells (Jansen *et al.*, 2006). They also promote antibody-dependent cell-mediated cytotoxicity (ADCC) by supporting B cell production of IgG1 (Fevrier *et al.*, 2011).

Th2 secrete IL-4, 5, 6, 9, 13 and 17E that are responsible for strong antibody production, and eosinophil activation (Tabb *et al.*, 2013).

Th17 cells found in the lamina propria of the small intestine are characterized by secretion of the pro-inflammatory cytokines including IL-17, IL-1, IL-6, IL21, IL-22 and TNF-α (Fevrier *et al.*, 2011). These cells mediate inflammation but also confer protection against extracellular bacterial and fungal infections by inducing the production of antibacterial defensins (Valverde-Villegas *et al.*, 2015). Th17 cells are involved in the control of epithelial integrity of the gastrointestinal barrier and microbial invasion, by inducing the proliferation and survival of epithelial cells (Okoye & Picker, 2013, Tabb *et al.*, 2013).

Tfh cells are predominantly located within the germinal centers of secondary lymphoid tissues and regulate the development of efficient neutralizing and non-neutralizing antibody responses in HIV infection (Saez-Cirion *et al.*, 2014, Phetsouphanh *et al.*, 2015, Zuniga *et al.*, 2015).

Treg cells are important in the maintenance of immune homeostasis and the control of immune activation by downmodulating the effector activity of other immune cells (Okoye & Picker, 2013). They also facilitate early protective responses to local viral infection by allowing a timely entry of immune cells into infected tissue and diminishing pro-inflammatory chemokine levels in the lymph nodes (Tiemessen *et al.*, 2007).

### b. HIV mechanisms to escape the $CD4^+$ T cells mediated action

Several factors are thought to contribute to the impairment of HIV-specific (Allan *et al.*, 2008) $CD4^+$ T cell responses in progressive HIV disease or in the absence of antiretroviral therapy. Possible mechanisms include direct infection, antigen-induced cell death and virus-induced anergy. $CD4^+$ T cells represent the main target of HIV because of high expression of CD4 receptor and chemokine receptors CCR5 and CXCR4 at their surface (Phetsouphanh *et al.*, 2015). Upon HIV entry, CCR5 tropic HIV strains

preferentially infect the CD4 memory cells ($CD4^+$ TEM) located at the gastrointestinal tract, while infection by CXCR4 tropic HIV isolates occurs in the late stage (Paranjape, 2005, Okoye & Picker, 2013). Central memory $CD4^+$ T cells ($CD4^+$ TCM), found in the lymph nodes and other inductive lymphoid tissues represent the largest reservoir of infected $CD4^+$ T cells in HIV-1 infection, as they are longer-lived, self-renewing cells that maintain $CD4^+$ T-cell homeostasis by replenishing the pool of effector memory $CD4^+$ T cells (Tabb *et al.*, 2013). Infection and depletion of Central memory $CD4^+$ T cells ($CD4^+$ TCM) cells are thought to contribute to the establishment of chronic immune activation more significantly than does infection of $CD4^+$ TEM cells (Tabb *et al.*, 2013). In addition to the virus-induced cytolysis, HIV proteins Nef, Env and Tat may upregulate CD95 (Fas) on $CD8^+$ T cell and CD95L (FasL) on HIV infected $CD4^+$ T cell, thus enhancing the susceptibility to Fas mediated killing (Fevrier *et al.*, 2011). Moreover, during the course of HIV infection, Treg cells accumulate in the gut and suppress Th17 activation, resulting in a loss of Th17/Treg balance. The massive loss of Th17 cells compromise intestinal mucosal integrity, resulting in microbial translocation from the lumen of the gut into peripheral blood. This contributes to immune activation and inflammation with deleterious consequences on viral replication, dysfunction of several immune cells, and increased activation-induced $CD4^+$ T cell death (Okoye & Picker, 2013, Cummins & Badley, 2014, Saez-Cirion *et al.*, 2014, Valverde-Villegas *et al.*, 2015). The decrease in CD4 T cell count affects the $CD4^+$ T cell responses, such as the inability to induce HIV-specific $CD8^+$ T cell and antibody responses as well as response to recall antigens (Paranjape, 2005, Fevrier *et al.*, 2011). On the other hand, HIV itself has developed several mechanisms to protect infected $CD4^+$ T cells from cell death and to extend the viral reservoir. For instance, HIV-1 Nef may downregulate the expression of HLA class I molecule in infected $CD4^+$ T cells resulting in their escape from HIV-1 specific CTLs. It can also inhibit the expression of proteins involved in apoptosis including ASK1, caspase 8 and caspase 3 (Kumar & Herbein, 2014). On the other hand, Nef stimulates the release of soluble factors ICAM and CD23 which makes uninfected $CD4^+$ T cells more susceptible to HIV infection (Fevrier *et al.*, 2011).

### I.5.2.2 Interaction between HIV and CD8+ cytotoxic T cells

#### a. Mechanisms of CD8+ cytotoxic T cells action

Cytotoxic T lymphocytes (CTLs) are generated in acute infection and persist during the chronic phase of HIV infection. These cells recognize HIV antigenic determinants, expressed on APCs in association with HLA class I molecules, and lyse HIV-infected cells. For that, CTLs can insert perforins into the membrane of an HIV-1 infected cell, thus facilitating the passage of granzymes B, which activate caspases that induce apoptosis (Fig. 9). They can also bind to Fas (CD95) expressed on the surface of the infected cell via the Fas ligand (CD95L). Binding of CD95 on the target cells will induce sequential caspase activation, leading to apoptosis of the infected cell (Broere *et al.*, 2011).

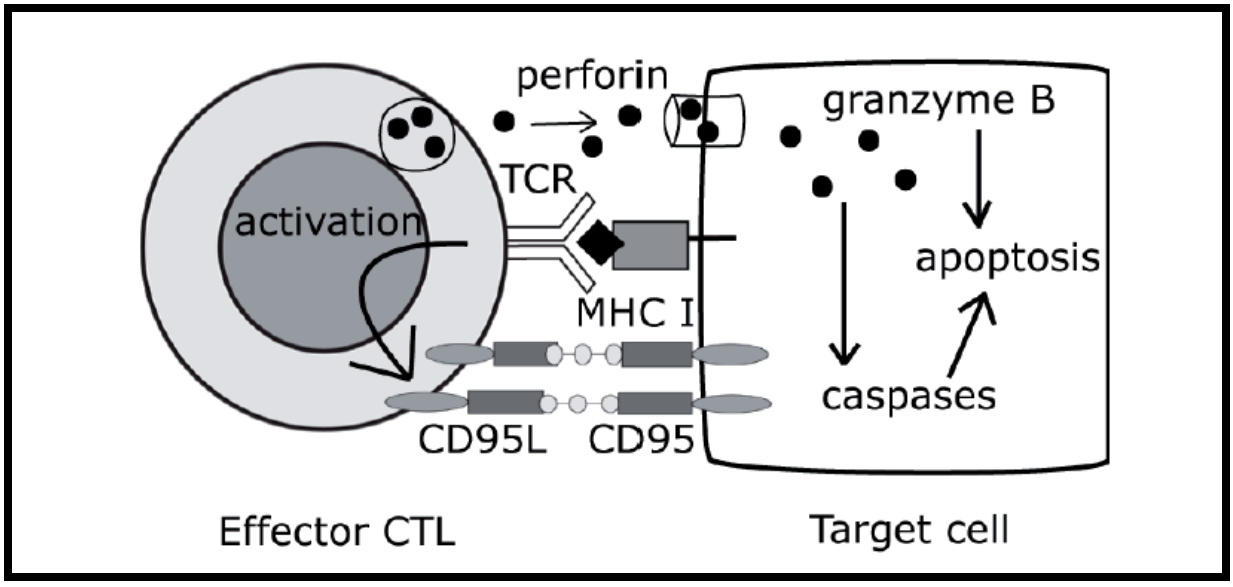



**Figure 9**: antiviral functions of HIV-specific CD8+T cells (Broere *et al.*, 2011). *Cytotoxic lymphocytes can lyse HIV-infected cells by secreting perforin and granzyme B from cytolytic granules. Perforin creates pores in the membrane of the target cell to enable granzyme B entry into the cell. Granzyme activates caspases that induce apoptosis. The second mechanism is via interaction between CD95 (Fas) and CD95L (FasL). TCR-mediated activation induces CD95L expression on the CTL. Binding of CD95 on the target cells will induce sequential caspase activation, leading to apoptosis.*

#### b. HIV mechanisms to escape CD8+ T cells action

Although HIV-1 replication can be predominantly controlled by HIV-specific CD8+ T cells, the effectiveness of CD8+ T cell responses appears to be affected by the progressive depletion of CD4+ T helper cells in advanced HIV disease. Interestingly,

antigen-specific $CD8^+$ T cells can be generated in the absence of $CD4^+$ T cell help, but the secondary expansion on antigen reencounter is inefficient (Streeck & Nixon, 2010b). In addition, $CD8^+$ T cells become progressively exhausted under repetitive antigenic stimulation or following down-regulation of MHC class I expression in APCs by HIV Nef (Davenport & Petravic, 2010, Yan *et al.*, 2013). This exhaustion is characterized by the loose of the ability to proliferate and to secrete different cytokines and chemokines. Exhausted $CD8^+$ T cells also fail to differentiate into potent memory cells and can die by apoptosis (Streeck & Nixon, 2010b, Saez-Cirion *et al.*, 2014). Finally, CTLs lose their ability to recognize HIV's genetic sequences due to the high level of viral turnover and mutation (Yan *et al.*, 2013).

### I.5.2.3 Interaction between HIV and B cells

#### a. Mechanisms of B cells action

Non-neutralizing antibodies (non-NAbs) are first to appear and generally consist of simultaneous IgM and IgG antibodies. They are unable to bind to virion Env and prevent the virus entry (Yu *et al.*, 2010). However, they are able to recognize HIV-1 Env on the surface of HIV-1–infected cells at the time of virus entry or virus assembly/budding and mediate antiviral activity through Fc effector functions (Ferrari *et al.*, 2017). Non-NAbs directed against Env gp41 antibodies are generated very rapidly whereas gp120 antibody responses are delayed as shown in Fig.10 (Alter & Moody, 2010). Later, neutralizing antibodies (NAbs) specific for the gp41 membrane proximal external region, the gp120-gp41 interface, the CD4-binding site, variable regions 1 and 2 (V1/V2) glycan, the fusion domain, and variable region 3 (V3) glycan are generated (Ferrari *et al.*, 2017). These Abs can neutralize free virions by preventing receptor engagement, or by interfering with the fusion process (Baum, 2010). They can also prevent uncoating of the viral genome in endosomes or cause aggregation of virus particles (Yu *et al.*, 2010). Unfortunately, these neutralizing antibodies are often specific for the initial native virus. Therefore, broadly neutralizing antibodies that can neutralize a wide range of HIV-1 isolates have been found in only few HIV-1 infected individuals (Baum, 2010). Both nAbs and non-nAbs are able to trigger complement-mediated lysis of free virus particles and infected cells via specific Ab-antigen binding events and complement activation. They can also bind to and coat viruses to mediate opsonization and phagocytosis by macrophages and other cells (Yu *et al.*, 2010).

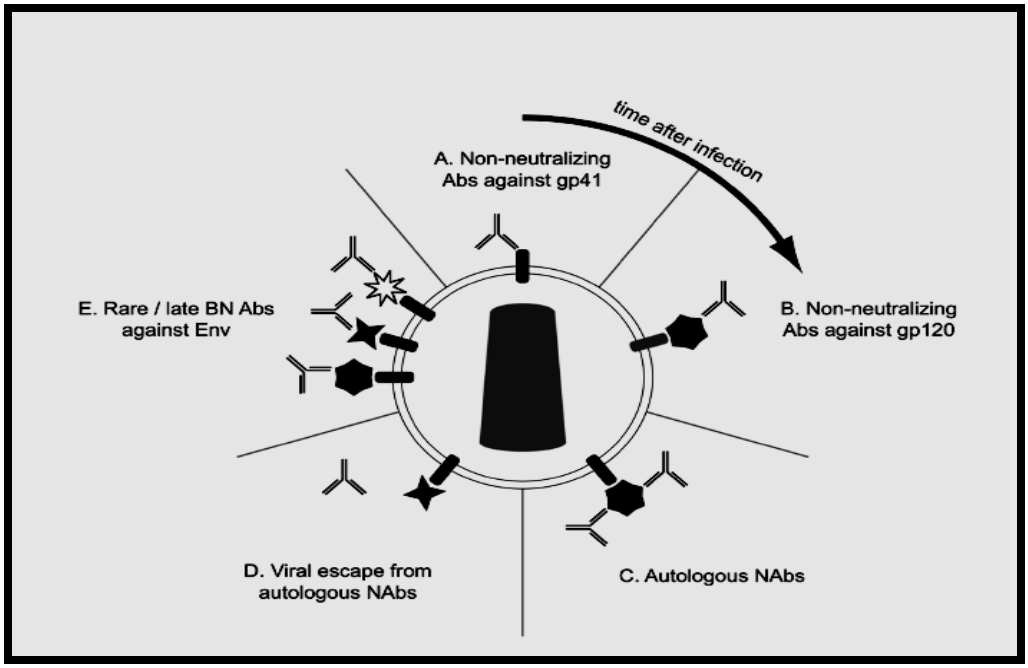



**Figure 10**: The antibody response to HIV-1 (Alter & Moody, 2010). *A, Initial antibody response to HIV-1 is non-neutralizing and directed against gp41. B, Non-neutralizing antibodies directed against gp120 arise soon thereafter. C, After a delay of weeks to months, autologous neutralizing antibodies arise that apply selection pressure on the virus. D, Viral mutation results in neutralization escape by HIV-1, represented here by a change in the shape of gp120. E, In some patients, antibodies that can neutralize a wide range of HIV-1 isolates arise, represented here by a variety of shapes of gp120. Mixing of envelope shapes on a single virus particle is shown for illustrative purposes only. BN Abs, broadly neutralizing antibodies.*

#### b. HIV mechanisms to escape B cells action

HIV-1 utilizes several mechanisms to escape nAb responses, including masking putative neutralizing epitopes with extensive glycosylation and rapid rearrangement of the glycan shield. Moreover, rapid mutation and recombination of the viral genome, replacement of typical wild-type gp120-gp41 trimers by monomeric gp120 and gp41 stumps on the viral surface prevent the recognition by induced nAbs. Although B cells are not readily infected by HIV, they serve as extracellular reservoirs for HIV-1. B cells possess the capability of circulating in peripheral blood and migrating through tissues where they can potentially interact with and pass virus to T cells (Yu *et al.*, 2010).

### I.5.3 HIV-associated chronic immune activation and inflammation

Systemic chronic immune activation is considered as the major driving force of $CD4^+$ T-cell depletion and the inability of immune system to control a wide range of potential pathogens (Lawn *et al.*, 2001). Among the causes of systemic immune

activation, there are the virus and its products, translocated bacteria or opportunistic infections (Appay & Sauce, 2008, Deeks *et al.*, 2013, Tabb *et al.*, 2013, Erlandson & Campbell, 2015, Korolevskaya *et al.*, 2016). Exposure of monocytes and macrophages to these microbial products can up-regulate the expression of TLR-2 and TLR-4. The stimulation of TLRs induces the activation of intracellular nuclear factor kappa B (NF-$\kappa$B), with subsequent production of pro- inflammatory cytokines (TNF-α, IL-1 $\beta$, IL-6 and IL-8) and chemokines (CCL2, CCL3, CCL4) responsible for attracting monocytes and other immune cells to sites of infection, which are new targets for HIV-1 infection (Appay & Sauce, 2008, Sassé *et al.*, 2012, Tabb *et al.*, 2013). In addition, IL-1$\beta$ and TNF-$\alpha$ may also decrease trans epithelial resistance in mucosal tissues, therefore promoting microbial translation and further activation (Appay & Sauce, 2008). A direct consequence of immune cell activation is the increase of NF-$\kappa$B levels, which enhance the transcription of integrated virus and therefore the production of new virions that will infect new targets (Sassé *et al.*, 2012). A vicious cycle is therefore established, during which HIV-1 replication promotes immune activation and immune activation promotes HIV-1 replication, resulting in chronic immune activation (Appay & Sauce, 2008, Sassé *et al.*, 2012, Tabb *et al.*, 2013).

Another important factor is the accelerated viral evolution, provided by an excessively high viral mutation rate and alteration in cellular tropism, resulting in progression from a pool of CCR5-tropic to dual tropic or dominantly CXCR4 tropic strains with increased virulence and broader target cell tropism (Mogensen *et al.*, 2010). Recently, immune complexes have been shown to contribute in the immunocompetent cells' activation. As shown in Fig. 11, immune complexes, together with TLR-ligands, viral antigens, translocated bacteria interact with macrophages. As a result, activated macrophages block IL-12 production and start synthesizing IL-10. High level of this cytokine slows down the development of Th1-response while up-regulate the production of antibodies. Newly synthesized antibodies' binding to viral antigens leads to continuous formation of the immune complexes capable of interacting with antigen presenting cells (Korolevskaya *et al.*, 2016). Overall, the chronic immune activation leads to general immune dysfunction and massive cellular destruction, in particular $CD4^+$ T cells through direct infection or apoptosis (Appay & Sauce, 2008, Sassé *et al.*, 2012).

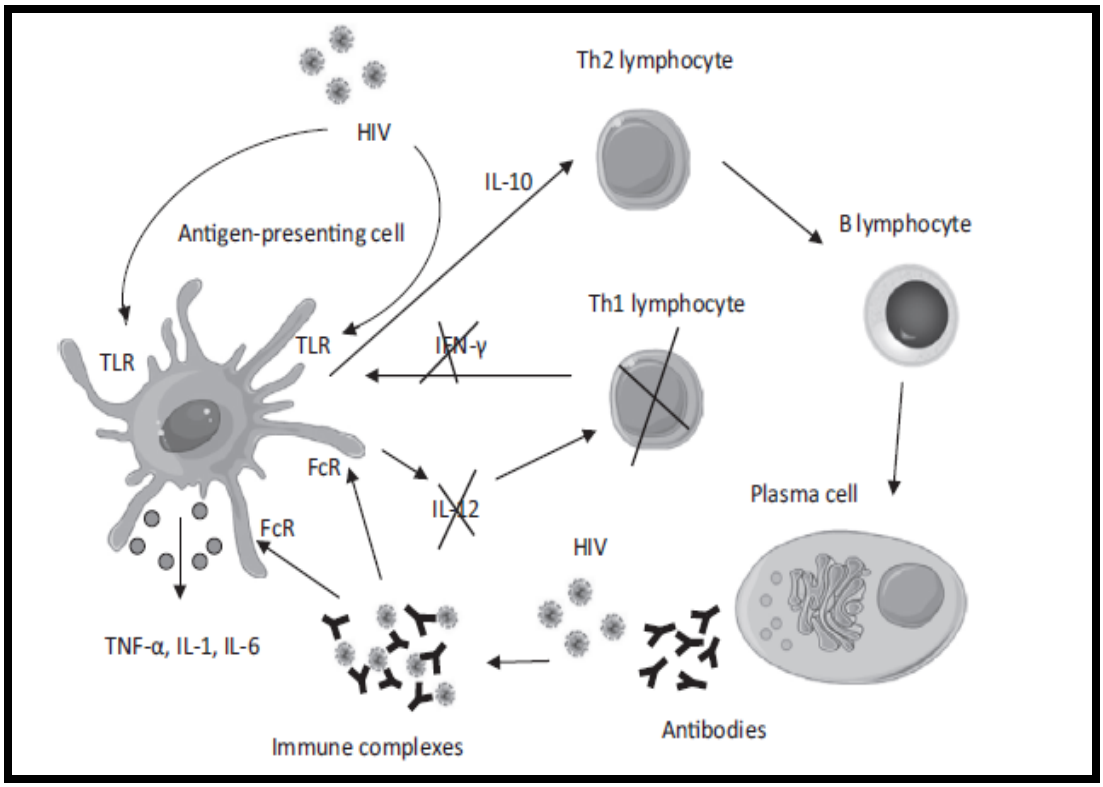



**Figure 11:** Activation of immune cells with immune complexes in HIV-infected patients (Korolevskaya *et al.*, 2016). *Immune complexes formed in the chronic phase of HIV-infection in cooperation with HIV antigens and microbial products entering the bloodstream from the damaged intestine influence the macrophages. These events result in IL-12 synthesis cessation and IL-10 production resumption. Macrophages are transformed into type II activated forms. This hinders the Th1-lymphocyte formation and biases antiviral response towards serogenesis. Plasma cells secrete sufficient amount of antiviral antibodies, which, upon binding with HIV antigens, maintain the immune complexes level capable of interacting with antigen-presenting cells.*

## I.6 HIV AND ANTIRETROVIRAL DRUGS

The current therapeutic treatment against HIV-1 includes a combination of three or more antiretroviral drugs named the highly active antiretroviral therapy (HAART), which target critical steps of the viral life cycle (Fig.12). Depending upon the steps they target, these drugs are termed as entry inhibitors, Fusion inhibitors, reverse transcriptase inhibitors, Integrase inhibitors and Protease inhibitors (Volberding & Deeks, 2010, Deeks *et al.*, 2013). HAART reduces plasma HIV RNA concentrations to undetectable values, followed by gradual reconstitution of the immune system (Volberding & Deeks, 2010, Deeks *et al.*, 2013). However, in spite of the specificity of action of these drugs, HAART fails to completely eradicate the virus in vivo (Noe *et al.*, 2005). This is due to the HIV-1 genetic variability and the high rate of HIV-1 replication, generating an estimated $10^{10}$ virions per day in an infected individual. In addition, the accumulation of archived proviral variants during the course of HIV-1 infection, and the

frequent genetic recombination lead to antiretroviral resistance (Deforche *et al.*, 2006, Konings *et al.*, 2006a, McCutchan, 2006). In addition, new infections more often result from the transmission of strains that are already resistant to one or more antiretroviral drugs (Lwembe *et al.*, 2007). Another important aspect is the inaccessibility of these antiretroviral agents to the viral reservoirs, that support ongoing low-level HIV-1 replication (Lawn *et al.*, 2001) leading to persistent immune activation and inflammation (Tabb *et al.*, 2013). On the other hand, HAART has several long-term side effects including kidney, liver and pancreatic problems, as well as changes in fat metabolism, which result in elevated cholesterol and triglyceride levels and an increased risk for strokes and heart attacks (Volberding & Deeks, 2010, Deeks *et al.*, 2013). Therefore, the development of a safe and effective prophylactic HIV-1 vaccine would be the best for the ultimate elimination of the AIDS pandemic.

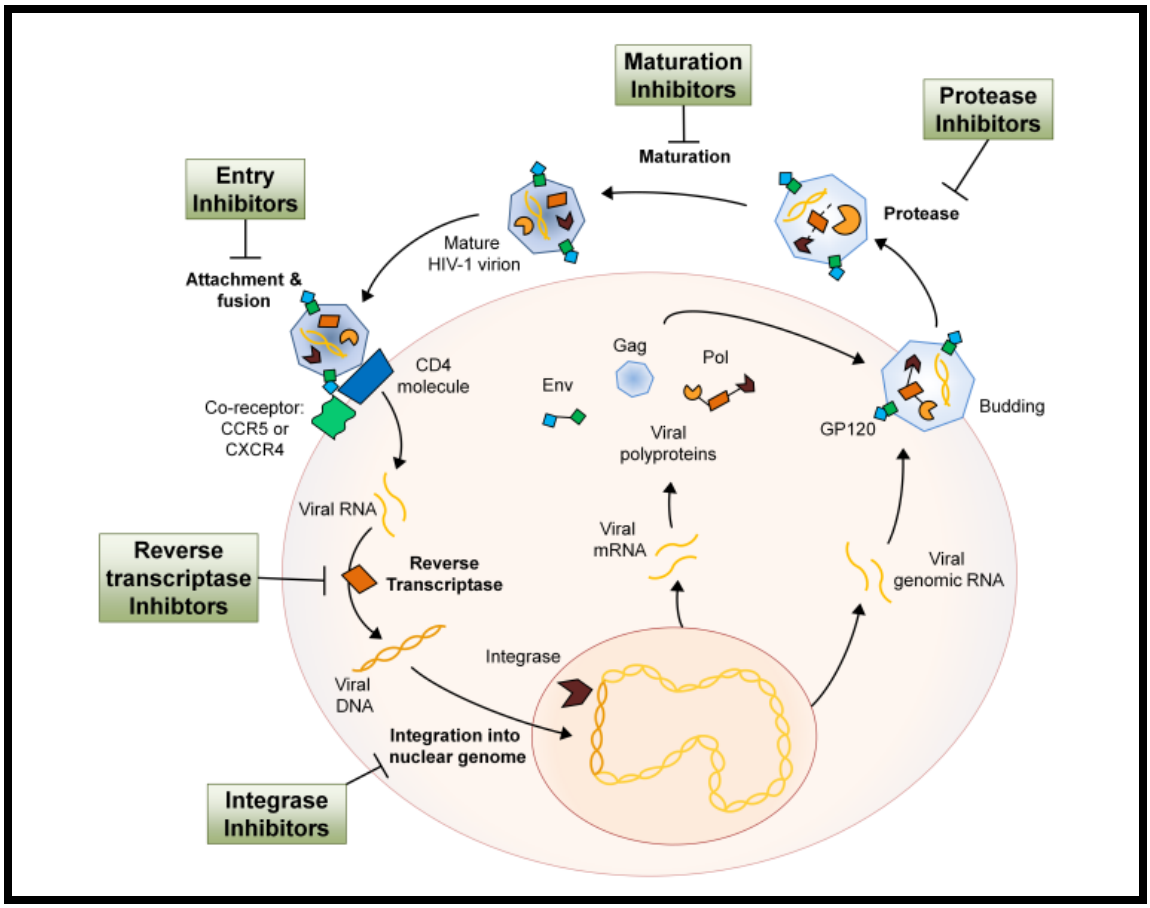



**Figure 12**: Antiretroviral therapy (Smith *et al.*, 2012). *There are currently six different classes of anti-HIV drugs.The major classes include the Entry Inhibitors, the Nucleoside Reverse Transcriptase Inhibitors, the Non-Nucleoside Reverse Transcriptase Inhibitors, and the Protease Inhibitors. The additional two anti-HIV drug classes are the Maturation Inhibitors and Integrase Inhibitors, of which most compounds are still in clinical development.*

The setting up of such a vaccine requires a well understanding of the immune functions during the HIV-1 infection. This raises up the importance of $CD4^+$ Treg cells, which are supposed to dampen immune responses following immune activation.

## I.7 FOXP3$^+$REGULATORY T CELLS

### I.7.1 Definition

Regulatory T cells are a sub-population of $CD4^+$ T cells that play a critical role in maintaining self-tolerance and homeostasis within the immune system as well as in limiting chronic inflammatory responses directed against pathogens (Sakaguchi, 2003, Li & Turka, 2010, Himmel *et al.*, 2013). Initially characterized by the constitutive expression of the IL-2 receptor alpha chain component CD25 (Sakaguchi, 2003, Zorn *et al.*, 2006, Duhen *et al.*, 2012), Treg cells were shown in further studies to express the transcription factor forkhead box P3(FoxP3), which acts as a master regulator for their development and suppressive function (Fontenot *et al.*, 2003, Sakaguchi, 2003, Chatila, 2005, Yang *et al.*, 2015). In 2006 Liu and colleagues demonstrated that FoxP3 interacts with the IL-7 receptor alpha chain (CD127) promoter, contributing to the reduced expression of CD127 in T reg cells(Liu *et al.*, 2006). Additional markers have been implicated in Treg cell suppressive function, including expression of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor family-related gene (GITR), lymphocyte activation gene 3 protein (LAG-3), ectonucleoside triphosphate diphosphohydrolase 1 (CD39), Galectin-9 (Paust *et al.*, 2004, Presicce *et al.*, 2011, Yang *et al.*, 2015). In addition, CD31, a platelet endothelial cell adhesion molecule-1, integrin $\alpha_E\beta_7$ (CD103), L-selectin (CD62L), CC chemokine receptor 7 (5CCR7) and lymphocyte function-associated antigen-1 (LFA-1) have been shown to mediate the rolling, adhesion, and extravasation of Treg cells through the high endothelial venules in peripheral lymph nodes and mucosal lymphoid organs (Simonetta & Bourgeois, 2013).

### I.7.2 Foxp3$^+$ regulatory T cell subsets

The FoxP3$^+$ Treg cell population can be divided into distinct subsets based on their development, the expression of cell surface markers and their specific tissue localizations.

### I.7.2.1 Regulatory T cell subsets based on their development

The FoxP3⁺ regulatory T cell population can be subdivided into natural and induced Treg cells based on their origin.

#### a. Natural regulatory T cells

Natural regulatory T (nTreg) cells also called thymic-derived regulatory T cells develop directly from CD4⁺ T cell precursors in the thymus during positive selection, after recognizing self-peptides in association with MHC class II with intermediate affinity (Jonuleit & Schmitt, 2003). Fully matured FOXP3⁺ nTregs exit the thymus and migrate to secondary lymphoid organs (Fig.13) where they account for 5-10% of peripheral CD4⁺ T cells (Chatila, 2005, Schmitt & Williams, 2013).

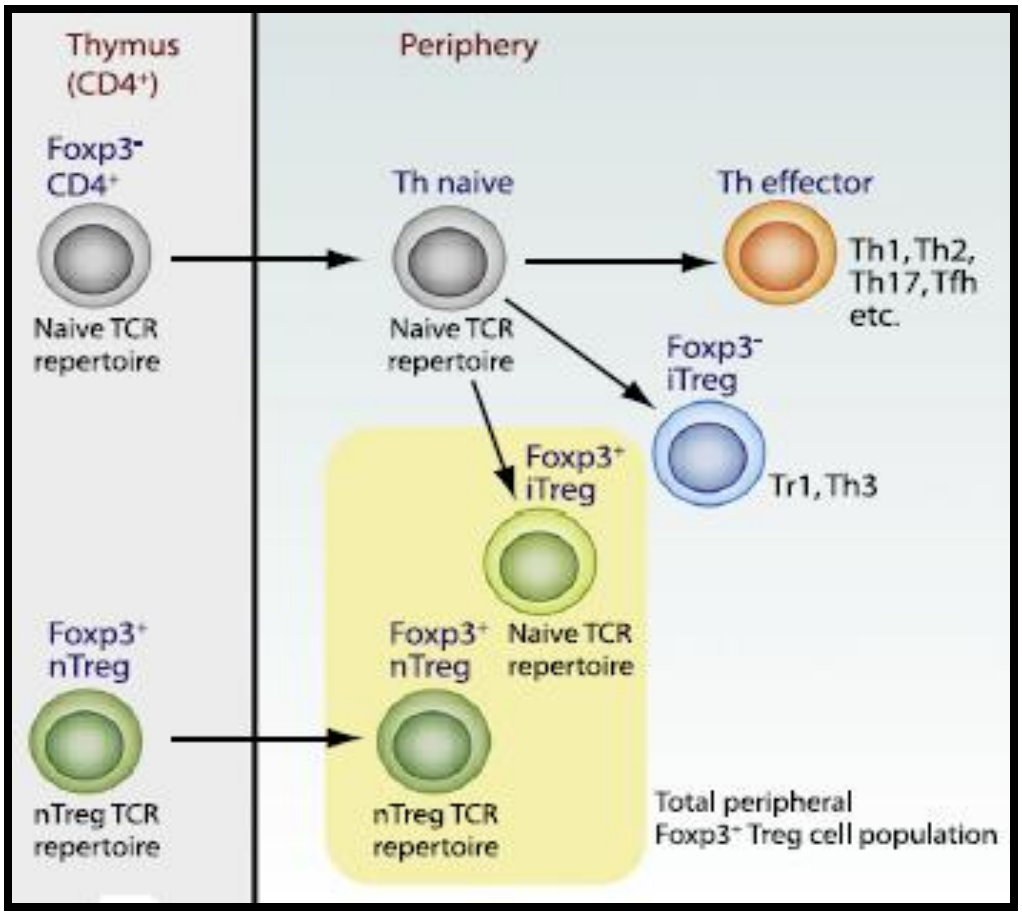



**Figure 13**: Thymic and peripheral generation of FoxP3⁺ T reg cells (Curotto de Lafaille & Lafaille, 2009). *Natural Treg (nTreg) cells differentiate in the thymus and migrate to peripheral tissues. Adaptive Foxp3⁺ Treg (iTreg) cells differentiate from mature peripheral naive CD4⁺ cells in secondary lymphoid organs and tissues. The peripheral population of Foxp3⁺ Treg cells comprises both nTreg and iTreg cells.*

### b. Induced regulatory T cells

Unlike nTreg cells, induced Treg (iTreg) cells differentiate in secondary lymphoid organs and tissues from mature $CD4^+$ T cells in response to specific antigens (Aandahl *et al.*, 2004, Schmitt & Williams, 2013). Induction of Foxp3 expression in peripheral naive $CD4^+$ T cells is facilitated by high amounts of transforming growth factor-β (TGF-β), IL-2 and retinoic acid (Toda & Piccirillo, 2006, Josefowicz *et al.*, 2012, Yang *et al.*, 2015). TGF-β limits DNA methyltransferase I recruitment to the Foxp3 locus, a molecule that normally blocks Foxp3 induction after TCR stimulation. IL-2 has a role in Treg cell survival, proliferation, and stability. IL-2 and retinoic acid function to limit the polarization of activated $CD4^+$ T cells into the Th17 lineage under inflammatory conditions (Sakaguchi *et al.*, 1995, Curotto de Lafaille & Lafaille, 2009, Kushwah & Hu, 2011, Yang *et al.*, 2015).

## I.7.2.2 Regulatory T cell subsets based on their specific tissue localizations

Tissue-resident Treg cells specify those Treg cells that have a long-term residence in non-lymphoid tissues. So far, four types of tissue-resident Treg cells have been identified: T-$bet^+$, Bcl-$6^+$, $STAT3^+$ and PPAR-$\gamma^+$ Treg cells (Josefowicz *et al.*, 2012, Yang *et al.*, 2015).

### a. T-box gene encoded transcription factor expressing $Foxp3^+$Treg cells

T-box gene encoded transcription factor (T-$bet^+$) $Foxp3^+$Treg cells are generated in tissues such as the skin or the lung, from activation of STAT1 in response to IFN-γ. These cells express CXCR3 and CCR4 which enable them to migrate, proliferate and accumulate at the type 1 inflammation sites (Josefowicz *et al.*, 2012). $Tbet^+$ $Foxp3^+$Treg Treg cells can then inhibit either Th1-cell differentiation or IFN-γ synthesis through TGF- β1 production (Sojka & Fowell, 2011, Zhang *et al.*, 2014).

### b. B cell lymphoma protein- 6 expressing $Foxp3^+$Treg cells

B cell lymphoma protein (Bcl)-6 expressing Treg cells also called follicular Treg cells are found within the germinal center (Vanderleyden *et al.*, 2014). These cells are able to regulate the quality and quantity of B cells, thus favoring the selective expansion of antigen-specific B cells (Josefowicz *et al.*, 2012, Yang *et al.*, 2015).

**c. Signal transducer and activator of transcription 3 expressing Foxp3⁺Treg cells**

Signal transducer and activator of transcription 3 (STAT3) expressing Treg cells are found in the gut. These cells express CCR6 and are recruited to Th17 inflammation sites in order to suppress Th17 responses (Sakaguchi *et al.*, 2009).

**d. Peroxisome proliferator-activated receptor gamma expressing Foxp3⁺Treg cells**

Peroxisome proliferator-activated receptor gamma (PPAR-γ) expressing FoxP3⁺ Treg cells are generated in adipose tissue. The chemokine receptors for trafficking of adipose tissue Treg cells are unknown. The homeostatic mediators for adipose tissue Tregs include lipids and long chain fatty acid (Yang *et al.*, 2015).

### I.7.2.3 Subsets based on the cell surface markers

FOXP3-expressing Treg cells can be divided into naïve, effector and memory subsets according to the expression of cell surface markers.

**a. Naive regulatory T cells**

Naive Treg cells are generated from the thymus and preferentially migrate to the periphery where they represent approximately 20–30% of total CD4⁺Foxp3⁺ Treg cells. These cells are essential to control immune responses via continuous replenishment of the fully-suppressive activated-Treg pool with memory phenotype (Silva *et al.*, 2016). Naive Treg cells highly express CD45RA, CD62L, CCR7, CD27 and CD31 markers, until they encounter their cognate Ag and become activated in the periphery (Miyara & Sakaguchi, 2011, Himmel *et al.*, 2013). They then acquire phenotypic features of effector or memory Treg cells (Campbell & Koch, 2011).

**b. Effector regulatory T cells**

Effector regulatory T (eTreg) cells constitute a minor fraction of Treg cells in the circulation and secondary lymphoid organs, which express low levels of CD45RA, CD62L, CCR7 and CD27 but high levels of CD103 (Miyara & Sakaguchi, 2011, Yang *et al.*, 2015). These cells are highly proliferative in vivo, and their increased number has been shown to be associated with disease progression (Simonetta & Bourgeois, 2013). Antigen-stimulated eTreg cells potently suppress the proliferation and cytokine

production of responder T cells *in vitro* and they are prone to die by apoptosis (Simonetta *et al.*, 2012). Effector Treg cells comprise Inducible Costimulatory molecule (ICOS) and HLA-DR involved in cell activation. Differential expression of ICOS has been shown to delineate $ICOS^+$ and $ICOS^-$ Treg cells that preferentially produce IL-10 and TGF-β, respectively (Zhang *et al.*, 2014). ICOS expressing eTreg cells are highly observed in untreated viremic chronically infected individuals (Simonetta & Bourgeois, 2013). Expression of HLA-DR helps also to delineate $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ effector Treg cells (Miyara & Sakaguchi, 2011). In chronically HIV-1 infected people, HLA-DR is usually co-expressed with CD39 and both are involved in the suppression of HIV-specific responses as well as in the control of HIV-1 viral replication (Simonetta & Bourgeois, 2013).

**c. Memory regulatory T cells**

According to the location, memory Treg cells are divided into central and effector memory cells. The central memory cells express CD45RO, CD27, CCR7 and CD62L and reside in lymphoid organs. Some of these have been found to migrate into certain inflammation sites depending on the expression of chemokine receptors such as CCR4, CCR6, and CXCR3. The other $CCR7^-$ subset with low CD62L and CD27 expression represents effector memory cells. Upon contact with the appropriate Ag, effector memory Treg cells can execute effector functions instantly, whereas central memory Treg cells can rapidly proliferate, expanding and acquiring effector functions (Broere *et al.*, 2011).

## I.7.3 Functions of $Foxp3^+$ regulatory T cells

$FOXP3^+$ Regulatory T cells are essential for maintaining immune homeostasis by shutting down immune responses after they have successfully eliminated invading organisms (Rudensky & Campbell, 2006). In addition to adaptive immune cells such as T and B cells, Treg cells also inhibit the function of innate immune cells including antigen-presenting cells, such as macrophages and DCs (Yang *et al.*, 2015). They also play active role in preventing autoimmune, immunopathological and allergic diseases by establishing and maintaining tolerance to self and foreign antigens (Keynan & Rubinstein, 2008). For instance, defective Treg cells resulted from mutations of the FoxP3 gene have been associated with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is characterized by severe multi-organ

autoimmune diseases, allergy and inflammatory bowel disease (Miyara & Sakaguchi, 2011). Beneficial roles for Foxp3$^+$ Treg cells have also been described in limiting asthma, as well as damage caused by inflammatory responses to infectious agents (Keynan & Rubinstein, 2008), in tissue repair, in regulating transplantation reactions (Yamazaki, 2006) and in maternal tolerance to the fetus (Sakaguchi *et al.*, 2009). In addition, Foxp3$^+$ Treg cells have protective roles in host defense against specific viruses, such as herpes simplex virus 2, West Nile virus, Dengue virus, parasites like *Plasmodium berghei*, *Toxoplasma gondii* and fungi as *Candida albicans*. They also protect commensal bacteria from elimination by the immune system (Rowe *et al.*, 2011). However, Foxp3$^+$ Treg cells mediated suppression have been shown to impede host defense following infection with bacterial pathogens like *Listeria monocytogenes*, *Salmonella enteric*, *Francisella tularensis* (Rowe *et al.*, 2011) or *Mycobacterium tuberculosis* (Li & Wu, 2008). In the same way, T reg cells have been demonstrated to suppress antigen-specific CD4$^+$ and CD8$^+$T cell responses directed against tumors, as well as NK-cell mediated cytotoxicity, thus contributing in immune evasion by certain malignancies (Taams *et al.*, 2005).

## I.7.4 Mechanisms of Foxp3$^+$ regulatory T cells action

The suppressive function of Treg cells in the maintenance of self-tolerance and prevention of the development of autoimmune and chronic inflammatory diseases is mediated by different mechanisms such as direct cell-cell contact, secretion of inhibitory cytokines, or competition for growth factors.

### I.7.4.1 Cell-cell contact – dependent mechanisms

The cell-cell contact dependent mechanism relies on interaction of receptor–ligand pairs on Treg cell and target cell (Sojka *et al.*, 2008) and involves different pathways including:

- Direct cytolysis via releasing of granzyme and perforin molecules;
- Delivering a negative signal to responder T cells via up-regulating intracellular cyclic adenosine monophosphate (cAMP) by CD39 and CD73;
- Modulating APC function via Treg–cytotoxic T-lymphocyte antigen-4 (CTLA-4) engagement of B7 on DCs.

### a. Granzyme and perforin dependent pathways

During Treg-effector cell interaction, Treg cells produce granzymes and perforins. Once released from the Treg cell, perforin molecules insert themselves into the lipid membrane of the target cell and polymerize in the presence of calcium ions to form a pore through which granzymes enter the cell. Once within the target cell, Granzyme B could induce apoptosis (Fig. 14) by caspase-dependent or independent mechanisms, thus decreasing the number of effector cells and controlling the immune response (Arce-Sillas *et al.*, 2016a). While granzyme A acts by facilitating migration of Treg cells through extracellular tissues, granzyme B is required for cytolytic regulation of inflammation by Treg cells during acute viral infection (Cao *et al.*, 2009).

### b. Production of cyclic adenosine monophosphate by CD39 and CD73

In the immune system, extracellular adenosine triphosphate (ATP) functions as a natural adjuvant or danger signal that activates the immune system. It can stimulate monocytes to release IL-1β, which mediates the acute phase response or the maturation of DCs (Lopez-Abente *et al.*, 2016). It is also an indicator of tissue destruction by activating pro-inflammatory responses, and inducing local pain. High doses of ATP are toxic for most cells, it seems to induce both necrosis as well as apoptosis (Borsellino *et al.*, 2007).

Treg cells are particularly sensitive to ATP and they respond to high doses of ATP by producing the CD39 or ectonucleoside triphosphate diphosphohydrolase-1 (NTDPase 1), which hydrolyzes the pro-inflammatory ATP to adenosine monophosphate (AMP) (Fig. 14) which in turn is converted to adenosine by the ecto-5'-nucleotidase CD73 (Deaglio *et al.*, 2007). Adenosine produced by Treg cells can then bind to the adenosine 2A receptor (A2AR) on the target cells, consequently inducing the adenylyl cyclase activity and, therefore, increasing the intracellular cyclic AMP (cAMP) level (Lopez-Abente *et al.*, 2016). Increased levels of cAMP elicit inhibitory functions in DCs and activated T cells (Sojka *et al.*, 2008, Jenabian *et al.*, 2012, Lopez-Abente *et al.*, 2016).

### c. Modulation of Antigen Presenting Cell function

Regulatory T cells express several surface molecules which allow them to adhere, interact and modulate DCs activity. These molecules include neuropilin-1, LFA-1, LAG-3 and CTLA-4. These molecules can help Treg cells to aggregate around DCs in a

CTLA-4-independent fashion and to prevent the access of responder T cells to DCs (Sakaguchi *et al.*, 2009). They can also inhibit the maturation and co-stimulatory capacity of DCs in a CTLA-4-dependent fashion (Josefowicz *et al.*, 2012). The interaction between CTLA-4 on Treg cells and the co-stimulatory receptor, B7 on DCs can condition DCs to express IDO which catalyzes the conversion of tryptophan to kynurenine (Fig.14), a pro-apoptotic metabolite (Paust *et al.*, 2004). This provides immunosuppressive effects in the local environment of DCs by cytotoxicity or by the *novo* generation of Treg cells (Velavan & Ojurongbe, 2011). Other molecules such as T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif domains (TIGIT) and galectin-1 have been shown to interact also with receptors on DCs. This interaction results in cell cycle arrest, apoptosis, inhibition of pro-inflammatory cytokines production and induction of immunosuppressive cytokines production, such as IL-10 and TGF-β by DCs (Josefowicz *et al.*, 2012).

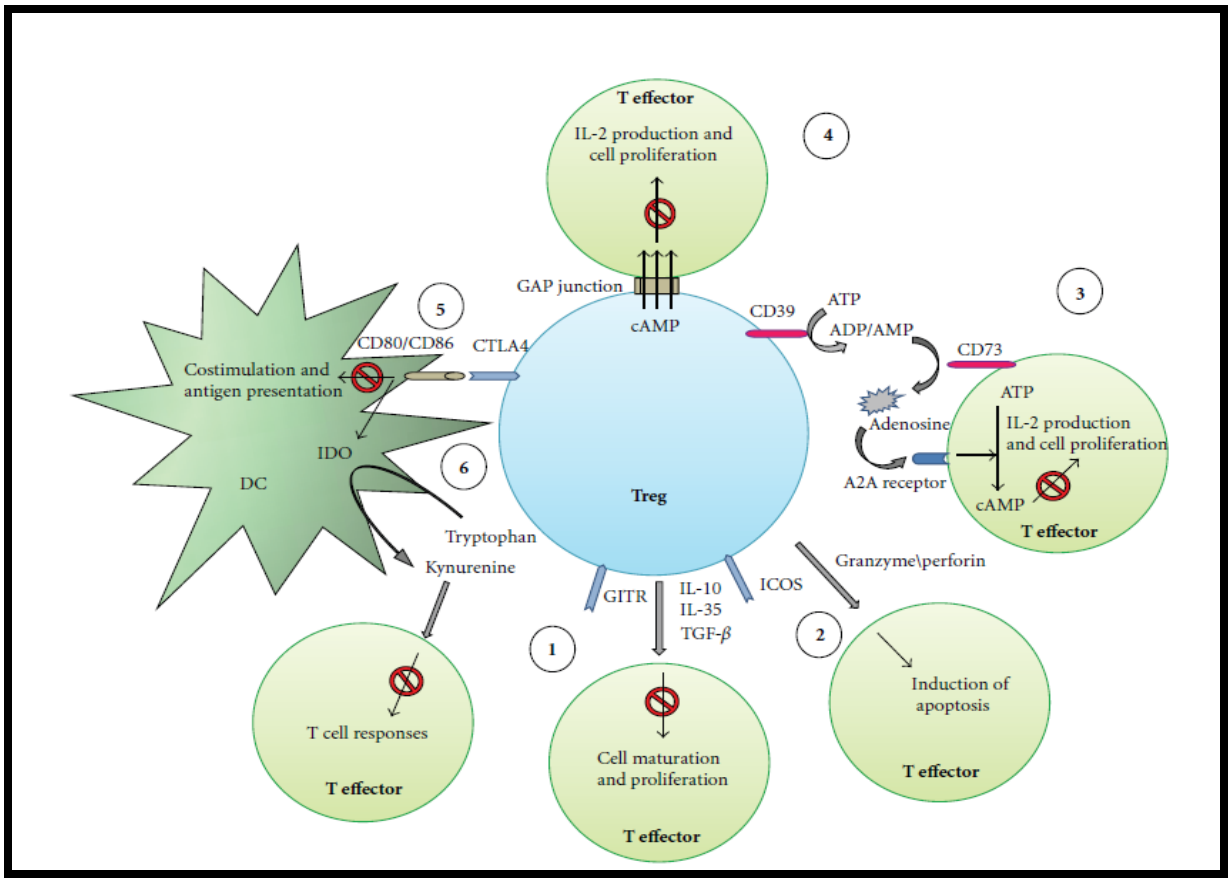



**Figure 14**: Mechanisms of Treg cells action (Jenabian *et al.*, 2012).*The ability of Treg cells to induce immune suppression is mediated via (1) inhibitory cytokines; (2) induction of apoptosis by granzyme/perforin pathway; (3) CD39/CD73/adenosine pathway; (4) direct transfer of cAMP via GAP junction; (5) inhibition of DC function by CD80/CD86 and CTLA-4 interaction; and (6) the catabolism of tryptophan via IDO enzyme*

### I.7.4.2 Secretion of inhibitory cytokines

Regulatory T cells can exert their immunosuppressive activity by directly secreting cytokines such as IL-10 and TGF-β or by inducing APCs to secrete such factors.

#### a. Interleukin-10

The anti-inflammatory cytokine, IL-10 inhibits the maturation and activation of DCs by downregulating the MHC class II and costimulatory molecules on DCs as shown in Fig. 15 (Rudensky & Campbell, 2006, Velavan & Ojurongbe, 2011). In addition, IL-10 suppresses Th1/2 cell proliferation and INF-$\gamma$ production via inhibition of IL-2. It also promotes the phagocytic activity, increasing the removal of cellular debris at the inflammation site (Arce-Sillas *et al.*, 2016a). In 2009, Sakaguchi and colleagues reported that IL-10-dependent mechanism is important for mucosal (colon and lungs) immune homeostasis.

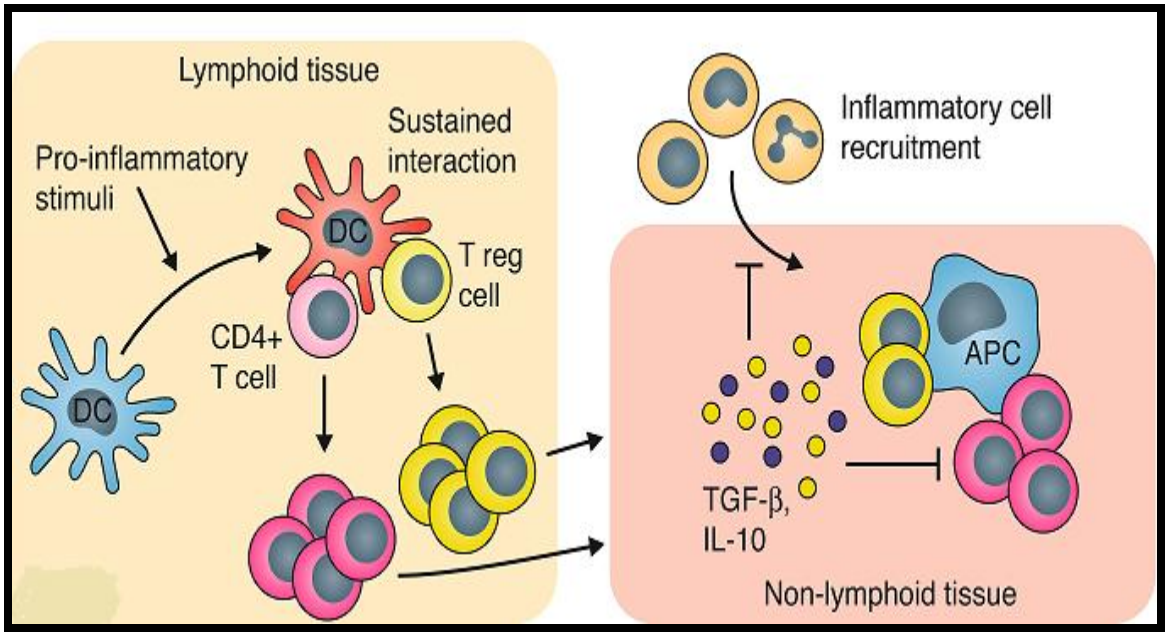



**Figure 15:** IL-10 and TGF-β mediated suppression (Rudensky & Campbell, 2006). *Upon priming and migration to nonlymphoid tissues, production of antiinflamatory cytokines, such as IL-10 or TGF-β, by T reg cells may limit effector T cell function and inhibit recruitment of inflammatory myeloid cells such as neutrophils, eosinophils, and monocytes.*

#### b. Transforming Growth Factor- β

Transforming growth factor-β (TGF-β) may act as a mediator of suppression either in a soluble form or in a membrane-bound form by conditioning responder T cells to be sensitive to suppression (Sakaguchi *et al.*, 2009). Like IL-10, TGF- β also down regulates the MHC class II expression and co-stimulatory molecules on DCs (Velavan &

Ojurongbe, 2011). In addition, TGF-β can suppress T -cell proliferation (Fig. 15) by inhibiting the production of IL-2, through direct inhibition of IL-2 promoter activity (Sojka *et al.*, 2008). However, this activity of TGF- β has minimal effects on activated T cells when compared with naive T cells, probably due to reduced TGF-β receptor II expression on activated T cells (Wan & Flavell, 2007a).

Upon priming and migration to non-lymphoid tissues, both IL-10 and TGF-β may limit effector T cell function and inhibit recruitment of inflammatory myeloid cells such as neutrophils, eosinophils, and monocytes (Rudensky & Campbell, 2006).

**c. Interleukin- 35**

IL- 35 is a heterodimer composed of the p35 and Ebi3 subunits (Collison *et al.*, 2007, Arce-Sillas *et al.*, 2016b). It acts on its target cells following binding to the IL-35 receptor composed of IL-12Rβ2 and gp130. While gp130 is expressed in most cell types, IL-12R β2 is expressed predominantly on activated T cells, NK cells, and to a lesser extent DCs and B cells. IL-35 is able to suppress $CD4^+$ and $CD8^+$ T-cell proliferation, antibody responses and Th17 differentiation and function in many inflammatory diseases such as encephalomyelitis and inflammatory bowel disease. IL-35 can also promote the expansion of IL-10 producing iTregs (Olson *et al.*, 2013).

### I.7.4.3 Inhibition of effector T cells activity by Competition

Regulatory T cells can compete with effector T cells for growth factors or for APC co-stimulation.

**a. Competition for growth factors**

Regulatory T cells do not secrete IL-2 but are dependent on the provision of this cytokine by paracrine sources to expand in the periphery and activate their immunosuppressive function. The constitutive expression of CD25, the IL-2 receptor α chain by Treg cells gives them an initial competitive advantage for the consumption of IL-2 over naive T cells, which express CD25 only after TCR stimulation. In turn, IL-2-uptake activates Treg cells and may also modulate their activity through enhanced IL-10 production (Sojka *et al.*, 2008). Thus, IL-2 consumption from the surroundings by Treg cells has profound consequences on the activation (Sakaguchi *et al.*, 2009) and proliferation (Josefowicz *et al.*, 2012) of other effector T cells. It may also induce their death by apoptosis via Bcl-2 interacting mediator of cell death.

### b. Competition for APC co-stimulation

CD28 is the most important co-stimulatory receptor on naive T cells which interacts with its ligands, CD80 (B7.1) and CD86 (B7.2) during T-cell activation by DCs, while CTLA-4 expressed on Treg cells is a negative regulator opposing CD28 stimulation (Yamazaki, 2006). CTLA-4 binds to the same ligands as CD28, albeit with a 20-fold (for CD80) and even 100-fold (for CD86) higher affinity than CD28 (Hunig, 2007). The interaction between CTL-4 on Treg cells and B7 on DCs down regulates CD80 and CD86 expression induced by antigen-specific effector cells (Fig. 16). As a consequence, the capacity of DCs to stimulate naive T cells through CD28 is limited, resulting in specific immune suppression and tolerance (Wing *et al.*, 2008).

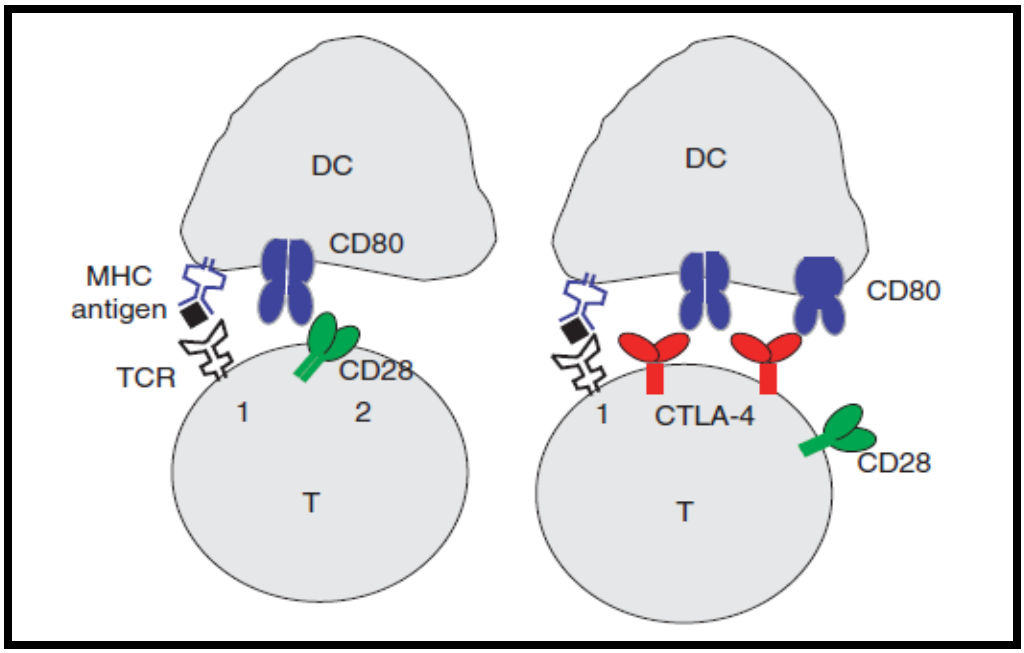



**Figure 16**: Co-stimulation of the T-cell response by CD28 and its counter regulation by CTLA-4 (Hunig, 2007). *Besides the displacement of CD28 by bivalent CTLA-4 molecules, intracellular signaling events contribute to counterregulation by CTLA-4.*

## I.7.5 Foxp3⁺ regulatory T cells in HIV infection

Based on their immunoregulatory properties, Treg cells are expected to dampen excessive immune activation caused by HIV-1 infection, thus ensuring normalcy. However, previous reports present conflicting pictures of the role of Treg cells during the course of HIV-1 infection (Antons *et al.*, 2008, Cao *et al.*, 2009, Moreno-Fernandez *et al.*, 2009, Macatangay & Rinaldo, 2010).

### I.7.5.1 Susceptibility of regulatory T cells to HIV-1 infection

As all $CD4^+$ T cells, Treg cells express chemokine receptors CCR5 and CXCR4 required for the entry of HIV-1 into the host cells and are therefore, potential targets for the virus. Brezar and colleagues have developed an HIV-1 infection model using humanized mice and found that $Foxp3^+$ Treg cells were preferentially infected and depleted by apoptosis, indicating that Treg cells are primary target cells for HIV-1 infection (Brezar *et al.*, 2016). By assessing the susceptibility of Treg cells to different HIV strains, Antons *et al.* (2008) have demonstrated a 10-fold higher HIV infection rate of naive Treg cells compared with conventional $CD4^+$ T cells with R5 tropism viruses during the early stages of infection. In contrast, Moreno-Fernandez *et al.* (2009) found a lower susceptibility of Treg cells to R5 viruses compared to non-Treg cells at both early and late time points of infection. This was explained by a decreased activation state of Treg cells compared with conventional $CD4^+$ T cells, as the activation state of the target cell markedly affects the efficiency of the early steps of HIV replication. However, Treg cells were more susceptible to X4 viruses at early time points compared to the late time points of the virus life cycle. Instead of using R5 or X4 tropic HIV-1 strain, Jiang and colleagues used the more pathogenic X4/R5 dual-tropic HIV-1 strain (Jiang *et al.*, 2008). They showed that this strain infects and replicates in $CD4^+FoxP3^+$Treg cells more efficiently than in $CD4^+FoxP3^-$ T cells, particularly in lymphoid tissues during acute infection when most human $CD4^+$ T cells are quiescent. Later in 2013, Simonetta & Bourgeois reported that, the susceptibility of Treg cells to HIV infection depends on the Treg cells phenotype. While both naive and effector Treg cells were similarly susceptible to CXCR4-tropic HIV-1 strain, effector Treg cells showed more susceptibility than naive Treg cells to *in vitro* HIV infection by CCR5-tropic HIV-1 strain. The differences in viral strains infection susceptibility was explained by the fact that, effector Treg cells expressed high levels of the HIV co-receptor CCR5 and lower levels of CXCR4 when compared to naive Treg cells.

As a consequence of direct HIV-infection, the absolute number of peripheral Treg cells will decline proportionally with the $CD4^+$ T cell population in the absence of therapy (Kared *et al.*, 2008, Macatangay & Rinaldo, 2010, Angin *et al.*, 2012, Arruvito *et al.*, 2012). In contrast, increased proportions of Treg cells in the peripheral blood from untreated HIV-1 infected individuals were reported (Cao *et al.*, 2009, Macatangay & Rinaldo, 2010).

This was explained by the high rate of conversion from conventional $CD4^+$T cells to Treg cells, resulting from intensive T cell activation associated with HIV-1 infection (Kinter *et al.*, 2004). In addition, the relative expansion of Treg cells has been attributed to a longer lifespan of Treg cells as well as the sparing of Treg cells from the activation-induced apoptosis of uninfected cells due to their low proliferation capacity (Cao *et al.*, 2009).

### I.7.5.2 Role of regulatory T cells in HIV infection

HIV infection is hallmarked by a chronic immune activation, which is correlated with $CD4^+$ T-cell depletion, several immune dysfunctions, and disease progression (Keynan & Rubinstein, 2008). One of the immune mechanisms capable of controlling the activation and expansion of immune cells is the suppressive function exerted by Treg cells. In 2008, Chase and collaborators observed that Treg cells frequency and function were preserved among elite controllers, suggesting a role of these cells in limiting immune activation(Chase *et al.*, 2008). In the same line, Jiao *et al.* (2009) observed a significant decrease of Treg cell numbers during HIV disease progression in the progressor group compared to long-term non-progressor individuals. Other studies reported that, during primary HIV-1 infection, Treg cells can increase in number and then regulate HIV-induced immune hyperactivation by suppressing both effector molecules production in response to HIV and proliferation of HIV- specific T cells (Weiss *et al.*, 2004, Velavan & Ojurongbe, 2011, Angin *et al.*, 2012). This activity of Treg cells, thus contributes in reducing the availability of target cells for HIV replication, limiting tissue damage, preventing the apoptosis of uninfected $CD4^+$ T cells and allowing sufficient immune pressure against the pathogen (Moreno-Fernandez *et al.*, 2009, Owen *et al.*, 2010, Velavan & Ojurongbe, 2011). Therefore, in some HIV-infected individuals where Treg cells are depleted, the active suppression of effector T cells decreases leading to an excessive T cell hyperactivation and subsequently to rapid disease progression (Antons *et al.*, 2008). A recent study by Brezar *et al*. (2016) showed that Treg cell depletion from HIV-chronically infected humanized mice significantly induced enhanced human T cell activation and increased HIV-1 viral replication, suggesting that Treg cells actively suppressed immune activation as well as HIV-1 replication during the chronic phase of HIV-1 infection.

Beside the beneficial effect of the suppressive activity of Treg cells, the proliferation of these cells may hamper the establishment of HIV-specific $CD4^+$ T cell responses,

especially during acute HIV infection. As a consequence, the immune system will fail to eradicate HIV-1 and other pathogens, resulting in a rapid progression to AIDS. This was well illustrated by Brezar and colleagues who showed a significant decline in HIV-1 infection when Treg cells were depleted before HIV-1 infection using the humanized mouse model (Brezar *et al.*, 2016). This result suggested that Treg cells may contribute to the suppression of anti-HIV immunity and to the efficient HIV-1 acute infection. In the same line, Macatangay and colleagues (Macatangay & Rinaldo, 2010, Macatangay *et al.*, 2010) demonstrated that, the increase in Treg frequency and activity affected the ability of $CD8^+$T cells to secrete cytokines in response to DC-HIV-1 peptide vaccine. In contrast, the increased polyfunctionality of the $CD8^+$ T cell response to Gag peptide was observed after Treg depletion. Indeed, Treg cells expansion in untreated HIV-1 patients has been associated with an increased activation of $CD8^+$ T cells, again supporting the detrimental effect of Treg cells in the course of the infection (Macatangay & Rinaldo, 2010). Considering that Treg cells are susceptible to HIV, they may constitute a potent reservoir and also have its function and phenotype profile altered, thus leading to consider infected Treg cells as an obstacle to efficient control of HIV infection. Several studies showed that Treg cells infected with HIV-1 were less potent in suppressing autologous $CD8^+$ and $CD4^+$ cell proliferation as compared to uninfected Treg cells. This impairment on Treg cells function was associated with decrease of FoxP3 and CD25 expression (Pion *et al.*, 2013). Another study published by Kared and colleagues (Kared *et al.*, 2008) suggested that HIV is a persistent antigen that might trigger continuously long-life HIV-specific memory Treg cells, resulting in an immune tolerance to HIV *in vivo*.

Although the suppressive activity of Treg cells may contribute to robust viral replication in the acute phase of HIV-1 infection, Treg cells may also be important in controlling immune activation and HIV-1 replication during chronic HIV-1 infection.

### I.7.5.3 Regulatory T cells as a potential target for HIV immunotherapy

The role of Treg cells in HIV infection is complex. They play a dual role by decreasing immune activation, which is beneficial for HIV-infected individuals, but also by suppressing HIV-specific immune responses. Their expansion or depletion should be therefore, exploited for therapeutic purposes. However, it may be ideal to identify the specific mechanisms used by Treg cells to suppress HIV -specific immune responses and the effect of this suppression activity at each stage of HIV infection in order to test

strategies for enhancing immune control and to prevent the enhancement of autoimmunity. During the acute phase of HIV infection, Treg cell suppression is necessary to limit the excessive immune activation. For instance, both *ex vivo* expansion with adoptive transfer and *in vivo* manipulation to expand and increase the function of endogenous Treg cells can be exploited. Previous studies have reported that HIV-specific Treg cells can be generated and expanded *in vitro* from naive $CD4^+$ T cells in the presence of a peptide HIV-antigen (Taams *et al.,* 2005, Allan *et al.*, 2008, Singer *et al.*, 2014). These cells can then be administered in the periphery with the knowledge that they will migrate to the anatomic location required for optimal function (Allan *et al.*, 2008). Another strategy could be the treatment of HIV-infected individuals with IL-2, which leads to the proliferation of Treg cells capable of suppressing HIV-specific T-cell proliferation (Jenabian *et al.,* 2012). In contrast, during chronic HIV-infection, the suppressive activity of Treg cells could be inhibited in order to boost HIV-specific T effector responses. Several studies have shown that, blockade of IL-2 receptor alpha chain component could inhibit Treg cell activity and therefore, boost HIV-specific T effector responses. In the same way, the administration of anti-CTLA-4 antibody and the IDO inhibitor (1-methyl-D-tryptophan) may result in increased anti-HIV responses but also decreased viral RNA levels in lymph nodes (Macatangay & Rinaldo, 2010).

Whatever the stategy used, additional efforts are required to surmount significant barriers including Treg cell functional stability, Treg cell plasticity, isolation and storage of Treg sub-populations, as well as off-target effects.

A prerequisite for this approach is therefore to characterize Treg cells in terms of phenotype, quantity and function in the methodological context presented below.

# CHAPTER II. MATERIAL AND METHODS

## II.1 TYPE, DURATION AND SITE OF STUDY

This study was a cross-sectional study carried out from March 2015 to January 2017 in the Microbiology and Immunology Laboratory of the Chantal Biya International Reference Centre (CIRCB) for research on HIV/AIDS Prevention and Management.

## II.2 STUDY POPULATION

The study participants were selected from the CIRCB African HIV-1 dendritic cell targeted vaccine (AFRODEC) cohort established since 2012 within the frame work of an HIV Vaccine Development Program. Members of the AFRODEC cohort were expected to visit CIRCB at least twice for clinical, immunological and virologic monitoring. The control group were age matched HIV-1 negative individuals who were randomly selected from the general population and invited to voluntarily participate in the study.

### II.2 .1 Inclusion criteria

HIV-1 infected participants were:

- naive to anti-retroviral treatment;
- aged between 21 and 65 years old;
- not pregnant for women;
- free of other infections like Tuberculosis (TB), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), malaria, and Dengue virus.

Age-matched Control participants consisted of people who were:

- HIV negative;
- free of the aforementioned infections;
- not pregnant for women.

### II.2.2 Sample size

The sample size was estimated using the following formula (Lwanga *et al.*, 1991):

$$n = Z^2_{1-\alpha/2} \frac{1-P}{\varepsilon^2 P} \qquad n = (1.96)^2 \frac{1 - 0.70}{(0.15)^2 \times 0.70} = 73,$$

where, **n** corresponds to the required sample size; $\mathbf{Z_{1-\alpha/2}}$, the confident level at 95% and equal to 1.96; **P**, the estimated prevalence of ART-naive HIV-1 infected people in Cameroon (70%) in 2015 (CEIC, 2016), and **ε**, the relative precision =0.15.

## II.3 METHODS

### II.3.1 Screening

The point-of-care (POC) testing for the detection of HIV, HBV, HCV, Dengue virus infection as well as Malaria diagnosis through POC testing and microscopy were performed.

#### II.3.1.1 Blood sample collection

Twenty milliliters (20 mL) of peripheral blood were collected from each consenting participant in Ethylene Diamine Tetra Acetic acid (EDTA) tubes by an experienced nurse. The tubes were then placed in a securely sealed zip lock plastic bag and transported to the Laboratory where they were stored at room temperature. Analyses were performed within 1-2 hours of blood collection.

#### II.3.1.2 General Principle of the POC Tests

The POC tests were immunochromatography tests based on the interaction of an antigen (Ag) and its cognate antibody (Ab) conjugated to the colloidal gold (colored labelled) microparticles forming soluble immune complexes. These migrate by capillary flow within the nitrocellulose membrane till the detection zone, where an anti-species antibody immobilized on this membrane captures them thereby forming a visible band. Another control Ab to the conjugate binds the excess colloidal dye conjugate and acts as the control line (Fig. 17).

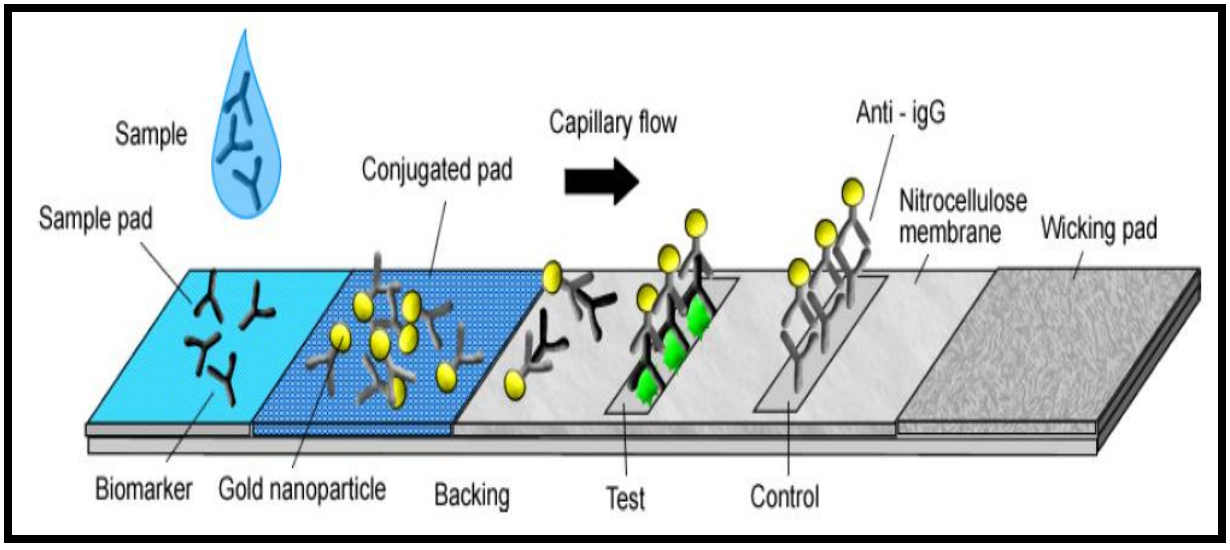



**Figure 17**: Representation of a typical immunochromatographic rapid test (Arroyo-Ornelas *et al.*, 2012).

### II.3.1.3 Screening for HIV infection

The HIV status of consented participants was initially obtained from their clinical files at the CIRCB medical section. This status was next confirmed using the Shanghai HIV (1+2) Ab test. Screening of HIV negative participants followed the agreed national algorithm which consisted of an initial screening with Alere Determine™ HIV-1/2 followed by the Shanghai HIV (1+2) Ab confirmatory test.

#### II.3.1.3.1 Alere Determine™ HIV-1/2

The Alere DetermineTM HIV-1/2 test allowed the detection of antibodies to HIV-1 and HIV-2 in plasma. The test device contains a nitrocellulose membrane strip coated with a conjugate to selenium colloid and HIV-1 and HIV-2 recombinant antigens and synthetic peptides.

- **Procedure**

The whole blood sample obtained from each participant was centrifuged at 1500 rpm for 10 min at 21°C ± 1°C. 50µL of the resulting plasma were then applied to the sample pad of the strip placed at room temperature. The test and control windows were observed after 15 min.

- **Interpretation**

- The appearance of two red lines in both the control and test windows of the strip indicated a reactive test (Fig. 18);
- the appearance of only one red line in the control window of the strip indicated a non-reactive or negative test;
- the absence of a line in the control window of the strip, irrespective if the test line appeared or not indicated an invalid test.

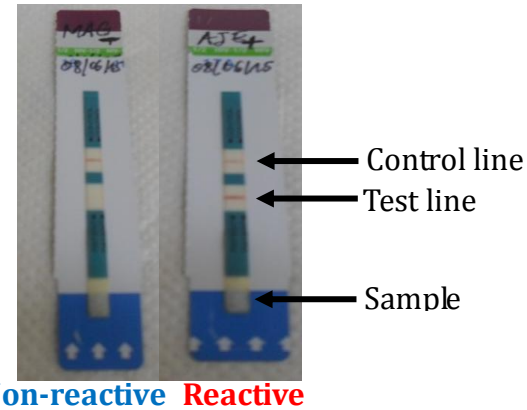



**Figure 18**: Determine HIV-1/2: examples of non-reactive and reactive tests (Photo credit: Ambada Georgia).

#### II.3.1.3.2 Shanghai HIV (1+2) antibody test

The Shangai HIV (1+2) Ab test (KHB® Bio-engineering Co., Ltd) uses a nitrocellulose strip with a conjugate site containing HIV-1 gp160 and HIV-2 gp36 conjugated to colloidal gold dye particles and a capture site containing the recombinant gp36 and gp41 antigens. An Ab against gp160 is immobilized in the control area.

- **Procedure**

Forty microliters (40 µL) of plasma sample and one drop of sample diluent were applied to the specimen area of the test strip. Following an incubation of 15 min at room temperature, the test and control windows were observed.

- **Interpretation**

- The appearance of two red lines in both the control and test areas of the test cassette indicated a positive result (Fig. 19);
- the appearance of only one red line in the control area of the test cassette indicated a negative test, and thus an indeterminate result;
- the absence of line in the control area, irrespective if the test line appeared or not indicated an invalid test.

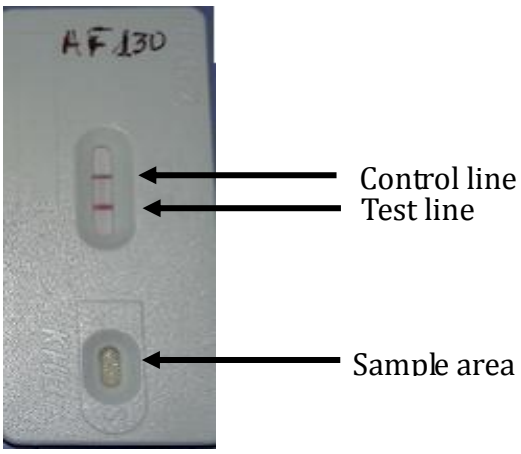



**Figure 19**: Shanghai HIV (1+2) antibody confirmatory test: example of a positive test (Photo credit: Ambada Georgia).

#### II.3.1.4 Screening for HBV infection

The SD BIOLINE hepatitis B surface antigen (HBsAg) test (Standard Diagnostic, INC) was used. This test utilizes a nitrocellulose strip with a conjugate site containing

the mouse monoclonal Abs reactive to HbsAg conjugated to colloidal gold. Anti-HbsAg and anti-IgG antibodies are immobilized respectively in the test and control zones.

➢ **Procedure**

One hundred microliters (100µL) of plasma sample were added into the sample well of the test device. After 20 min of incubation at room temperature, the test and control windows were observed.

➢ **Interpretation**

- The appearance of red lines at both control and test windows indicated a reactive test to HBs Ag (Fig. 20);
- the appearance of a red line at only the control window indicated a non-reactive test;
- the absence of a red line at the control window indicated an invalid test .

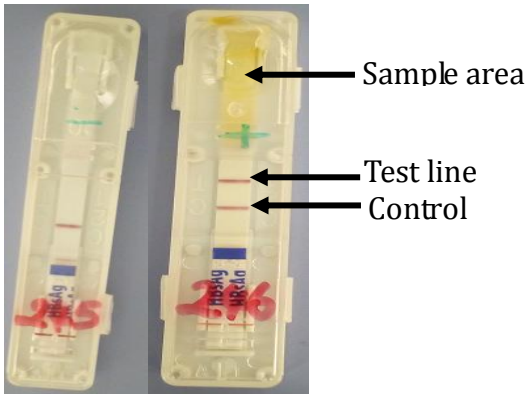



**Figure 20**: Hepatitis B surface antigen detection: example of non-reactive and reactive tests (Photo credit: Ambada Georgia).

### II.3.1.5 Screening for HCV infection

The SD BIOLINE HCV rapid test (Standard Diagnostic, INC) was used. This device is equipped with a nitrocellulose strip, which is coated with the protein A-colloid gold conjugate on the sample pad and the recombinant HCV antigens (core, NS3, NS4 and NS5) on the test region. Anti-IgG antibodies are immobilized in the control zone.

➢ **Procedure**

Ten microliters (10 µL) of plasma and 4 drops of assay diluent were dispensed into the sample well of the test device. Following 15 min of incubation at room temperature, the test and control windows were observed.

➢ **Interpretation**

- The appearance of red lines at both control and test windows indicated a reactive test to HCV antibodies (Fig. 21);
- the appearance of a red line at only the control window indicated a non-reactive test;
- the absence of red line at the control window indicated an invalid test.

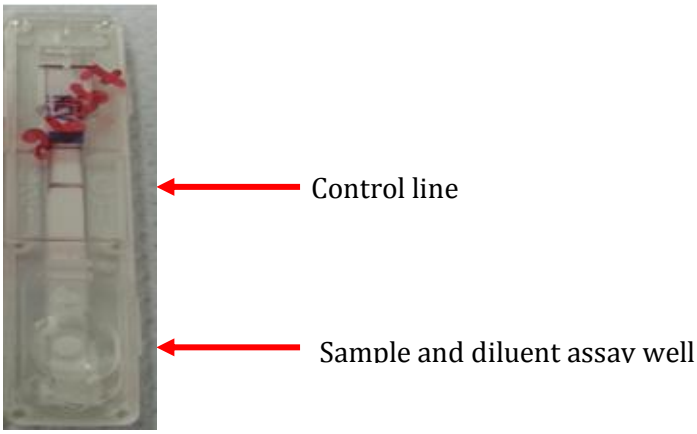



**Figure 21**: Detection of antibodies to HCV: example of a non-reactive test (Ambada, 2015).

### II.3.1.6 Screening for Dengue virus infection

The SD BIOLINE Dengue Duo rapid test (Standard Diagnostic, INC) allowed the detection of both NS1 Ag and differential Ig G/Ig M antibodies to Dengue virus in plasma using two test cassettes. The first one (left-side) consists of: 1) a conjugate pad containing mouse monoclonal anti-Dengue NS1 conjugated with gold colloid, 2) a nitrocellulose membrane strip pre-coated with mouse monoclonal anti-Dengue NS1 at the Test (T) line and the goat anti-mouse IgG at the Control line. The second one (right-side) includes: 1) a conjugate pad with recombinant Dengue virus envelope protein conjugated with gold colloid, 2) a nitrocellulose membrane strip pre-coated with the mouse monoclonal anti-human IgG and IgM respectively at the 'G' and 'M' test lines and the rabbit anti-Dengue IgG at the Control line.

➢ **Procedure**

One hundred (100µL) of plasma were dispensed into the sample well of the Dengue Ag cassette. In parallel, Ten microliters (10µL) of plasma sample and 4 drops of sample diluent were introduced respectively into the sample and buffer wells of the

Dengue IgG/IgM cassette. After 20 min of incubation at room temperature, the test and control windows were observed.

➢ **Interpretation**

- The appearance of a red G or M line indicated the presence of IgG or IgM anti-Dengue virus;
- the appearance of a red T line indicated the presence of Dengue Ag;
- the appearance of only a red control line indicated that neither anti-dengue virus antibodies nor dengue virus antigens were detected (Fig. 22);
- the absence of a red control line indicated that the test was invalid.

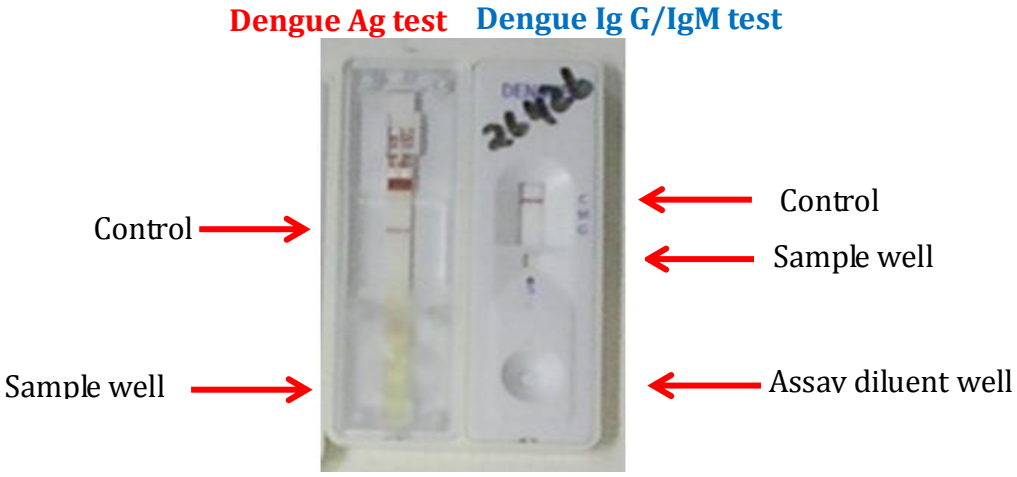



**Figure 22**: Detection of Ig G/ Ig M anti-dengue virus and Dengue antigen: example of non-reactive test (Photo credit: Ambada Georgia).

### II.3.1.7 Screening for Malaria infection

Malaria diagnosis was done by both POC test and microscopy.

#### II.3.1.7.1 POC testing for *Plasmodium* species infection

The SD BIOLINE Malaria Antigen *P.f*/Pan test (Standard Diagnostics, INC.) was used. It consists of a Conjugate pad, which is dispensed with antibodies conjugated to colloidal gold, which are specific to *Plasmodium falciparum* histidine-rich protein II (*P.f* HRP-II) and pan to the lactate dehydrogenase (pLDH) of *Plasmodium* species [*P. falciparum, P. vivax, P. malariae (PM) and P. ovale*]c. The membrane strip is pre-coated with two monoclonal antibodies: One (test line 1) is specific to *P.f* HRP-II, the other one (test line 2) is pan to the lactate dehydrogenase of *Plasmodium* species. A recombinant pPM-LDH Ag is bound at the control line.

➢ **Procedure**

Five microliters (5µL) of whole blood were introduced into the sample well (S) and then, 4 drops of assay diluent were added into the square assay diluent well (A). Following an incubation of 15 min at room temperature, the test and control windows were observed.

➢ **Interpretation**

- The appearance of a red line at the 'p.f'' test window indicated a reactive test to P. falciparum (Fig. 23);
- the appearance of a red line at the 'pan' test window indicated a reactive test to P. vivax or P. malariae or P. ovale;
- the appearance of only one red line at the control window indicated a non-reactive test;
- the absence of a red line at the control window indicated an invalid test.

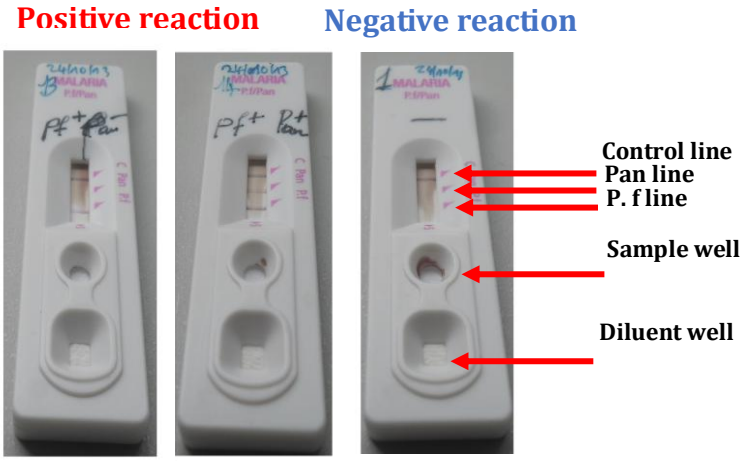



**Figure 23:** Malaria Antigen P.f/Pan detection: example of non-reactive and reactive tests (Photo credit: Ambada Georgia).

### II.3.1.7.2 Microscopy

The direct visualization of malaria parasites on the thick or thin blood smears was used to determine if parasites were present and to estimate parasitemia in one hand and to confirm the plasmodium species on the other hand.

➢ **Procedure**

It was performed in three steps including the preparation of blood films, the slide staining and the microscopic observation.

✓**Thick and thin blood film preparation**

- To make a thick film, 6µL of blood were placed in the center of a pre-cleaned labeled slide and spread with a pipette tip in one circular direction.
- For the Thin smear, 3µL of blood were placed near the frosted end of the same slide and spread toward the unfrosted end using a cover slide . The Thin film was fixed with absolute methanol (Sigma-Aldrich Chemie GmbH) and the slide was air dried for 1 hour.

✓**Staining slides**

Three drops of 10% Giemsa solution (New Delhi-110002, India) were poured on the slide. After 15 min of incubation at room temperature, the slide was rinsed in tap water and dried.

✓**Microscopic observation**

The slide was read using a binocular microscope under a 100X objective. The parasitaemia was obtained using the following formula:

$$P = \frac{N \times WBC}{L},$$

where **P** is the number of parasites per µL, **N**, the number of parasites counted per L leucocytes, **WBC**, patient's leukocyte number obtained by complete blood count, and assumed to be 8000/ µL, **L**, the number of leukocytes counted =200 (WHO, 2016).

➢ **Interpretation**

- After reading at least 20 power fields, the slide was considered negative;
- after observing at least one parasite in a power field, the slide was considered positive.

## II.3.2 Purification of regulatory T cells

Regulatory T cells were partially or totally purified from a known concentration of PBMCs' suspension using the Magnetic -Activated Cell Sorting (MACS) separation technique.

### II.3.2.1 Isolation of peripheral blood mononuclear cells

Peripheral Blood Mononuclear Cells (PBMCs) composed mainly of lymphocytes and monocytes were isolated from peripheral blood (obtained in II.3.1.1) using Ficoll-Paque Plus solution.

- **Principle**

The isolation of PBMCs using Ficoll-Paque Plus solution is based on the principle of density gradient centrifugation. Under centrifuged force, the components of blood are separated into different layers according to their size, shape and density. Erythrocytes, which have been aggregated by the Ficoll sediment completely and form a bottom layer, followed by granulocytes, Ficoll-Paque Plus, PBMCs and plasma (Fig. 24).

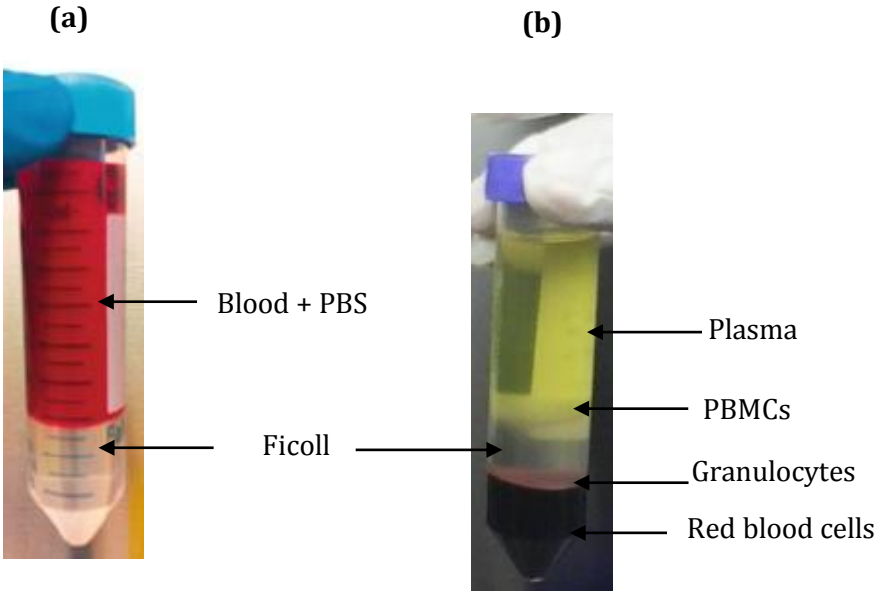



**Figure 24:** Isolation of PBMCs by density gradient centrifugation in Ficoll (Photo credit: Ambada Georgia). *Sample before Ficoll centrifugation is shown in (a) and different layers after Ficoll centrifugation are observed in (b).*

- **Procedure**

Fifteen milliliters (15mL) of whole blood were diluted with an equal volume of 1X Phosphate Buffered Saline (PBS) without Ca and Mg ions (Mediatech, corning). The mixture was then carefully overlaid on 15mL of Ficoll-paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) in a 50mL conical tube and centrifuged at 2000rpm for 20 min at 21°C ± 1°C without brake. Using a 10mL serological pipette, the plasma was aspirated and discarded. The mononuclear cells rich interface was transferred to a 15mL sterile tube, washed twice with 1X PBS without $Ca^{2+}$ and $Mg^{2+}$ for removal of platelets and resuspend in 10mL of the same buffer .

### II.3.2.2 Counting of peripheral blood mononuclear cells

A viable cell count is a prerequisite for cell separation, cell culture, and cell staining. This was performed here using the trypan blue exclusion method and a bright line hemocytometer (improved Neubauer, 0.100mm deep, 0.0025 mm2; Hausser scientific, USA), formed by two counting chambers (Fig. 25 a). Each chamber contains four white blood cells counting squares, each containing 16 squares (Fig. 25b).

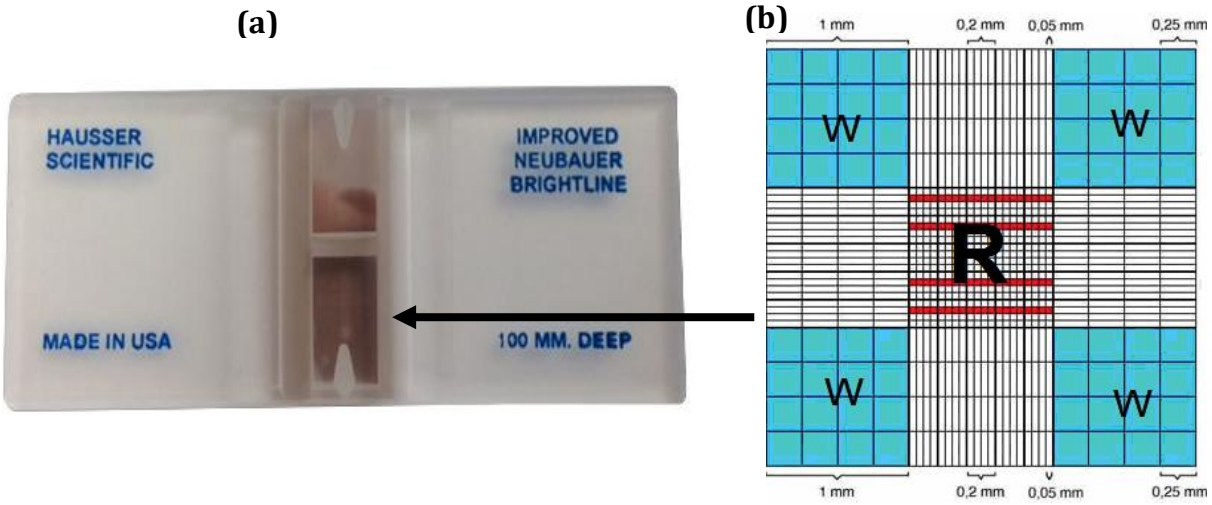



**Figure 25:** Neubauer hemacytometer (a), Neubauer counting chamber (b)(Biotech, 2006).

- **Principle**

This method is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not.

- **Procedure**

Ten microliters (10µL) of cell suspension were mixed with 90 µL of 0.4% trypan blue. The counting chamber was then filled with 10µL of this mixture and observed at a microscope with a 10X objective. Using the hand tally counter, cells were counted in 16 squares and the number of viable cells/mL was obtained using the formula: number of cells/ mL = Number of cells counted in 16 squares x dilution factor (10) x multiplication factor (10 000) x original volume of cell suspension (Abcam, 2016).

### II.3.2.3 General principle of the MACS separation

This technique involves the attachment of small magnetic beads to target cells via specific antigen-antibody attraction. When the beads coupled to specific antibodies are added to a mixed suspension containing the desired cells, they bind to their desired target forming an antigen-antibody complex. When a magnetic field is used, the tagged cells will be attracted to the magnet and are separated from the unlabeled cells. After eliminating the magnetic field, the magnetically retained cells can easily be eluted as the positively selected cell fraction.

### II.3.2.4 Partial purification of regulatory T cells

$CD4^+CD25^+$Treg cells were isolated from PBMCs using the BD Imag human regulatory T lymphocyte separation kit (BD Biosciences, USA) following an optimized protocol.

- **Procedure**

It was done in two steps: the negative selection of CD4+ T lymphocytes and the positive selection of CD25+ cells.

- **Negative selection of $CD4^+$ cells**

Peripheral Blood Mononuclear Cells were re-suspended in 5 µL of human regulatory T lymphocyte separation cocktail per 1 $x10^6$ cells and incubated at room temperature for 15 min. After washing away excess antibody with MACS buffer, cells were incubated with 7.5 µL of Streptavidin Particles Plus at room temperature for 30 min. The labeling volume was brought up to 1 mL with MACS buffer, transferred to a 12x75mm BD FACS tube placed within the magnetic field of the BD Imagnet (Cat. No. 552311) and depleted of labeled cells (Fig. 26). This negative selection was repeated twice to increase the yield of the enriched fraction ($CD4^+$ T cells). After removing 10 µL of cell suspension for counting (as described in II.3.2.2), the rest of sample was centrifuged at 1200 rpm for 10 min and the supernatant was discarded.

- **Positive selection of $CD4^+CD25^+$cells**

The $CD4^+$cell pellet was re-suspended in 5 µL of the BD Imag Anti-APC particles per 1 $x10^6$ cells and incubated at room temperature for 30 min. The labeling volume was brought up to 1 mL with MACS buffer and the tube was placed within the magnetic field

of the BD Imagnet for 8 min (Fig. 26). The supernatant was carefully aspirated and discarded. The positive selection was repeated three times to increase the purity of the positive fraction. The tube was then removed from the BD Imagnet, and the isolated CD4+CD25+cells Treg cells were washed twice in R10 medium. Cells were counted following the procedure described in II.3.2.2 and the samples were adjusted to a concentration of $2x10^5$ cells per 200µL of R10 medium before resting for 3 hours at 37ºC, 5%CO2.

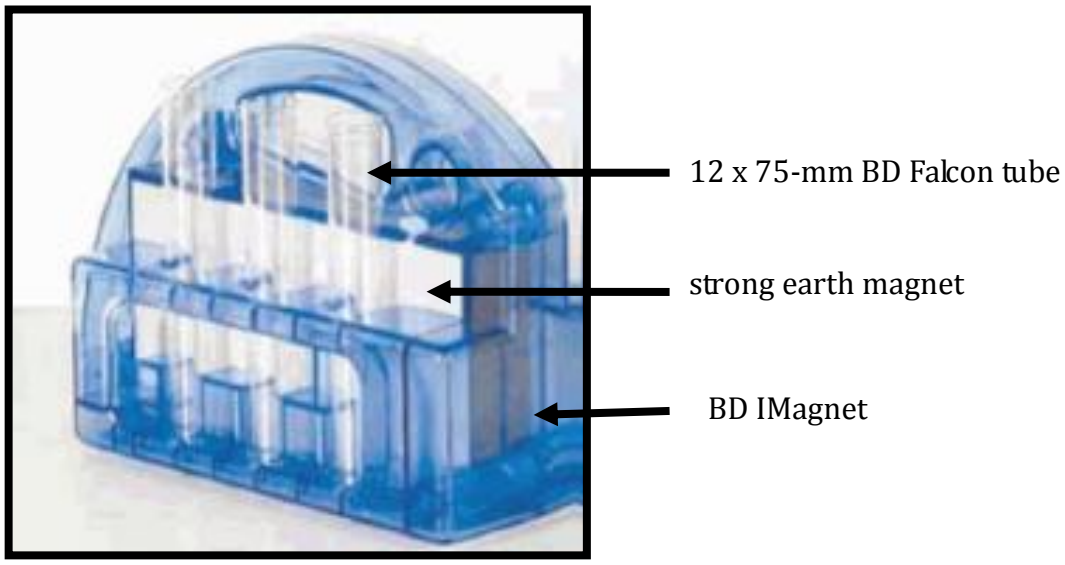



**Figure 26:** BD Imagnet cell separation device (BD Biosciences, USA).

### II.3.2.5 Total purification of regulatory T cells

The transcription factor Forkhead box P3 (FoxP3) is the key marker for Treg cell identification. However, its intranuclear expression limits it use for the isolation of live Treg cells. Therefore, we used the IL7-receptor α-chain (CD127) whose expression on Treg cells has been demonstrated to inversely correlate with Foxp3 (Liu *et al.*, 2006, Seddiki *et al.*, 2006, Horta *et al.*, 2013). This was achieved with the $CD4^+CD25^+$ $CD127^{dim/-}$ Treg cell isolation kit II (Miltenyi biotech, Bergish Gladbach) following an optimized protocol.

- **Procedure**

It is a two-step procedure that combined both negative ($CD4^+$ T cell enrichment) and positive ($CD25^+$ cell isolation) selection.

- **Negative selection of $CD4^+$ $CD127^{dim/-}$ T cells**

Peripheral blood mononuclear cells were washed with cold MACS buffer [MACS Bovine Serum Albumin (BSA) stock solution 1:20 autoMACS rinsing solution; miltenyi biotec, Bergish Gladbach]. Afterwards, $10^7$ cells were resuspended in 40 µL of MACS buffer and incubated with 10 µL of $CD4^+CD25^+CD127^{dim/-}$ T cell biotin- antibody cocktail II for 20 min at $4^oC \pm 1^oC$. A volume of 30 µL of MACS buffer and 20 µL of anti-biotin microbeads were then added. The stained cells were incubated at $4^oC \pm 1^oC$ for 20 min, washed with MACS buffer and re-suspended in 500 µL of the same buffer. This cell suspension was applied onto the Miltenyi LD column placed in the magnetic field of a MACS separator as shown in Fig. 27. The flow-through fraction formed by pre-enriched CD4+ CD127dim/-T cells was collected into a 15 mL conical tube. The column was washed two times with 1 mL of MACS buffer and $CD4^+$ $CD127^{dim/-}$ T cells were counted as described in II.3.2.2. The cell suspension (≃ 2.5 mL) was centrifuged at 1200 rpm for 10 min and the supernatant was aspirated completely using a 2 mL serological pipette.

- **Positive selection of $CD4^+CD25^+CD127^{dim/-}$ regulatory T cells**

The $CD4^+$ $CD127^{dim/-}$ T cells were re-suspended in MACS buffer (90 µL per $10^7$ cells) and incubated with 10 µL of CD25 microbeads II for 20 min at $4^oC \pm 1^oC$. After washing cells with 2 mL of cold MACS buffer, the supernatant was completely removed and cells were re-suspended in 500µL of cold MACS buffer. This cell suspension was applied onto the Miltenyi LS column and the flow-through fraction containing only the $CD4^+CD25^-$ cells was collected into a 15 mL conical tube. The column was washed three times with 3 mL of pre-chilled MACS buffer, then removed from the separator and placed on a 15 mL conical tube (Fig. 27). 5 mL of cold MACS buffer were introduced into the column and the magnetically labeled cells ($CD4^+CD25^+$ $CD127^{dim/-}$ Treg cells) were flushed out by firmly pushing the plunger into the column. After removing 10µL of cell suspension for counting (as described in II.3.2.2), the tube was centrifuged at 1200rpm for 10 minutes at 4 °C and the supernatant was discarded. Cells were washed twice in $R_{10}$ [RPMI 1640 medium supplemented with glutamine, 10% heat inactivated FBS and 1% penicillin (5,000 units/mL)- streptomycin (5,000µg/mL)]. $CD4^+CD25^+CD127^{dim/-}$ Treg cells were resuspended at $2x10^5$ cells per 200µL of $R_{10}$ medium and rested for 3 hours at 37ºC, 5%$CO_2$ prior to phenotypic and functional analyses.

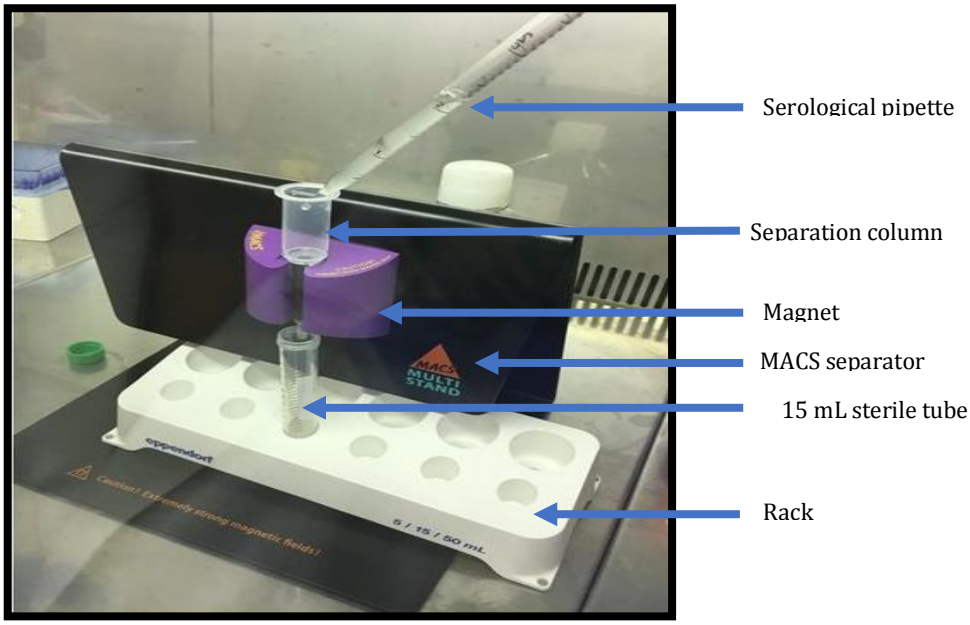



**Figure 27:** Magnetic cell separation device.

### II.3.2.6 Purity check of isolated Treg cells

To assess the purity of partially and fully purified Treg cells, multiparametric flow cytometry analysis was carried out.

#### II.3.2.6.1 Flow cytometry analysis

This was achievd by using a BD Fortessa X-20 cytometer.

##### II.3.2.6.1.1 Principle of flow cytometry

Flow cytometry is based upon the light-scattering features of the cells being labelled with fluorochrome-conjugated antibodies. Inside the flow cytometer, labelled cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an analysis point where the laser beam (light source) intersects the stream of flowing cells (Fig. 28a). The fluorescently labelled cell components are excited by the laser and emit light at varying wavelengths. The fluoresecence can then be measured to determine the amount and type of cells present in a sample. The Forward Scatter measures the size, whereas the Side Scatter measures the inner complexity of the cell particle (Fig. 28b) (Adan *et al.*, 2017).

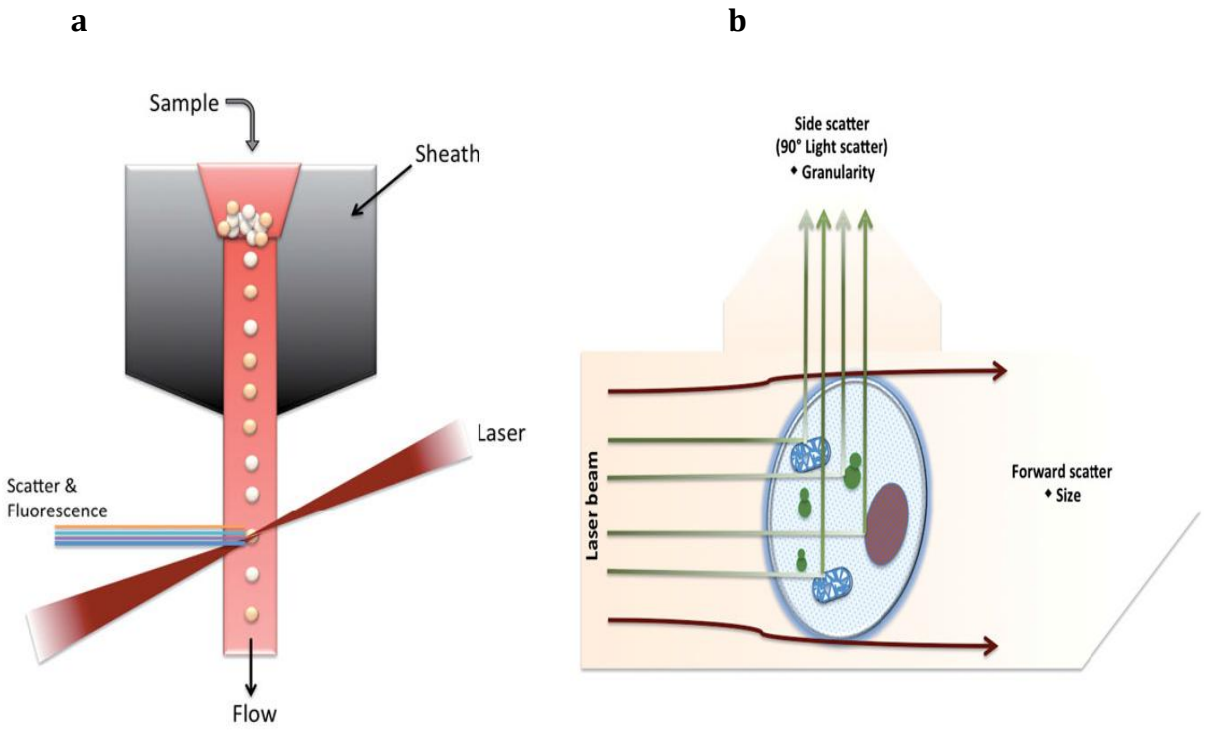



**Figure 28:** Principle of flow cytometry (Adan *et al.*, 2017).

#### II.3.2.6.1.2 Monoclonal antibodies

They included Allophycocyanin (APC) Vio770-conjugated anti-CD3 (clone BW 264/56) from Miltenyi Biotec, Phycoerythrin (PE)- Texas Red conjugated anti-CD4 from Beckman coulter (Brea, CA), Fixable Viability stain (FVS)- 510 and Brilliant Violet (BV)-421 labeled anti- CD127 (clone 9HIL-7R-M21) from BD Biosciences. PE conjugated anti-CD25 was provided by BD pharmingen and Phycoerythrin Cyanine Seven (PECY7) labeled anti-FOXP3 (clone PCH101) was supplied by eBiosciences.

The functions of these antibodies are shown on table I.

**Table I**: Immunophenotypic markers commonly used for regulatory T cells identification

| Markers | Functions |
|---|---|
| CD3 | T cell specific marker, necessary to differentiate T cells from other populations (Rathore *et al.*, 2014, Singer *et al.*, 2014) |
| CD4 | Identifies $CD4^+$ lymphocyte subset, interacts with MHC class II molecules on APCs and amplifies TCR signals (Dalgleish *et al.*, 1984, Lifson & Engleman, 1989). |
| CD25 | IL-2 receptor component, essential for Treg function and maintenance (Sakaguchi *et al.*, 2009) |
| FoxP3 | Transcription factor, co-ordinates expression of various genes required for Treg cell development and function (Wan & Flavell, 2007a). |
| CD127 | IL-7 receptor component expressed at low levels on Treg cells. It is an excellent marker of human Treg cells, especially when combined with CD25. (Liu *et al.*, 2006, Presicce *et al.*, 2011). |

Each antibody was titrated and the different fluorochromes were compensated for a best signal.

#### II.3.2.6.1.3 Titration of fluorochrome-conjugated antibodies

Monoclonal antibodies were titrated in order to determine the antibody amount and concentration resulting in the brightest signal with the lowest background (Hulspas *et al.,* 2009).

- **Principle**

The detemination of the maximum amount of Ab that allows for the minimum increase in background fluorescence represents the total number of Ab molecules available in the sampe for epitope binding. This amount is then used to determine the Ab concentration that produces the best discrimination between positive and negative cells.

- **Procedure**

A suspension of $1x10^6$ PBMCs per 100 µL of Fluorescence Activated Cell Sorting (FACS) buffer [1x PBS with $Ca^{2+}$ and $Mg^{2+}$ + 2% heat inactivated Fetal Bovine Serum, Mediatech, corning] was distributed in BD FACS tubes. The dilution series of the antibody were prepared starting with the concentration recommended by the manufacturer. Fifty microliters (50 µL) of each dilution were added to the corresponding tube. Unstained cells were used as negative control. The tubes were gently vortexed for 3 to 5 seconds, protected from the light with aluminum foil and incubated for 15 min at room temperature. After that, cells were washed with 2mL of FACS buffer, re-suspended in 400 µL of the same buffer and acquired on BD FACSCanto II cytometer using BDFACS Diva Software. Data were analyzed using the FlowJo software version 9.8.5 and the staining index was calculated as follows (Bushnell, 2015):

$$\text{Staining Index} = \frac{\text{Geometric Mean of the positive population} - \text{Geometric Mean of the negative population}}{2 \times \text{Standard deviation of the negative population}}$$

The dilutions used for different mAbs are shown on table II:

Table II: Panel of antibodies and dilutions for purity check

| | **Antibody** | **Dilution** |
|---|---|---|
| **Surface** | APC-Vio770 CD3 | 1:100 |
| | PE- Texas Red CD4 | 1:100 |
| | PE CD25 | 1:100 |
| | BV421 CD127 | 1:100 |
| | Fixable Viability stain 510 | 1:1000 |
| **Intracellular** | PECY7 FOXP3 | 1:100 |

### II.3.2.6.1.4 Fluorescence compensation

One consideration when performing multiparametric flow cytometry analysis, is the possibility of spectral overlap between fluorophores. Therefore, there is need to

adjust the photomultiplier tube of the cytometer to enable the specific detection of each fluorescent particle according to their individual fluorescence.

- **Principle**

The fluorophores used in flow cytometry emit photons of multiple energies and wavelengths which can overlap during excitation or emission. To adjust for spectral overlap, fluorescence values of each fluorophore is measured separately in single color controls for each detection channel (detector). The spillover values are then placed in a symmetrical matrix by an inbuilt compensation software which calculates all overlaps and makes adjustments separating the individual peaks based on fluorochrome selection. This matrix algebra essentially calculates the simultaneous solution of the equations for the contributions of the spectral overlaps of each of the colors into every detector. Finally, the compensation values are used by the flow cytometer compensation software to eliminate the contribution of non-primary colors overlapping into a given detector (BD biosciences, 2009).

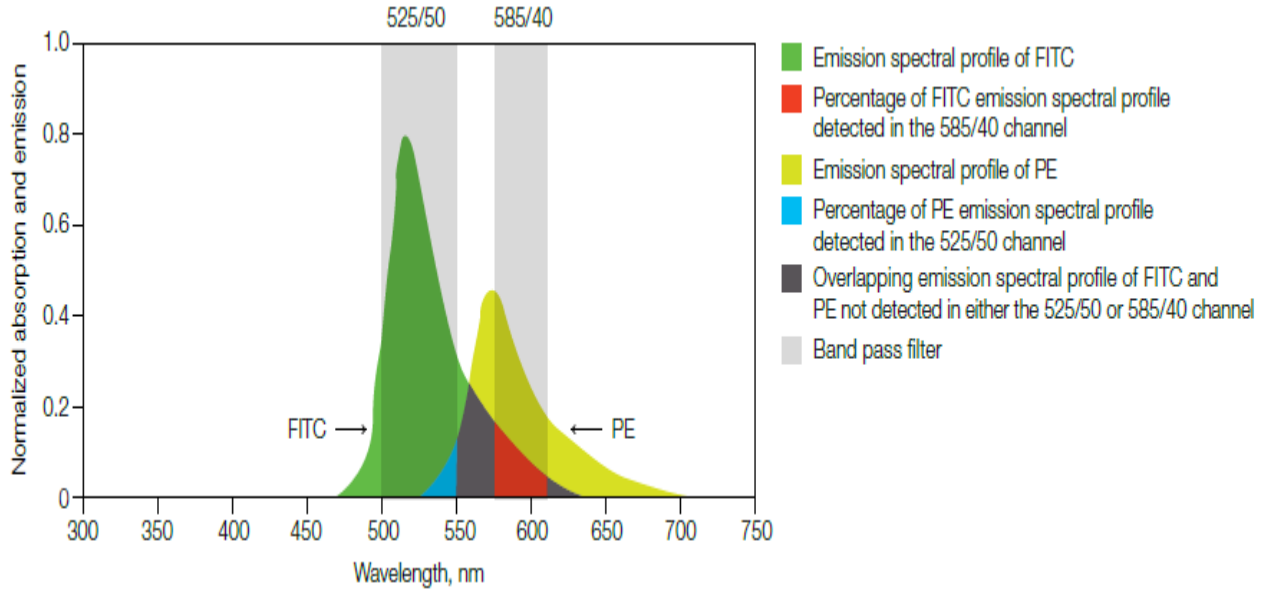



**Figure 29:** Fluorescence compensation(BIO-RAD, 2016).

- **procedure**

A single-stained control tube was prepared for each fluorophore to be used in the experiment. An unstained sample was also prepared to determine the size and location of the population of interest. In brief, $1.10^6$ PBMCs were distributed in the well

labeled 12x75mm BD FACS tubes. Each fluorochrome-conjugated antibody was added to the corresponding tube. All the tubes were then capped, vortexed for 3 to 5 seconds, wrapped with aluminum foil and incubated for 15 min at room temperature. Following incubation, cells were washed with 2mL of FACS buffer, re-suspended in 400 µL of the same buffer and stored at 4ºC while waiting for acquisition on BD Fortessa X-20 cytometer using BDFACS Diva Software. The unstained tube was first acquired in order to adjust the gate on the population of interest P1 (lymphocytes), as well as the forward scatter (FSC) and size scatter (SSC) gates. The auto fluorescent signals were also adjusted on each parameter to form peaks within the first decade. Once all the adjustments were made, a right-click on P1 was done to Apply all the changes on the remaining compensation Controls. The stained control tubes were then acquired and the compensation was calculated automatically by the BD FACSDIVA software.

#### II.3.2.6.1.5 FoxP3 intracellular staining

Following 3 hours of incubation at 37ºC, 5%$CO_2$, sorted Treg cells ($1.10^6$) and bulk PBMCs ($1.10^6$) were plated in a V-bottom 96 well plate (Costar, Corning incorporated, USA), pelleted following centrifugation at 2000 rpm for 2 min. The supernatant was discarded by flipping the plate and cells were washed twice with 180 µL of FACS buffer. Cells were then incubated 20 min at 4°C ± 1°C in the dark, with 1% FcR blocking solution (miltenyi biotec) diluted in FACS buffer. After incubation, the cells were pelleted by centrifugating at 2000 rpm for 2 min, washed once with cold FACS buffer and stained on the surface with a cocktail of fluorochrome-labeled antibodies to CD3, CD4, CD25, CD127and FVS-510 for 20 minutes at 4°C ± 1°C in the dark. After two washes in 180 µL of FACS buffer, cells were resuspended in 100 µL of FoxP3 fixation/permeabilization buffer (e-Bioscience, USA) for 45 min at 4°C ± 1°C. The permeabilized cells were washed with 180 µL of 1x FoxP3 permeabilization buffer (e-Bioscience) and stained intracellularly with anti-Foxp3 for 30 min at 4°C ± 1°C protected from the light. After intracellular staining, cells were washed with 180 µL of 1x FoxP3 permeabilization buffer and re-suspended in 400 µL of FACS buffer. Samples were acquired on BD Fortessa X-20 cytometer using BDFACS Diva Software. For each sample, 1,000 events were collected within a gate set on $CD4^+CD127^{lo}$ cells.

### II.3.3 Phenotypic characterization of regulatory T cells

In addition to CD25, FoxP3 and CD127, which are key markers for Treg cells identification (Fontenot *et al.*, 2003, Presicce *et al.*, 2011, Duhen *et al.*, 2012), other differentiation markers were added to refine the phenotypic characterization of Treg cells on totally purified Treg cells.

#### II.3.3.1 Antibodies

The mAbs used to differentiate Treg cell subsets included BV-421 labeled anti-CD127 (clone 9HIL-7R-M21), BV-650 labeled anti-CD39 (clone TU66), BV605 conjugated anti-CD62L (clone DREG-56) and FVS 510 all purchased from BD Biosciences (BD Bioscience, USA). Alexa-Fluor 700 labeled anti-HLA-DR (clone LN3), peridin-chlorophyll protein (PerCP)-eFluor 710 conjugated anti-CCR7 (clone 3D12) and fluorescein isothyocyanate (FITC) labeled anti-CD27 (clone LG.7F9) were obtained from eBiosciences (San Diego, CA, USA). PE conjugated anti-CD25, phycoerythrin cyanine seven (PECY7) labeled anti-CD73 (clone AD2), phycoerythrin cyanine five (PECY5) labeled anti-CD45RA (clone HI100) and Allophycocyanin (APC) labeled anti-CD38 were provided by BD pharmingen (San Diego, CA, USA). APC Vio770-conjugated anti-CD3 (clone BW 264/56) and PE- Texas Red conjugated anti-CD4 were obtained from Miltenyi Biotec and Beckman coulter (Brea, Fullerton, CA, USA), respectively.

The functions of these antibodies are shown on table III.

**Table III**: Functions of markers used for Treg cells differentiation

| Markers | Functions |
|---|---|
| CD39 | cell surface-located prototypic member of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, anti-inflammatory effect by hydrolyzing pro-inflammatory ATP to ADP and AMP (Deaglio *et al.*, 2007, Dwyer *et al.*, 2007, Moncrieffe *et al.*, 2010). |
| CD73 | Ecto-5'-nucleotidase (CD73) is a glycosyl phosphatidylinositol-linked, membrane-bound glycoprotein which dephosphorylates AMP to adenosine, which plays an essential role in suppressing inflammatory reactions by controlling the immune cell activation and proliferation (Colgan *et al.*, 2006, Strater, 2006, Deaglio *et al.*, 2007). By so doing CD73 completes the degradation of ATP initiated through CD39 activity as described above. |
| CD62L | Cell adhesion molecule found on naive Treg cells and further distinguishes central memory ($CD62L^+$) from effector memory ($CD62L^-$) Treg cells. CD62L is important in Treg cells rolling along endothelial surfaces during trafficking to lymphoid tissues(Yang *et al.*, 2011, Singer *et al.*, 2014). |
| CCR7 | CCR7 expression is associated with naive phenotype. It is required for the homing of naive Treg cells to the lymph nodes, limits Treg circulation back to the thymus, (Appay & Sauce, 2008, Campbell & Koch, 2011) |
| CD45RA | Protein tyrosine phosphatase, receptor type, C; identifies naive and effector T cells (Singer *et al.*, 2014) |
| CD27 | TNF receptor-family member that is expressed on naive and subsets of memory T cells. It is lost on terminally differentiated effector T cells. It promotes survival of activated and memory Treg cells and is involved in generating the effector T cell pool (Mack *et al.*, 2009). |
| HLA-DR | Glycoprotein expressing on activated T cells, B cells, monocytes and DCs. It serves to identify a functionally distinct subpopulation of Treg cells (Xiao *et al.*, 2011) |
| CD38 | Glycoprotein expressing on activated T cells, B cells, monocytes and DCs. Its expression may define a highly suppressive subset of Treg cells (Xiao *et al.*, 2011, Feng *et al.*, 2017) |

The mAbs were titrated as described in II.3.2.6.1.3 and the different dilutions are shown on table IV.

**Table IV:** Panel of antibodies and dilutions for the differentiation of Treg cell subsets

| Antibody | Dilution | Antibody | Dilution |
|---|---|---|---|
| BV421 CD127 | 1:100 | PE CD25 | 1:100 |
| BV650 CD39 | 1:50 | PECY7 CD73 | 1:100 |
| BV605 CD62L | 1:100 | PECY5 CD45RA | 1:100 |
| Fixable Viability stain 510 | 1:1000 | APC CD38 | 1:100 |
| Alexa-Fluor 700 HLA-DR | 1:100 | APC Vio770 CD3 | 1:100 |
| PerCP-eFluor 710 CCR7 | 1:100 | PE- Texas Red CD4 | 1:100 |
| FITC CD27 | 1:100 | | |

### II.3.3.2 Surface cell staining

Sorted Treg cells ($1.10^6$) were transferred to a V-bottom 96 well plate (Costar, Corning incorporated, USA), pelleted following centrifugation at 2000 rpm for 2 min. The supernatant was discarded by flipping the plate and cells were washed twice with 180 µL of FACS buffer. Cells were then incubated 20 min at 4°C ± 1°C in the dark, with 1% FcR blocking solution (miltenyi biotec) diluted in FACS buffer. After incubation, the cells were pelleted by centrifugating at 2000 rpm for 2 min, washed once with cold FACS buffer and stained on the surface with a cocktail of fluorochrome-labeled antibodies to CD3, CD4, CD25, CD127, HLA-DR, CD38, CD45RA, CD27, CD39, CD73, CCR7, CD62L, CCR7, CD27 and Live/Dead. After incubation for 20 minutes at 4°C ± 1°C in the dark, cells were washed two times with 180 µL of FACS buffer and re-suspended in 400 µL of the same buffer. Samples were acquired on BD Fortessa X-20 cytometer using BDFACS Diva Software. For each sample, 1,000 events were collected within a gate set on $CD4^+CD127^{lo}$ cells.

## II.3.4 Evaluation of immunologic and virologic parameters of ART-naive HIV-1 infected participants

Helper $CD4^+$ T cell count and plasmatic HIV-1 viral load are the most important parameters to assess the immunological and virological status of HIV-1 infected people, respectively. These parameters were used to understand the functionality of Treg cells in the study population.

### II.3.4.1 Helper $CD4^+$ T cell count

The helper $CD4^+$ T cell count provides information on the overall immune function of an individual. It was determined for both $HIV^+$ and $HIV^-$ participants in fresh whole blood by BD multitest CD3/CD8/CD45/CD4 and TruCount tubes (BD biosciences, USA) following an optimized protocol.

- **Principle**

The helper $CD4^+$ T cell count is based on immunophenotypic identification of cells with fluorescently labeled monoclonal antibodies directed against CD4 antigen. When TruCount tubes are used, the lyophilized pellet contained in these tubes' dissolves during sample preparation, releasing a known number of fluorescent beads. By gating the bead population during sample acquisition, absolute cell counts (cells/ $mm^3$) can be automatically determined.

- **Procedure**

Five microliters of CD3/CD8/CD45/CD4 Multitest were introduced into the bottom of each labeled BD TruCount tube (BD Bioscience, USA) without touching the pellet and then, 50µL of well mixed, EDTA-treated whole blood were added. The tubes were capped, vortexed gently for 3 to 5 seconds, protected from the light and incubated for 15 min at room temperature. A second incubation of 15 min was done following addition of 450µL of 1x FACS lysing solution (BD Bioscience, San Jose, USA). The samples were then acquired on the clinical software using the BD FACS Canto II machine, which was first calibrated with the BD Cytometer Setup and Tracking Beads (BD Biosciences, San Jose, USA).

➢ **Interpretation of results**

- The helper $CD4^+$ T cell count below 200 cells/ µL indicated a severe immunosuppression;
- the helper $CD4^+$ T cell count ranged between 200–349 cells/ µL indicated an advanced immunosuppression;
- the helper $CD4^+$ T cell count ranged between 350–499 cells/ µL indicated a mild immunosuppression;
- the helper $CD4^+$ T cell count equal or above 500 cells/ µL indicated that there was no significant immunosuppression (WHO, 2005).

### II.3.4.2 Plasma HIV-1 RNA quantification

The plasmatic HIV-1 viral load is the most important indicator of HIV-1 disease progression. It was determined on the m2000rt machine using the Abbott Real-Time HIV-1 Assay protocol. The assay detection limit was 40 viral copies / mL.

➢ **Principle**

The Abbott Real Time HIV-1 assay is based on the principle of quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The RNA genome of HIV is first transcribed into complementary DNA, which is then used as a template for the RT-qPCR by reverse transcriptase to generate amplified products.

➢ **Procedure**

The samples and amplification Master mix were prepared manually before amplification and detection steps.

✓**Sample preparation**

The purpose of this step is to extract and concentrate the target RNA molecules to make them accessible for amplification.

The lysis solution was prepared by adding 500µL of internal control to the lysis buffer and by inverting 5-10 times for mixing.

Wash buffers 1 and 2 as well as elution buffer were also mixed by gently inverting 5 – 10 times until the crystals disappeared.

One hundred microliters (100 µL) of micro particles were introduced into each labeled 12x75mm tube and then 2.4 mL of lysis solution were added. 500µL of plasma were transferred to the lysis tubes by using aerosol barrier pipette tips. Sample was mixed with lysis buffer by up and down pipetting and incubated at 50°C ± 2°C for 20 min in a heating block. Following incubation, the tube was removed from the heating block and placed in the red magnetic capture stand for 2 min. The lysate was carefully removed using sterile disposable Pasteur pipettes without disturbing the captured magnetic particles. The tube was then transferred to a non-magnetic rack and the magnetic particles were re suspended with 700 µL of wash buffer- 1 by aspiration. The wash fluid and particles were transferred to a labeled 1.5 mL tube which was placed in a blue magnetic capture stand for 1min. The fluid was completely removed without disturbing the captured magnetic particles and tubes were transferred to a non-magnetic rack. Second wash-1 was performed and after removing the fluid, the tube was transferred to a non-magnetic rack. The magnetic particles were washed two times again with wash buffer. 2. 25 µL of elution buffer were added to re suspend the magnetic particles. The tube was placed in the 75°C heating block for 20 min. Following incubation, the tube was transferred to a non-magnetic rack, 63 µL of wash-2 were added to wash the particles by aspiration. The tube was placed in a blue magnetic capture stand for 1min. The eluate was removed and transferred to the Abbott 96 Deep-Well Plate.

✓**Preparation of the amplification Master mix**

Two hundred and seventy-one microliters (271µL) of the HIV-1 activation reagent and 949 µL of the HIV-1 oligonucleotide reagent were added in the thermostable rTth DNA polymerase enzyme bottle. The master mix was transferred from the enzyme bottle into a RNase/DNase-free tube and vortexed. The Abbott 96-well optical reaction plate was placed in the strata Cooler 96 and 50µL of the amplification master mix was dispensed into the 96-well plate. The uniform content of each well was verified and 50µL of sample eluate were transferred to the 96-well plate on the strata Cooler 96. The reaction was mixed by aspirating and rejecting 3-5 times.

After checking visually that the total content in each well is uniform (100µL), the 96-well plate was sealed with an optical adhesive cover, centrifuged for 5 min at

5000g and then placed into the Abbott m2000rt instrument (m2000rt; Abbott Molecular Inc.) for reverse transcription, PCR amplification and detection.

## II.3.5 Evaluation of regulatory T cells cytokine profile

To understand how Treg cells can exert their suppressive activity, we defined their cytokine profile following an intracellular staining.

### II.3.5.1 Antibodies

The mAbs used included BV-650 labeled anti-IL-10 (clone JES3-9D7), BV-421 labeled anti- CD127 (clone 9HIL-7R-M21), BV605 conjugated anti-CD25 (clone 2A3), PE-conjugated anti-TGF-β1 (clone TW4-9E7), APC-labeled anti-IL4 (clone 8D4-8) and FVS-510, all purchased from Becton Dickinson (BD Biosciences, USA). APC Vio770-conjugated anti-CD3 (clone BW 264/56) and PE- Texas Red conjugated anti-CD4 were obtained from Miltenyi Biotec and Beckman coulter (Brea, Fullerton, CA, USA), respectively. PerCP-CY5-5 labeled anti- IL-17A (clone N49-653) was provided by BD pharmigen (San Diego, CA, USA). PECY7 labeled anti-FOXP3 (clone PCH101) was supplied by eBiosciences.

The functions of cytokines produced by Treg cells are shown on table V.

**Table V:** Functions of cytokines used to assess Treg cell function

| **Cytokines** | **Functions** |
|---|---|
| IL-10 | IL-10 inhibits the production of inflammatory cytokines, causing a decrease in effector responses; it also promotes the phagocytic activity, increasing the removal of cellular debris at the inflammation site (Arce-Sillas *et al.*, 2016a). |
| TGF-β | Suppressive function; important for Treg trafficking to the gut; promotes the generation of Treg cells in the periphery by inducing the production of FoxP3 on conventional $CD4^+$T cells (Wan & Flavell, 2007a) |
| IL-17A | Inhibit the proliferation of $CD4^+$ effector T cells (Zhang *et al.*, 2014). |
| IL-4 | can boost CD25 and FOXP3 expression on Treg cells, prevent their apoptosis and increase their suppressive function *in vitro (Campbell & Koch, 2011)*. IL-4 Counterbalances the pro-inflammatory cytokines effects(Brites-Alves *et al.*, 2018) |

The dilutions of these cytokines are shown on table VI.

**Table VI:** Panel of antibodies and dilutions to assess the cytokine profile of Treg cells

| | **Antibody** | **Dilution** |
|---|---|---|
| **Surface** | APC-Vio770 CD3 | 1:100 |
| | PE- Texas Red CD4 | 1:100 |
| | PE CD25 | 1:100 |
| | BV421 CD127 | 1:100 |
| | Fixable Viability stain 510 | 1:1000 |
| **Intracellular** | APC IL4 | 1:100 |
| | PE TGF-β1 | 1:100 |
| | BV 650 IL-10 | 1:100 |
| | PerCP-CY5-5 IL-17A | 1:100 |
| | PECY7 FOXP3 | 1:100 |

### II.3.5.2 Intracellular staining

Treg cells were transferred in a V bottom 96-well plate, washed twice with FACS buffer and surface stained with a cocktail of fluorochrome-labeled antibodies to CD3, CD4, CD25, CD127 and live-dead. The cells were then incubated with a FoxP3 fixation/permeabilization buffer (e-Bioscience, USA) at 4°C ± 1°C for 45 min in the dark before intracellular staining with a cytokine cocktail consisting of Foxp3, IL-4, IL-10, IL-17A and TGF-β1. Next, the cells were washed with FoxP3 permeabilization buffer (e-Bioscience), re-suspended in FACS buffer and acquired on BD Fortessa X-20 cytometer using BDFACS Diva Software.

## II.3.6 Evaluation of the suppressive activity of regulatory T cells on activated monocytes

To assess the suppressive activity of Treg cells, they were cultured with autologous monocytes.

### II.3.6.1 Purification of $CD14^+$ Monocytes

Monocytes were isolated from PBMCs by MACS separation technique using the CD14 MicroBeads provided by Miltenyi Biotec (Miltenyi biotech, Bergish Gladbach).

- **Procedure**

Peripheral Blood Mononuclear Cells re-suspended in MACS buffer at 80 µL per 1 $x10^7$ cells were incubated with 20 µL of CD14 MicroBeads at 4°C ± 1°C for 15 min. The cells were then washed with MACS buffer, re-suspended in 500 µL of the same buffer and applied onto the Miltenyi LS column fixed on the MACS separator. The column was washed three times with 3 mL of pre-chilled MACS buffer, removed from the separator and placed on a sterile 15-ml conical tube. 5 mL of cold MACS buffer were added to the column and the magnetically labeled cells ($CD14^+$ cells) were flushed out by firmly pushing the plunger into the column. The cells were counted and pelleted following centrifugation for 10 min at 4°C ± 1°C, 1200rpm. After washing in a $R_{10}$ medium, $CD14^+$ cells were adjusted to a concentration of $2x10^5$ cells per 200 µL of $R_{10}$ and rested for 3 hours at 37°C, 5% $CO_2$. The purity of $CD14^+$ cells was assessed by flow cytometry using BD Fortessa X-20 (BD biosciences, USA) as described in II.3.2.6.

### II.3.6.2 Monocyte-Treg cell co- culture

Following 3 hours of rest, monocytes were cultured either alone or with autologous Treg cells in a 96-well U-bottom plate (Costar, Corming incorporated, USA) at a 1:1 ratio for 6 hours at 37ºC, 5%CO2. Poly-ICLC (1 µg/mL) was used to stimulate the cells for 1 hour before the addition of brefeldin A (1 µg/mL; BD Biosciences) for the last 5 hours.

### II.3.6.3 Evaluation of activation status and cytokine profile of monocytes using multiparametric flow cytometry

Surface and intracellular staining of monocytes were performed.

#### II.3.6.3.1 Antibodies

The mAbs used for surface and intracellular cytokine analysis included BV-421 labeled anti-IL-6 (clone MQ2-13A5), PECY7 labeled anti-TNFα (clone MAb11), PerCP-CY5-5 conjugated anti- CD14 (clone MØP9), PE-CF594 labeled anti-CD16 (clone 3G8), and FVS-510, all purchased from Becton Dickinson (BD Biosciences, USA). APC labeled

anti-CD38 and Alexa-Fluor 700 labeled anti-HLA-DR (clone LN3) were provided by BD pharmigen (San Diego, CA, USA) and eBiosciences (San Diego, CA, USA), respectively.

The functions and the dilutions of the cytokines used are presented on table VII and VIII, respectively.

**Table VII:** Functions of cytokines forming monocytes

| **Cytokines** | **Functions** |
|---|---|
| IL-6 | Soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis. Dysregulated continual synthesis of these cytokines plays a pathological effect on chronic inflammation(Walter *et al.*, 2013). |
| TNF-α | Inflammatory cytokine. Overexpression of this cytokine plays a pathological effect on chronic inflammation (Walter *et al.*, 2013) |

**Table VIII:** Panel of antibodies and dilutions for monocytes characterization

| | **Antibody** | **Dilution** |
|---|---|---|
| **Surface** | Alexa-Fluor 700 HLA-DR | 1:100 |
| | APC CD38 | 1:100 |
| | PE-CF594 CD16 | 1:100 |
| | PerCP-CY5-5 CD14 | 1:100 |
| | FVS- 510 | 1:1000 |
| **Intracellular** | PECY7 TNFα | 1:100 |
| | BV421 IL-6 | 1:100 |

### II.3.6.3.2 Intracellular staining

Following co-culture of Treg cells and autologous monocytes, cells were transferred in a V bottom 96-well plate washed twice with FACS buffer and surface stained with a cocktail of fluorochrome-labeled antibodies either to CD14, CD16, HLA-DR, CD38 and live-dead. The cells were then fixed and permeabilized before intracellular staining with a cytokine cocktail of IL-6 and TNF-α. Cells were first washed with FoxP3 permeabilization buffer (e-Bioscience), then with FACS buffer. The later was used to re-

suspend cells before acquisition on BD Fortessa X-20 cytometer using BDFACS Diva Software.

#### II.3.6.4 Determination of the stimulation and suppression indexes

To assess the suppressive activity of Treg cells, we first determined the capability of monocytes to be activated or to produce inflammatory cytokines following stimulation with poly-ICLC. The resulting stimulation index (SI) was obtained by dividing the Mean Fluorescence Intensity (MFI) of mAb expression after poly-ICLC treated monocytes by the MFI of mAb expression in unstimulated monocytes. The Treg cell suppression index (IS) was assessed based on the following formula: 1- (MFI of mAb expression of poly ICLC treated monocytes in the presence of Treg cells / MFI of Ab expression of poly ICLC treated monocytes in the absence of Treg cells).

### II.3.7 Ethical considerations

This study received ethical approval from the Cameroon National Ethics Committee for Human Health Research. (Protocol numbers: CIRCB/14-11/DROS631-1112; 2014/10/499/CE/CNERSH/SP; 2015/03/569/CE/CNERSH/SP). All participants provided written informed consent. Data were processed using specific identifiers for privacy and confidentiality purposes. Clinical data generated during the course of this study was provided free of charge to all participants. Copies of ethical clearance and consent form are provided in appendix section.

### II.3.8 Statistical analysis

Flow cytometric data obtained from surface and intracellular staining were analyzed using FlowJo software version 9.8.5. All statistical analyses were performed using GraphPad Prism version 5.0. The data were shown as median (25th percentile-75th percentile). Non-parametric tests were used. Comparisons of medians among two groups were performed by the U-Mann- Whitney test. Kruskal Wallis test with Dunn's multiple comparisons post-test were used to test for differences between more than two groups. Correlations were made with spearman test and p values less than 0.05 were considered to be statistically significant.

# CHAPTER III. RESULTS AND DISCUSSION

## III.1 RESULTS

### III.1.1 Characteristics of the study population

Overall, 110 participants were recruited for this study as reported in table IX. ART- naive HIV-1 infected participants consisted of 49 (68%) females and 23 (32%) males with a median age of 37 (30-42) and 40 (37-46) years old, respectively. Their sex-matched HIV-negative controls (19 females and 19 males) had significantly lower median ages, 30 (26-37) and 30 (27-34) years old, respectively for females (p=0.013) and males (p=0.0002).

Median CD4 counts were lower in $HIV^+$ participants than healthy controls: 424 cells/$mm^3$ (201– 568) versus 936 cells/$mm^3$ (798-1115) in males (p<0.0001) and 444 cells/$mm^3$ (309 – 633.5) versus 963 cells/$mm^3$ (678-1120) in females (p<0,0001). The majority of $HIV^+$ participants (31%) showed no significant immune suppression (CD4≥500 cells/$mm^3$), 17% had mild immunosuppression (350-499 cells/$mm^3$), 13 % had advanced immunosuppression (200-349 cells/$mm^3$) and 11% had severe immunosuppression (<200 cells/$mm^3$).

Comparatively, HIV-1 infected females showed a lower plasmatic viral load [3.66 Log (2.67-5.03) than males [5.07 Log (3.56-5.5); p<0.05].

There was no significant difference (P= 0.12) in the duration of HIV-1 infection in both males [4 (2-6) years] and females [5 (4-6) years].

**Table IX:** Characteristics of study population

| **Variable** | **HIV negative participants (n=38)** | | **HIV-1-infected participants (n=72)** | |
|---|---|---|---|---|
| Gender | Male | Female | Male | Female |
| Participants (%) | 19 (50) | 19 (50) | 23 (32) | 49 (68) |
| Median age (IQR) | 30 (27-34) *** | 30 (26-37) * | 40 (37-46) | 37 (30-42) |
| Median CD4 count (cells/$mm^3$) | 936 (798-1115) | 963 (678-1120) | 424 (201-568) *** | 444 (309-633.5) *** |
| CD4≥500 cells /$mm^3$ (n/%) | NA | NA | 9 (13) | 22 (31) |
| 350-499 cells /$mm^3$ (n/%) | NA | NA | 6 (8) | 11 (15) |
| 200-349 cells /$mm^3$ (n/%) | NA | NA | 3 (4) | 10 (14) |
| CD4<200 cells /$mm^3$ (n/%) | NA | NA | 5 (7) | 6 (8) |
| Median viral load ($Log_{10}$ copies/mL) | NA | NA | 5.07 (3.56-5.5) | 3.66 (2.67-5.03) * |
| Duration of HIV-1 infection (years) | NA | NA | 4 (2-6) | 5 (4-6) |

** P<0.05, **P<0.001 and ***P<0.0001, using the Mann Whitney test. NA=Not Applicable, IQR=interquartile range, n=number of participants, %=percentage.*

### III.1.2 Analysis of FoxP3-expression in magnetically sorted Treg cells from participant's PBMCs using a combination of $CD25^+CD127^{lo}$ markers

Regulatory T cells defined as $CD4^+$ $CD127^{Lo}$ $CD25^+$ $FoxP3^+$ cells were analyzed using the gating strategy shown in Fig. 30(a). Following identification of lymphocytes based on forward and side scatter, $CD3^+$ $CD4^+$ T cells were selected from live lymphocytes and $CD127^{Lo}$ T cells were gated from this population. The purity of the magnetically sorted Treg cells ranged between 90 and 99.5% for PBMCs from both ART-naive HIV-infected and sero-negative participants. 1000 events were collected from the $CD127^{Lo}$ gate to detect cells that were positive for CD25 and FoxP3 expression. As shown in Fig. 30 (a, b, c), prior purification of Treg cells resulted in higher numbers of $CD4^+$ $CD127^{Lo}$ $CD25^+$ cells [852 cells/$mm^3$ (690-908)] relative to partially purified samples [364 cells/$mm^3$ (259-672); p<0.001] and analysis with bulk PBMCs [320 cells/$mm^3$

(173-514); p<0.0001]. This was also reflected in total number of FoxP3 expressing cells as fully purified group showed the highest numbers of FoxP3 expressing Treg cells [591 cells/$mm^3$ (415-636)] followed by partially purified samples [214 cells/$mm^3$ (126-401); p<0.001] and lastly by bulk PBMCs [138 cells/$mm^3$ (77-223); p<0.0001]. A similar trend was also observed in HIV negative controls (compare Fig. 30b, c). Next, we assessed the relationship between the surface expression of a combination of CD25 and CD127 and the transcription factor, Foxp3 which is the signature marker of Treg cells. To achieve this, we plotted the number of $CD4^+$ T cells expressing a combination of $CD25^+$ and $CD127^{Lo}$ against the number of cells positive for Foxp3. Using spearman correlation analysis, we could demonstrate a positive correlation between $CD25^+CD127^{low}$ and FoxP3 expression in all the categories listed above. This correlation was determined for PBMCs (r= 0.94; p<0.0001; Fig. 30d), partially purified Treg cells (r=0.86; p<0.0001; Fig. 30e) and fully purified Treg cells (r=0.71; p<0.0002; Fig. 30f).

Due to this strong correlation, we therefore selected $CD4^+CD25^+$ $CD127^{Low}$ expression for subsequent tracking of Treg cell phenotypes.

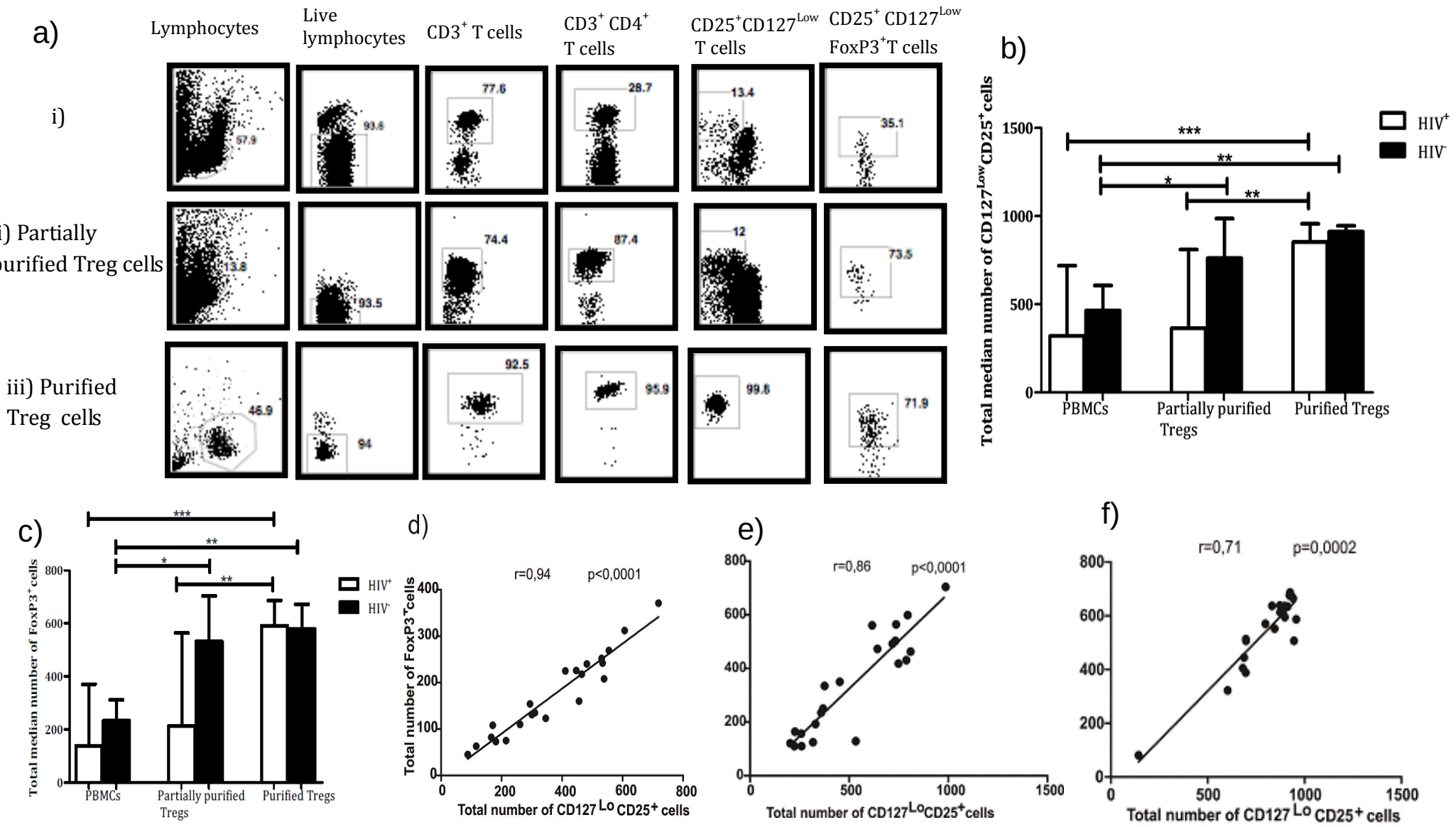
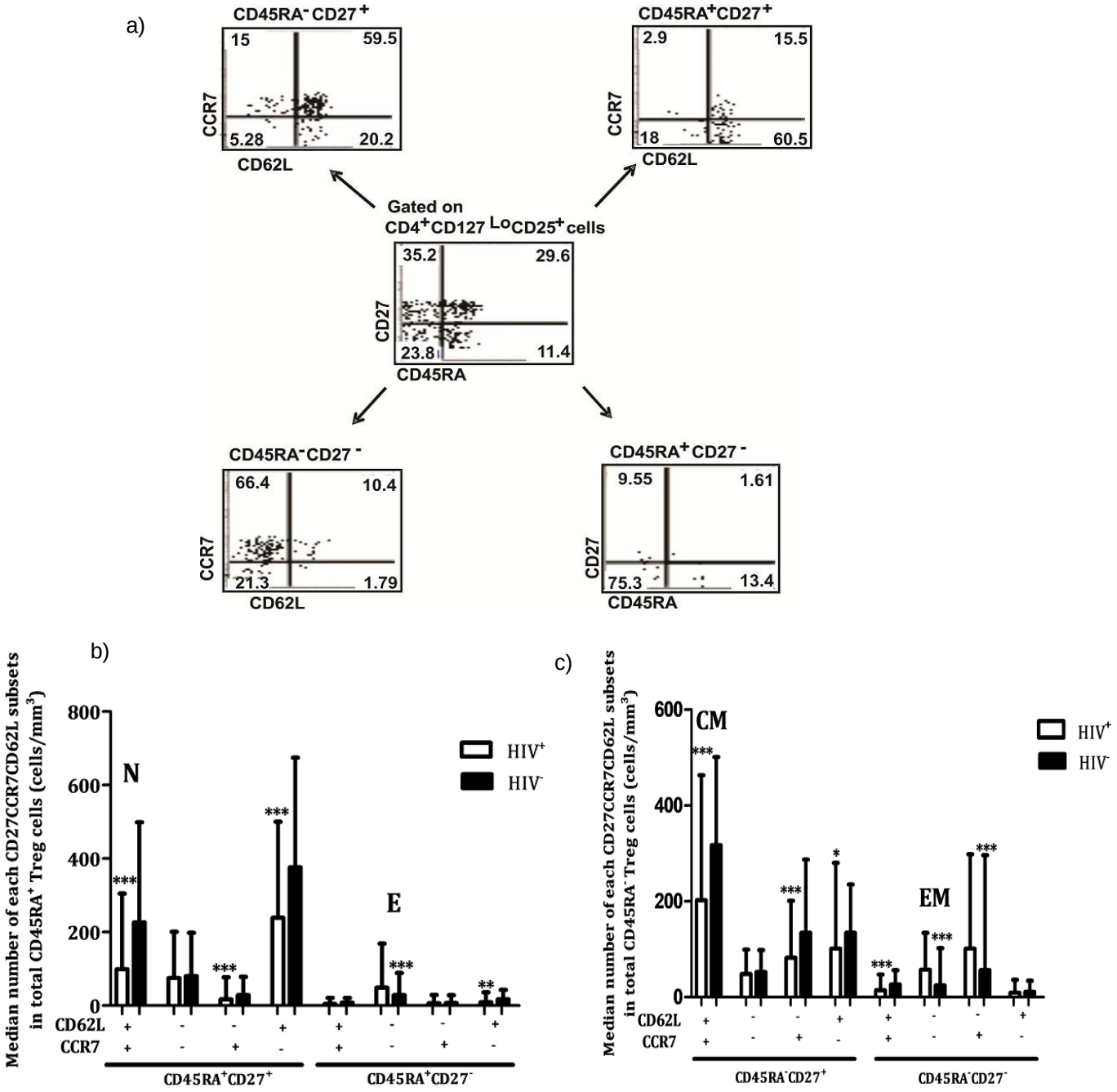



**Figure 30:** Tracking FoxP3 expressing cells using a combination of CD25+CD127Lo markers in either magnetically sorted Treg cells or bulk PBMCs. *(a) Representative dot plots of gating strategy for Treg cells stained directly in bulk PBMCs (i), in partially purified Treg cells (ii) or in totally purified Treg cells (iii). The numbers represent the percentage of gated cells in the indicated region. The Magnetic sorting of Treg cells resulted in a significant increase in the desired population in both HIV+ (n=16) and HIV- (n=6) participants (b and c). Vertical bars represent the median. d-f: There was a positive correlation between CD127LoCD25+ and FoxP3+ irrespective of whether staining was done with bulk PBMCs (d), partially purified (e) or fully purified Treg cells (f). * P<0.05, **P<0.001 and ***P<0.0001.*

### III.1.3 Phenotypic characterization of regulatory T cells during ART naive HIV-1 infection

Treg cells like all immune cells express several surface markers which are associated with their functions and as such could be used to predict their behavior. Thus using multiparametric FACS analysis, the expression profile of a number of surface markers including CD45RA, CD27, CD62L and CCR7 were analyzed upon purified Treg cells ($CD3^+CD4^+$ $CD25^+$ $CD127^{low}$ ). This resulted to the identification of sixteen Treg cell subsets (Fig. 31a, b, c) based on the combination of different surface markers. The gating schema for various Treg cell subsets is shown in Fig. 31a. However, we focused our analysis on naive ($CD45RA^+CD27^+CCR7^+CD62L^+$), effector ($CD45RA^+CD27^-CCR7^-CD62L^-$), central memory ($CD45RA^-CD27^+CCR7^+CD62L^+$) and effector memory ($CD45RA^-CD27^-CCR7^-CD62L^-$) Treg cells, as these subsets have been strongly associated with various Treg cell functions.

Relative to ART naive HIV-1 infected participants, uninfected people displayed predominantly naive [226 cells/$mm^3$ (176.5-303) versus 99 cells/$mm^3$ (78-198); $p<0.0001$] and central memory [317 cells/$mm^3$ (266.5-389) versus 202 cells/$mm^3$ (173-297); $p<0.0001$] phenotypes. In contrast, effector and effector memory Treg cell subsets increased significantly in ART naive HIV-1 infected participants compared with uninfected people: [49 (37-78) cells/$mm^3$ versus 28 (19-39) cells/$mm^3$; $p<0.0001$ and 57 (40-87) cells/$mm^3$ versus 24 cells/$mm^3$ (18-34.5); $p<0.0001$, respectively for effector and effector memory Treg cells].



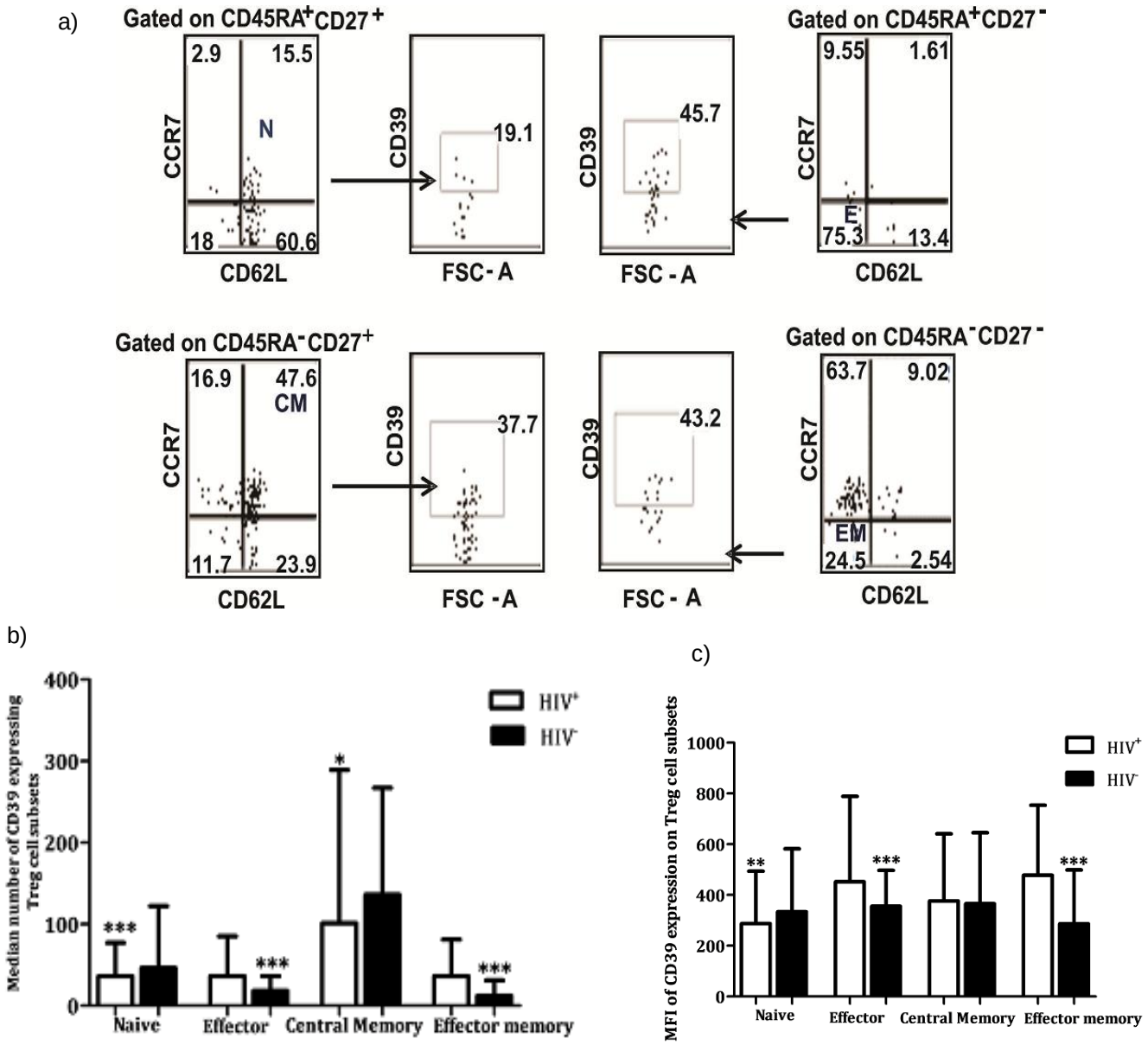

*N: naive; E: effector; CM: central memory; EM: effector memory*

**Figure 31:** Identification of regulatory T cell subsets in antiretroviral-naive HIV infection using CD45RA, CD27, CD62L and CCR7 cell surface markers. *(a) Representative dot plots of the gating strategy of different Treg cell subsets. Dot plots showing co-expression of both CD27 and CD45RA on CD4⁺ CD25⁺ CD127$^{low}$ Treg cells are represented in the middle panel. The data on CCR7 and CD62L are shown in each CD27 CD45RA subset. Distribution of Treg cell subsets analyzed on CD45RA⁺ or CD45RA⁻ Treg cells are shown in (b) and (c), respectively. Bars represent the median and Mann–Whitney U-test was used to compare each subset between HIV-infected and uninfected controls test (**P<0.001 and ***P<0.0001).*

We further performed an in-depth analysis of naive, effector, central memory and effector memory Treg cell subsets by examining other markers implicated in Treg suppressive functions such as CD39, CD73, HLA-DR and CD38.

### III.1.3.1 CD39 expression upon Treg cell subsets

The surface expression of CD39 was analyzed on naive, effector, central memory and effector memory Treg cell subsets (Fig. 32a). As shown in Fig. 32(b), there was a significant increase in the number of effector and effector memory Treg cells from ART naive HIV-1 infected participants expressing CD39 relative to seronegative controls [36 (27-45) versus 18 (14.5-25) cells/$mm^3$; P<0.0001 and 36 (29-45) versus 12 (9-17) cells/$mm^3$; p<0.0001, respectively]. In contrast, naive and central memory Treg cells from uninfected people displayed increased expression of CD39 compared with ART naive HIV-1 infected participants [46 (36.5-58) cells/$mm^3$ versus 36 (26-46) cells/$mm^3$; p=0.0002 and 136 (96.5-191.5) cells/$mm^3$ versus 101 (71-167) cells/$mm^3$; p=0.018, respectively]. The same tendency was also observed when the CD39 expression was analyzed in mean fluorescence intensity (MFI) as observed in Fig. 32(c). Effector and effector memory Treg cells from ART naive HIV-1 infected participants showed a significant increase in CD39 MFI (P<0.0001) relative to uninfected participants [452 (376-535) versus 355 (286-420) and 478 (379-574) versus 286 (178-376), respectively]. In contrast, the CD39 MFI on naive Treg cells from ART naïve HIV-1 infected participants were markedly lower [287 (222-376)] compared with uninfected participants [334 (293.5-400.5); p=0.0016]. There was no difference in the MFI of CD39 expressing central memory Treg cells between $HIV^+$ [376 (284-467)] and $HIV^-$ [365 (305.5-470.5)].




*MFI: mean fluorescence intensity*

Figure 32: CD39 expression on naive, effector, central memory and effector memory Treg cell subsets **.** *(a): Representative dot plots illustrating the gating strategy of CD39 expression. The median numbers of CD39 expressing Treg cell subsets are shown in (b). The differences between HIV⁺ (n=72) and HIV⁻ (n=38) participants were calculated using Mann–Whitney U-test. Effector and effector memory Treg cells from ART-naive HIV-1 infected participants showed significant increase in CD39 (P < 0.0001) expression. In contrast, CD39 was highly expressed instead on naive (p=0.0002) and central memory (p=0.018) Treg cells from uninfected individuals. A similar trend was observed when the MFI of CD39 expression was considered (c).*

### III.1.3.2 CD73 expression upon Treg cell subsets

In Fig 33 (a), a representative dot plots illustrating the gating strategy of CD73 expression on naive, effector, central memory and effector memory Treg cells are shown. Compared to seronegative controls, ART naive HIV-1 infected participants showed a significant increase in the number of CD73 expressing effector and effector memory Treg cells [31 (22-44) versus 18 (16-23) cells/$mm^3$; P<0.0001 and 31 (21-36) versus 18 (14.5-27) cells/$mm^3$; p<0.0001 respectively]. In contrast, naive and central memory Treg cells from uninfected people displayed increased expression of CD73 [51 (41.5-66.5) cells/$mm^3$ versus 37 (28-49) cells/$mm^3$ and 89 (76.5-99) cells/$mm^3$ versus 61 (46-87); p<0.0001, respectively, Fig. 33b]. The same tendency was observed when the expression was analyzed in MFI (Fig.33c). Effector Treg cells from ART naive HIV-1 infected participants express significantly higher CD39 MFI compared with uninfected participants [367 (187-426) versus 286 (154.5-356.5)]; p=0.0009]. Similarly, the MFI of CD39 expression increased significantly in effector memory Treg cells from $HIV^+$ participants relative to $HIV^-$ control group [368 (255-428) versus 237 (177-352); p<0.0001]. Conversely, naive and central memory Treg cell subsets from $HIV^+$ people showed lower levels of CD73 MFI compared with $HIV^-$ individuals [168 (119-194) versus 206 (164.5-295); p=0.0005 and 310 (185-362) versus 352 (275-379.5); p=0.02, respectively].

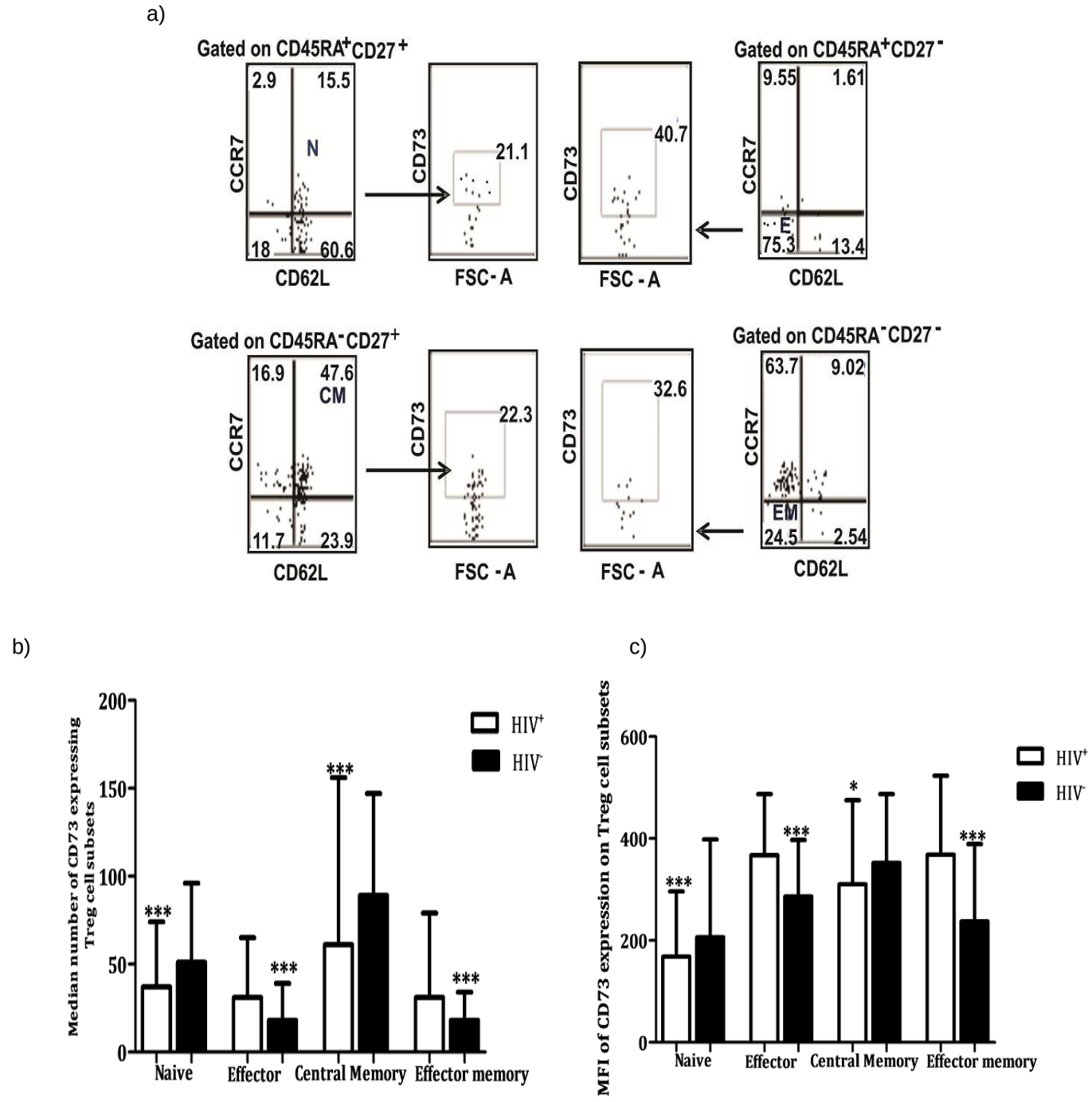
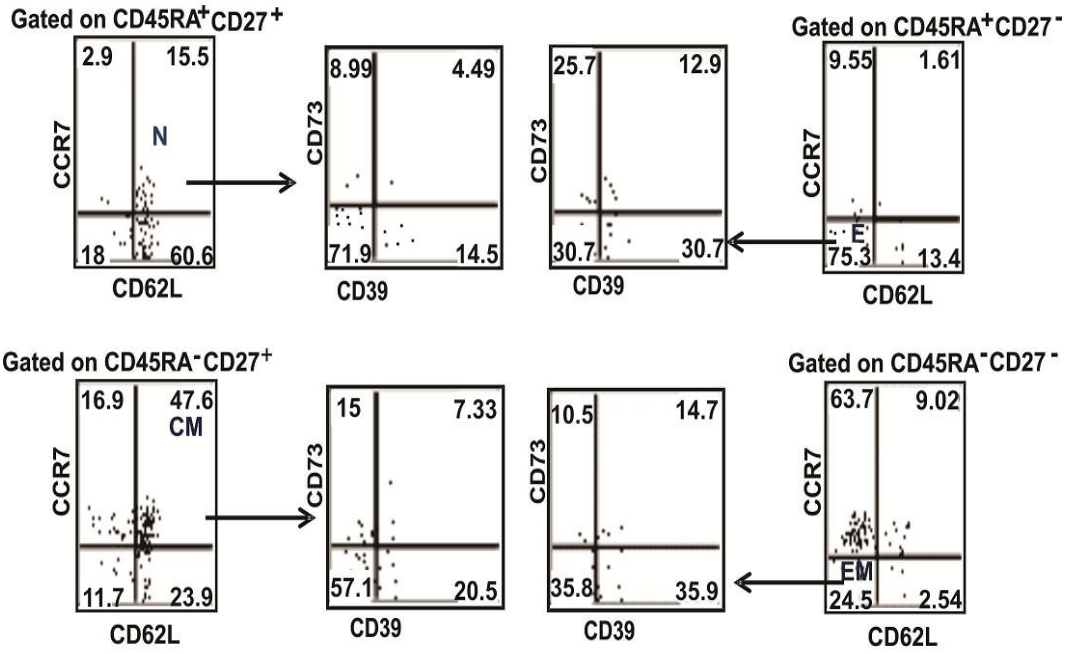



**Figure 33**: CD73 expression on naive, effector, central memory and effector memory Treg cell subsets. *(a): Representative dot plots illustrating the gating strategy of CD73 expression. The median numbers of each Treg subset expressing CD73 are shown in (b). The differences between HIV⁺ (n=72) and HIV⁻ (n=38) participants were calculated using Mann–Whitney U-test. Effector and effector memory Treg cells from ART-naive HIV-1 infected participants showed significant increase in CD73 (P < 0.0001) expression. In contrast, CD73 was highly expressed on naive and central memory Treg cells from uninfected individuals (P < 0.0001). A similar tendency was observed when the density of CD39 also referred as the MFI was considered (C).*

### III.1.3.3 Dual expression of CD73 and CD39 upon Treg cell subsets

Given that the synergistic action of both ectoenzymes CD39 and CD73 is required for the generation of adenosine, we further analyzed the concomitant expression of these markers on the surface of different Treg cell subsets. Although few cells co-expressed both CD39 and CD73, the combined expression of these markers increased significantly in effector and effector memory Treg cells from ART naive HIV-1 infected individuals ($p<0.0001$) compared with seronegative controls [23 (17-28) cells/$mm^3$ versus 12 (9-16) cells/$mm^3$ and 18 (14-23) cells/$mm^3$ versus 13 (10-16) cells/$mm^3$]. In contrast, both CD39 and CD73 decreased significantly on the surface of naïve and central memory Treg cells from $HIV^+$ participants ($p=0.0017$) compared with $HIV^-$ participants [17 (12-22) cells/$mm^3$ versus 20 (17-27) cells/$mm^3$ and 36 (25-53) cells/$mm^3$ versus 53 (32-69.5) cells/$mm^3$, respectively].

a)




b)

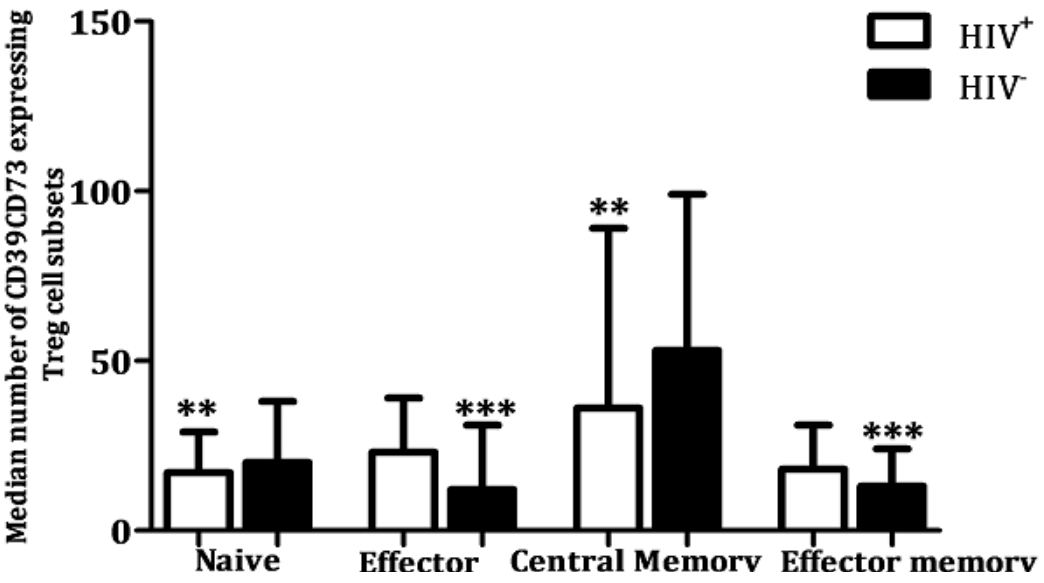



Figure 34: CD39 and CD73 co-expression in naive, effector, central memory and effector memory Treg cell subsets. *(a) Representative dot plots illustrating the gating strategy of the combined expression of CD39 and CD73. The median numbers of each Treg subset expressing both CD39 and CD73 are shown in (b). The differences between HIV⁺ (n=72) and HIV⁻ (n=38) participants were calculated using Mann–Whitney U-test. Effector and effector memory Treg cells from ART-naive HIV-1 infected participants showed significant increase in CD39 and CD73 co-expression (P < 0.0001). In contrast, these markers were highly co-expressed instead on naive and central memory Treg cells from uninfected individuals (P =0.0017).*

### III.1.3.4 Expression of HLA-DR upon Treg cell subsets

The activation status of Treg cell subsets from ART naive HIV-1 infected participants was assessed following HLA-DR expression analysis as determined by the number of HLA-DR positive cells (Fig. 35b) and the MFI of HLA-DR (Fig. 35c). Relative to $HIV^-$ participants, effector and effector memory Treg cells from $HIV^+$ participants showed significant increased expression of HLA-DR [ 39 (27-59) cells/$mm^3$ versus 20 (14-32.5) cells/$mm^3$ and 38 (30-48) cells/$mm^3$ versus 16 (12-19) cells/$mm^3$; $p<0.0001$, respectively, Fig. 35b]. However, HLA-DR expression by naïve and central memory Treg cells was not significantly different between $HIV^+$ and $HIV^-$ participants [ 36 (25-48) cells/$mm^3$ versus 39 (28.5-52) cells/$mm^3$ and 41 (28-67) cells/$mm^3$ versus 43 (35.5-77) cells/$mm^3$, respectively].

Similarly (compare Fig. 35b and c), the MFI of HLA-DR expression in effector and effector memory Treg cells from HIV+ and HIV- participants were 487 (297-684) versus 267 (162.5-393) and 498 (399-723) versus 245 (193.5-293) with $p<0.0001$, respectively. The MFI of HLA-DR expression in naive and central memory Treg cells from $HIV^+$ and $HIV^-$ participants were 396 (275-524) versus 436 (294-627.5) and 432 (275-678) versus 524 (355.5-907.5), respectively.

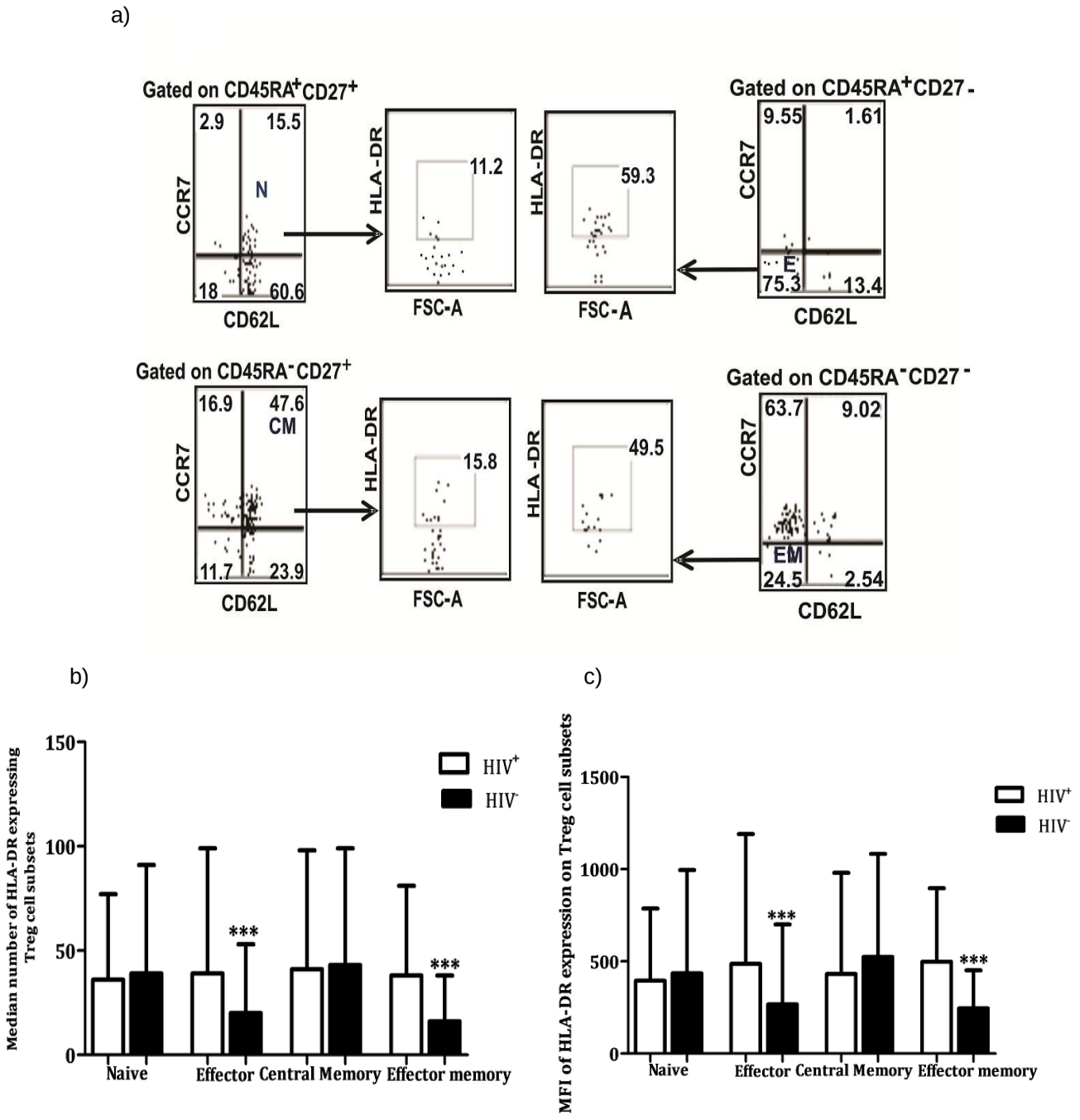



Figure 35: HLA-DR expression on naive, effector, central memory and effector memory Treg cell subsets. *In (a) the gating strategy for HLA-DR Treg cell surface expression is shown. The median values of the number of HLA-DR positive cells and the MFI of HLA-DR are represented as bars with range (b and c). The differences in HLA-DR expression for each Treg subset was assessed between HIV⁺ (n=72) and HIV⁻ (n=38) participants using Mann–Whitney U-test. HLA-DR was highly expressed by effector and effector memory Treg cells from antiretroviral therapy-naive HIV-infected participants compared with seronegative people (p<0.0001).*

### III.1.3.5 Expression of CD38 upon Treg cell subsets

We also used CD38 marker to evaluate the activation status of Treg cell subsets (Fig. 36a, b and c). In HIV-1 infected participants, CD38 expression was significantly higher in effector [43 (33-61) cells/$mm^3$] and effector memory Treg cells [38 (28-47) cells/$mm^3$] relative to seronegative controls [22 (18.5-31.5) cells/$mm^3$] and [18 (16-21) cells/$mm^3$], respectively ($p<0.0001$). However, we found no significant change in CD38 expressing naive and central memory Treg cell numbers from both $HIV^+$ and $HIV^-$ participants [38 (28-51) cells/$mm^3$ versus 41 (30.5-53.5) cells/$mm^3$ and 44 (32-69) cells/$mm^3$ versus 47 (35-75.5) cells/$mm^3$, respectively].

The same tendency was observed when the expression was analyzed in MFI as shown in Fig.36c [562 (385.5-750) versus 383.5 (267-465.3) and 577 (510.5-752) versus 367 (298-397), $p<0.0001$ respectively for effector and effector memory Treg cells]. For naive and central memory Treg cells from $HIV^+$ and $HIV^-$ participants, the MFI values were [452 (366-630) versus 533 (390-695.8) and 478 (379-721) versus 549.5 (423.3-698.5), respectively].

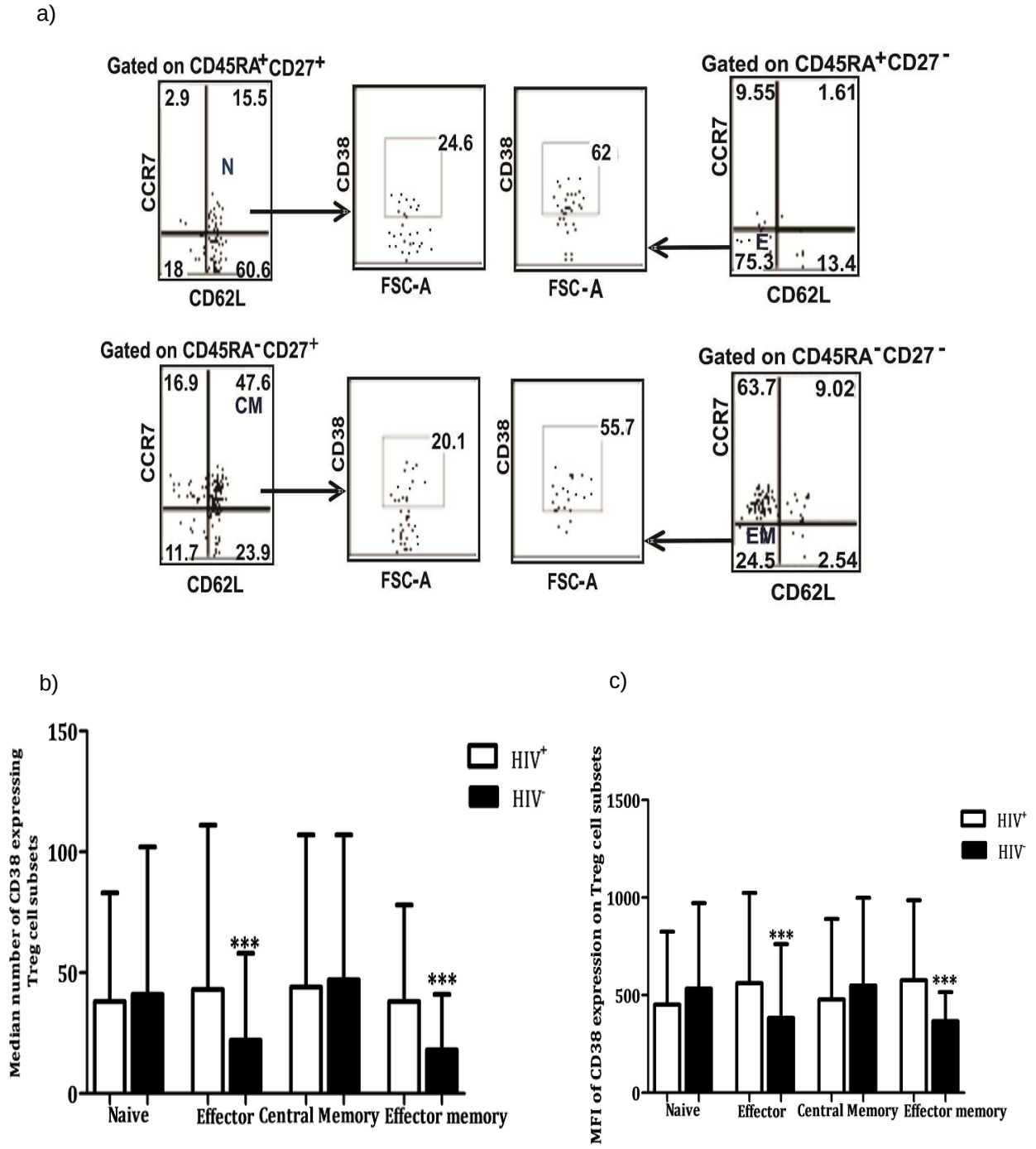



Figure 36: CD38 expression on naive, effector, central memory and effector memory Treg cell subsets. *In (a) the gating strategy for CD38 Treg cell surface expression is shown. The median values of the number of CD38 positive cells and the MFI of CD38 are represented as bars with range (b and c). The differences in CD38 expression for each Treg subset was assessed between HIV⁺ (n=72) and HIV⁻ (n=38) participants using Mann–Whitney U-test. While naive and central memory Treg cells showed no significant difference in CD38 expression between HIV⁺ and HIV⁻ participants, this marker was highly expressed by effector and effector memory Treg cells from ART naive HIV-1 infected participants (p<0.0001).*

### III.1.4 Relationship between helper CD4$^+$ T cell count, plasmatic HIV-1 viral load and regulatory T cells during ART naive HIV-1 infection

As conflicting data regarding the levels, and thus the role of Treg cells in HIV-1 infected individuals have been previously reported, we analyzed the relationship between helper CD4$^+$ T cell count, plasmatic HIV-1 viral loads and regulatory T cells during the course of ART naive HIV-1 infection. We first expressed Treg cells as either overall cell numbers or as a percentage of total CD4$^+$T cells, and then as Treg cell subsets.

#### III.1.4.1 Relationship between helper CD4$^+$ T-cell count and overall Treg cell numbers

CD4$^+$ T cells are the primary target of HIV-1 and the depletion of these cells is a hallmark of HIV-1 disease progression. As Treg cells are a subpopulation of CD4$^+$ T cells, we assessed whether total Treg cell (CD4$^+$CD25$^+$CD127$^{Low}$ FoxP3$^+$ cells) numbers are affected by HIV-1 infection. We did a Foxp3 staining in bulk PBMCs and observed significantly higher Treg cell numbers in HIV$^-$ participants 520.5 (456.8-571) cells/mm$^3$ compared to HIV$^+$ participants with CD4 counts ≥500 cells/mm$^3$ [372 (240-496) cells/mm$^3$, $p<0.001$], between 350–499 cells/mm$^3$ [298 (211-390) cells/mm$^3$, $p<0.0001$], between 200- 349 cells/mm$^3$ [223 (163.5-323.5) cells/mm$^3$, $p<0.0001$] and less than 200 cells/mm$^3$ [87 (62-173) cells/mm$^3$, $p<0.0001$] as shown in Fig. 37a. In addition, helper CD4$^+$ T-cell counts correlated positively with total Treg cell numbers in ART naive HIV-1 infected participants ($r = 0.4$, $P <0.0001$; Fig. 37b), thereby demonstrating that overall Treg cell numbers are depleted in the course of HIV infection.

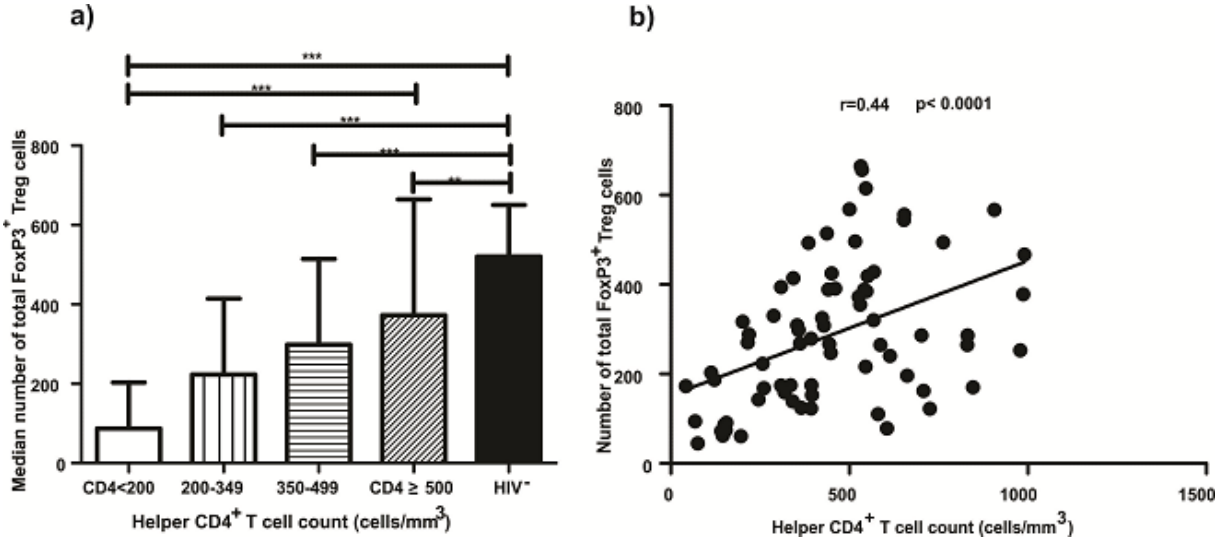



**Figure 37:** Relationship between helper CD4⁺ T-cell count and Treg cell numbers.

*HIV-1 infected participants were grouped into four categories according to their helper CD4⁺ T-cell counts [CD4 < 200 cells/mm³ (n=11); 200- 349 cells/mm3 (n = 13); 350–499 cells/mm3 (n = 17); ≥500 cells/mm3 (n = 31)]. When compared with uninfected participants (n = 38), the median number of total FoxP3⁺ Treg cells diminished proportionately with the helper CD4⁺ T-cell count (a). Moreover, Helper CD4⁺ T-cell count of HIV-infected participants correlated positively with the total number of FoxP3⁺ Treg cells (b). Bars represent the median values, which were compared using Kruskal–Wallis test followed by Dunn's multiple comparison test. Each dot represents a single HIV-1 infected individual and the correlation was calculated using Spearman test.*

### III.1.4.2 Relationship between helper CD4⁺ T-cell count and Treg cell percentages

Reduction in Treg cell numbers may reflect the impact of HIV in all CD4⁺ T cells. To determine whether Treg cells were differentially affected among CD4⁺ T cells, we analyzed Treg cell percentages in all HIV⁺ participants according to their immunological status. Unlike Treg cell numbers, Treg cell percentages increased as helper CD4⁺ T-cell counts decreased (Fig. 38a). A significant increase in Treg cell percentages was observed in ART-naive HIV-1-infected participants with helper CD4⁺ T-cell counts < 200 cells/ mm³ (7.03%; 4.44–8.58), between 200-349 cells/mm³ (5.76%; 5.28–6.53) and between 350-499 cells/mm³ (3.67%; 3.12–4.26) compared to those with helper CD4⁺ T-cell counts ≥500 cells/mm³ (2.05%; 1.33–2.81) and to uninfected participants (2.19%; 1.56–2.42) with p<0.0001. Moreover, helper CD4⁺ T-cell counts correlated negatively with Treg cell percentages (r = -0.82, P < 0.0001; Fig. 38b), suggesting a slower decline of Treg cells. These results demonstrate that Treg cells decline at different rates compared

with conventional CD4+ T cells, resulting in an increased regulator to helper ratio in ART-naive HIV-1 infected individuals with advanced immune system degradation.

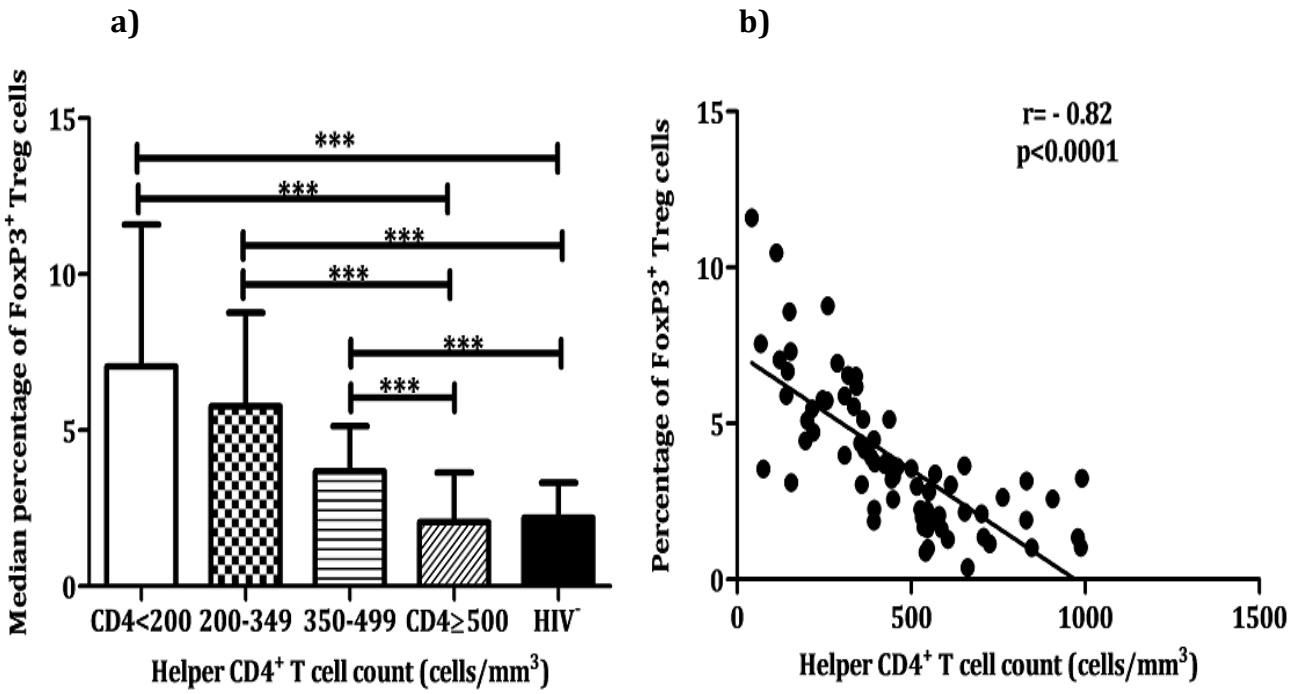



**Figure 38**: Relationship between helper CD4+ T-cell count and Treg cell percentages.

(a) *There was a significant increase in Treg cell percentages within total CD4+ T cells in ART-naive HIV-1 infected participants with CD4 counts < 500 cells/mm³ as compared to immunocompetent (CD4 counts ≥ 500 cells/mm³) and uninfected participants (P < 0.0001). Helper CD4+ T-cell counts of ART-naïve HIV-1 infected participants correlated negatively (r = -0.02; p<0.0001) with the percentages of Treg cells (b). Bars represent the median values, which were compared using Kruskal–Wallis test followed by Dunn's multiple comparison test. Each dot represents a single HIV-1 infected individual and the correlation was calculated using Spearman test.*

### III.1.4.3 Relationship between plasmatic HIV-1 viral loads and Treg cell numbers

In order to evaluate the relationship between plasmatic HIV-1 viral loads and the total number of Treg cells, participants were divided into three categories: those with plasmatic HIV-1 viral loads less than 3, between 3 and 4, and above 4 $Log_{10}$ copies/mL. We observed a progressive decrease in the total number of Treg cells when the plasmatic HIV-1 viral load increased: 388 (273.5-447.5) cells/mm³, 253 (123-385) cells/mm³ and 191.5 (140-315) cells/mm³, respectively for ART naive HIV-1 infected participants with plasmatic HIV-1 viral loads <3, 3-4 and >4 $Log_{10}$ copies/mL. However,

this reduction was statistically significant solely in participants with plasmatic HIV-1 viral loads above 4 $Log_{10}$ copies/mL (p=0.003; Fig 39a). Additionally, the total number of Treg cells correlated negatively with the plasmatic HIV-1 viral load (r = -0.29; P = 0.01; Fig. 39b)

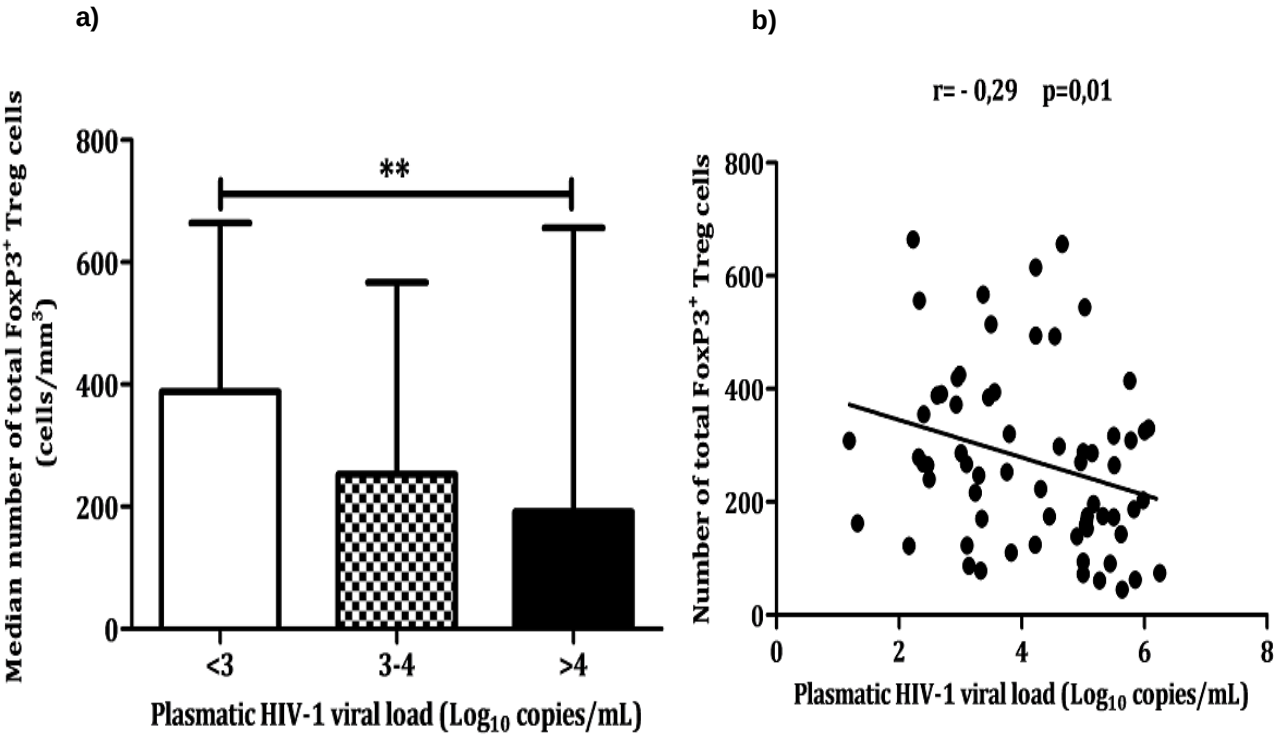



**Figure 39:** relationship between plasmatic HIV-1 viral loads and overall Treg cell numbers. *HIV-1 infected participants were divided into three groups according to their plasmatic HIV-1 viral loads [VL< 3 $Log_{10}$ copies/mL (n = 21); 3–4 (n = 15); > 4 (n = 36)]. The total number of Treg cells decreased significantly when the plasmatic HIV-1 viral load increased, using Kruskal–Wallis test (Fig.39a; p=0.003). In addition, plasmatic HIV-1 viral load correlated negatively with total Treg cell numbers (r = - 0.29; P = 0.01; b), using Spearman test.*

### III.1.4.4 Relationship between plasmatic HIV-1 viral loads and Treg cell percentages

Unlike Treg cell numbers, the percentages of Treg cells among total $CD4^+$ T cells increased significantly in HIV-1 infected participants with plasmatic HIV-1 viral loads > 4 $Log_{10}$ copies/mL (4.5%; 3.25-6.53) relative to participants with plasmatic HIV-1 viral loads between 3-4 $Log_{10}$ copies/mL (2.1%; 1.33-3.2; p<0.001) and< 3 $Log_{10}$ copies/mL (2.97%; 1.95-3.54; p<0.001). However, Treg cell percentages did not differ significantly

in HIV-1 infected participants with plasmatic HIV-1 viral load <3 and between 3-4 Log copies/mL (Fig. 40a). We also found that plasmatic HIV-1 viral loads correlated positively with Treg cell percentages (r = 0.5; P<0.0001; Fig.40b).

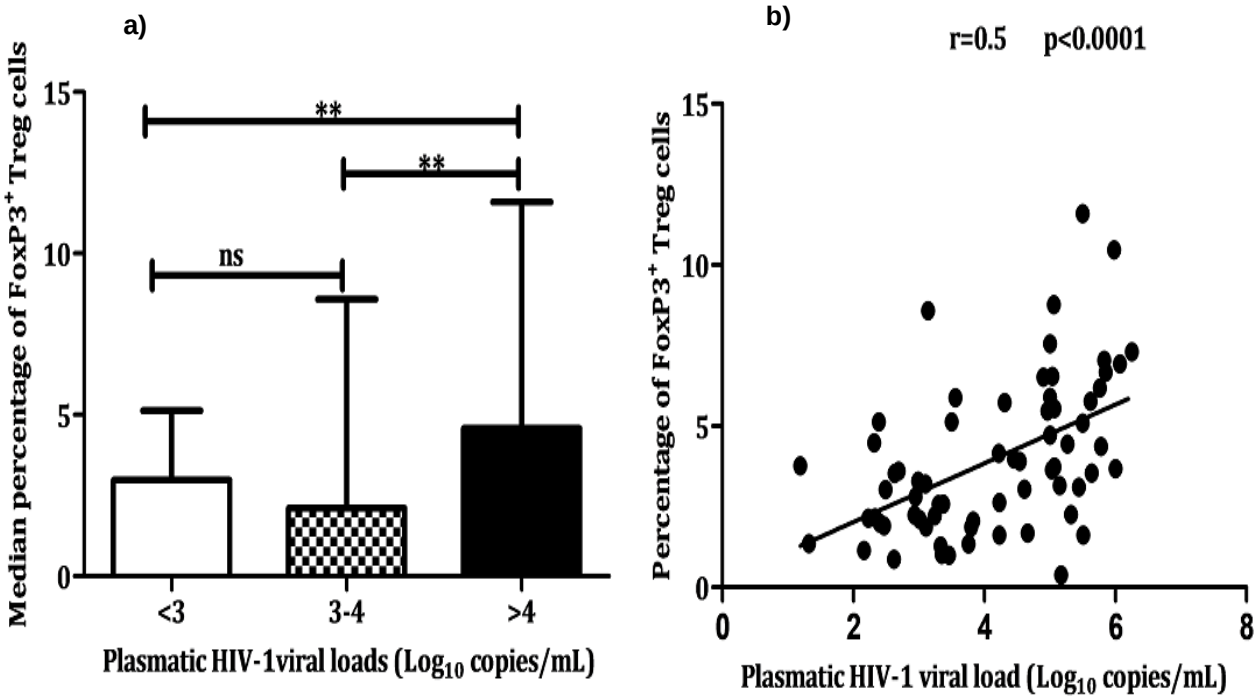



Figure 40: relationship between plasmatic HIV-1 viral loads and Treg cell percentages. *As shown in Fig. 39a, ART naive HIV-1 infected participants with plasmatic HIV-1 viral load >4 Log copies/mL showed higher percentages of Treg cells than those with plasmatic HIV-1 viral loads between 3-4 and < 3 Log copies/mL (p<0.001). Treg cell percentages correlated positively with plasmatic HIV-1 viral loads (r=0.5; p<0.0001; Fig. 39b). Bars represent the median values, which were compared using Kruskal–Wallis test followed by Dunn's multiple comparison test. Each dot represents a single HIV-1 infected individual and the correlation was calculated using Spearman test.*

### III.1.4.5 Relationship between naive, effector, central memory and effector memory Treg cells and helper CD4⁺ T cell count

Having established that the number of bulk Treg cells diminished with HIV disease progression, it was important to determine whether Treg cell subsets were differently affected during ART naive HIV-1 infection by assessing the relationships between naive, effector, central memory and effector memory Treg cells and helper CD4⁺ T cell count. We reported similar numbers of naive Treg cells (Fig. 41a) in HIV⁺

participants with CD4 count <200 cells/mm$^3$ [104 (81.25-137.8) cells/mm$^3$], between 200-349 cells/mm$^3$ [92(78.75-150.8) cells/mm$^3$], between 350-499 cells/mm$^3$ [116 (64-298) cells/mm$^3$] and ≥ 500 cells/mm$^3$ [121.5 (84.75-200.3) cells/mm$^3$]. In addition, naive Treg cell numbers did not correlate with helper CD4$^+$ T cell count (r=0.05; p=0.68) as shown in Fig.41c. Similarly, no significant difference was observed in the numbers of central memory Treg cells (Fig. 41e): 195.5 (175.3-262.8) cell/mm$^3$, 188 (138.3-266) cell/mm$^3$, 199(140.5-377) cell/mm$^3$ and 234 (175.3-347.8) cell/mm$^3$, respectively for HIV$^+$ participants with helper CD4$^+$ T cell count <200, between 200-349, between 350-499 and >500 cells/mm$^3$. However, we observed a positive correlation between central memory Treg cell numbers and helper CD4$^+$ T cell count (r=0.26; p=0.04, Fig. 41f).

In contrast to naive and central memory phenotypes, the numbers of effector Treg cells (Fig. 41c) decreased significantly in participants with CD4 count <200 cells/mm$^3$ [31 (27.25-43.5) cell/mm$^3$] and between 200-349 cells/mm$^3$ [32.5 (28-38.75) cells/mm$^3$] compared to those with CD4 counts between 350-499 cells/mm$^3$ [53 (41.5-72.5) cells/mm$^3$] and above 500 cells/mm$^3$ [67 (44.75-98.5) cells/mm$^3$, with p<0.0001. The same tendency was observed for effector memory Treg cells (Fig. 40h) with the lowest number in participants with CD4 count <200 cells/mm$^3$ [31(27-43) cells/mm$^3$], compared to those with CD4 counts between 200-349 cells/mm$^3$ [38 (29.5-50.25) cells/mm$^3$; p<0.05], 350-499 cells/mm$^3$ [59 (40.5-83) cells/mm$^3$; p<0.0001] and above 500 cells/mm$^3$ [78 (58.25-92) cells/mm$^3$; p<0.0001]. In addition, there was a positive correlation between helper CD4$^+$ T cell count with either effector (r=0.61, p<0.0001, Fig. 41d) or effector memory (r=0.74, p<0.0001, Fig. 40i) Treg cell numbers.

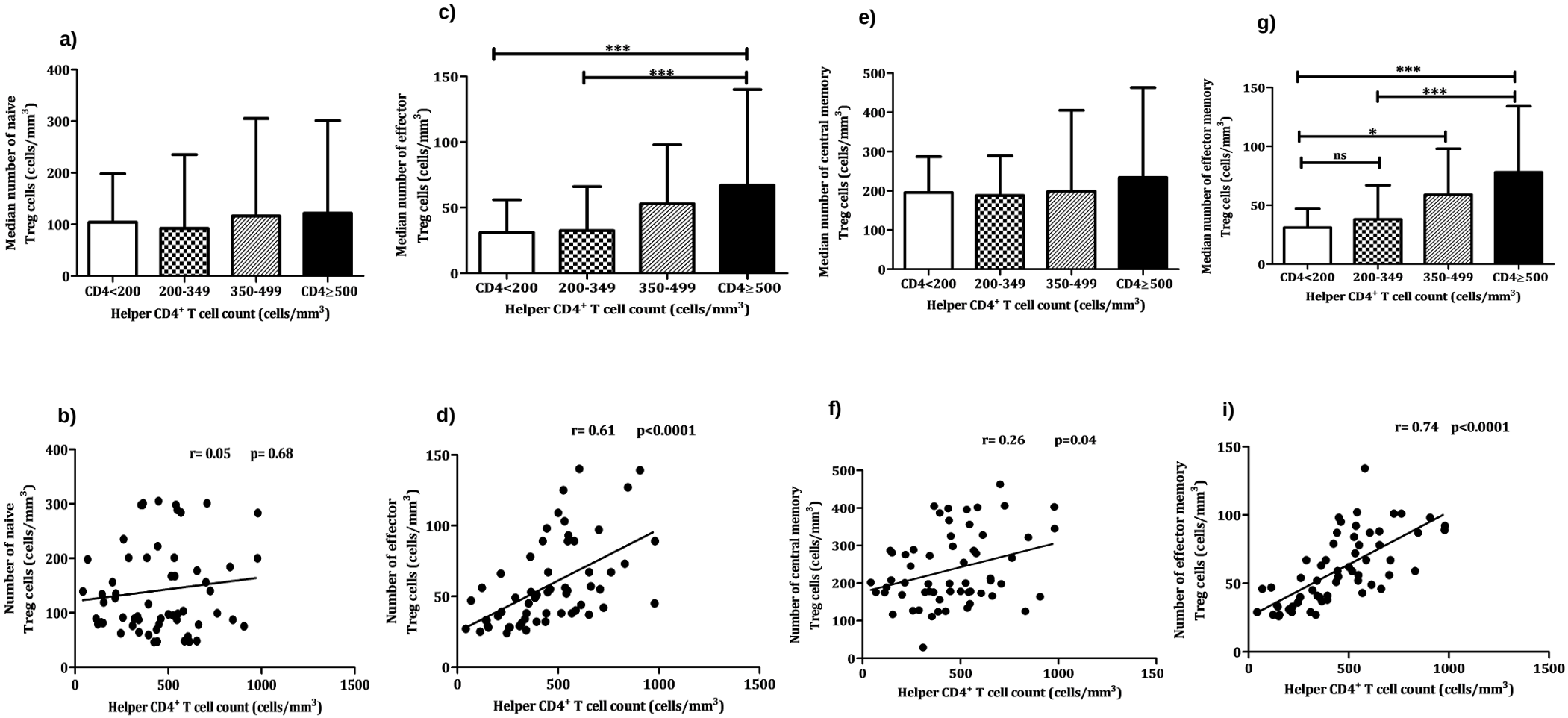



Figure 41: Relationship between naive, effector, central memory and effector memory Treg cells and helper CD4⁺ T cell count. *The median numbers of naive and central memory Treg cell subsets did not differ between all the groups of HIV-1 infected participants as shown in Fig. 41a, e. Moreover, Helper CD4⁺ T-cell count did not correlate with naive and central memory Treg cell numbers (Fig. 41b, f). In contrast, effector and effector memory Treg cell numbers were profoundly altered with immunosuppression (Fig. 40c, g; P<0.0001) and positively correlated with Helper CD4⁺ T-cell count (r=0.61, p<0.0001 and r=0.74, p<0.0001, respectively; Fig. 40d, h). Bars represent the median values, which were compared using Kruskal–Wallis test followed by Dunn's multiple comparison test. Each dot represents a single HIV-1 infected individual and the correlation was calculated using Spearman test.*

### III.1.4.6 Relationship between naive, effector, central memory and effector memory Treg cells and plasmatic HIV-1 viral loads

Regarding the association of Treg cell subsets and plasmatic HIV-1 viral loads, we observed a slight reduction of naive Treg cell numbers with HIV-1 disease progression: 128 (90.5-262.5) cells/$mm^3$, 103 (755-253) cells/$mm^3$ and 96 (78.75-171.3) cells/$mm^3$, respectively for $HIV^+$ participants with plasmatic HIV-1 viral loads <3 Log, between 3-4Log and >4Log (Fig, 42a); However, this trend did not reach statistical significance (r= -0.22, p=0.08, 42b). Relative to central memory Treg cells, an increase was observed in participants with plasmatic HIV-1 viral loads between 3-4$Log_{10}$ copies/mL [279 (168.5-360.5) cells/$mm^3$] compared to those with plasmatic viral loads <3 Log [231 (178-340.8) cells/$mm^3$], and >4Log [193.5 (132.5-273.8) cells/$mm^3$], although no significant difference was detected (Fig, 42e). This was also reflected by the lack of correlation between plasmatic HIV-1 viral loads and central memory Treg cell numbers (r=-0.16, p=0.22, Fig. 42f). In contrast, effector and effector memory Treg cell numbers decreased significantly with HIV-1 disease progression, the lowest values being observed in $HIV^+$ participants with plasmatic HIV-1 viral loads >4Log: 39.5 (31.5-53.75) cells/$mm^3$ and 41 (33-56.5) cells/$mm^3$, respectively for effector ($p<0.05$) and effector memory (p=0.0003) Treg cells. Participants with plasmatic HIV-1 viral loads between 3-4 Log had 53 (32-97) cells/$mm^3$ and 61 (42.5-92) cells/$mm^3$ of effector and effector memory Treg cells, respectively. Those with plasmatic HIV-1 viral loads <3 Log values had 56 (45-87) cells/$mm^3$ of effector and 78 (62.25-94.25) cells/$mm^3$ of effector memory Treg cells (Fig. 42c and g). Additionally, we observed an inverse correlation between plasmatic HIV-1 viral loads and either effector (r= -0.42, p=0.0009, Fig. 42d) or effector memory (r= -0.48, $p<0.0001$, Fig.42h) Treg cell numbers.

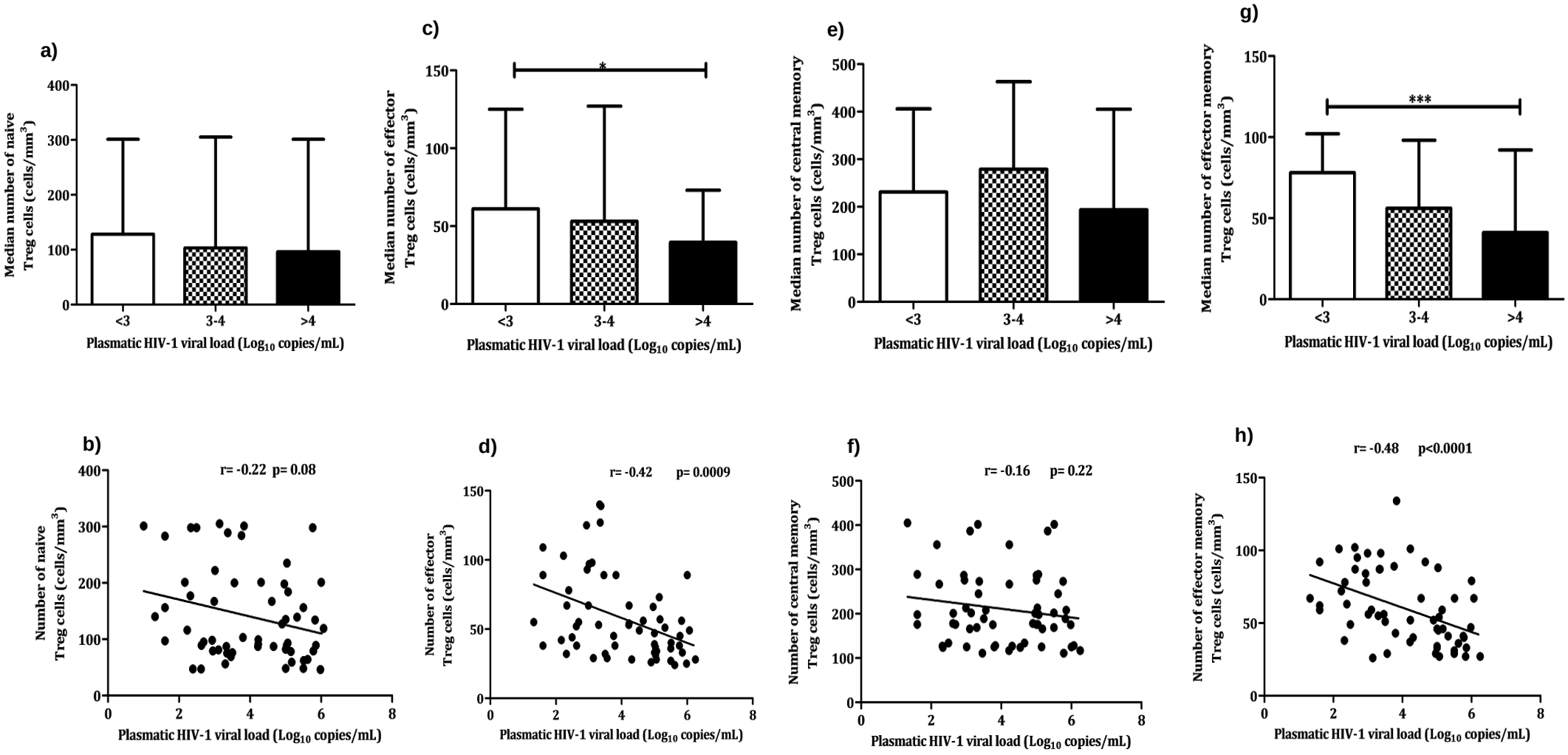



**Figure 42:** Relationship between naive, effector, central memory and effector memory Treg cells and plasmatic HIV-1 viral loads. *Despite a slight reduction of naive Treg cell numbers with HIV-1 disease progression, we did not observe a significant difference between the HIV-1 infected groups (plasmatic HIV-1 viral loads <3, between 3-4, >4 Log) as shown in Fig. 42a. Moreover, plasmatic HIV-1 viral loads did not correlate with naive Treg cell numbers (Fig. 42b). Similarly, the median numbers of central memory Treg cells did not correlate with plasmatic HIV-1 viral loads (Fig. 42e, f). In contrast, effector and effector memory Treg cell numbers decreased significantly when the plasmatic HIV-1 viral loads increased ($p<0.05$ and $p=0.0003$, respectively; Fig 42c, g). Additionally, there was an inverse correlation between plasmatic HIV-1 viral loads and either effector ($r= -0.42$, $p=0.0009$) or effector memory ($r= -0.48$, $p<0.0001$) Treg cell numbers (Fig. 42d, h).*

### III.1.5 Cytokine profile of regulatory T cells

The regulation of immune homeostasis involves the capacity of Treg cells to suppress excessive activation, proliferation and effector functions of adaptive and innate immune cells. For that, several strategies including direct cell to cell contact, the secretion of inhibitory cytokines or competition for growth factors are employed by Treg cells. To understand the nature of Treg cells in the context of ART naive HIV-1 infection we have assessed key cytokines associated with their functions.

### III.1.5.1 Single cytokine production profiles of Treg cells

In figure 43a, dot plots illustrating single cytokine expression of $CD4^{+}CD25^{+}CD127^{Low}$ $FoxP3^{+}$Treg cells is shown. ART naive HIV-1 infected participants with helper $CD4^{+}$ T cell counts ≥ 500 cells/$mm^3$ showed significantly higher numbers of TGF-β producing $FoxP3^{+}$Treg cells [389 (345-466) cells/$mm^3$] compared to people with helper $CD4^{+}$ T cell counts < 200 cells/$mm^3$ [267 (206-314) cells/$mm^3$, $p<0.05$] and between 200-349 cells/$mm^3$ [298 (250.3-322.8) cells/$mm^3$, $p<0.05$]. This elevated level of TGF-β expression was similar to that seen in participants with helper $CD4^{+}$ T cell counts between 350-499 cells/$mm^3$ [338 (208-373) cells/$mm^3$] and in uninfected individuals [423 (330.5-470.3) cells/$mm^3$] as shown in 43b.

With respect to IL-17A producing $FoxP3^{+}$ Treg cells, similar numbers were recorded in both $HIV^{+}$ and $HIV^{-}$ participants: 356 (298-409) cells/$mm^3$, 314 (266.3-450.8) cells/$mm^3$, 391.5 (263.8-415.5) cells/$mm^3$, 436 (367-457) cells/$mm^3$ and 385.5 (335.8-483.5) cells/$mm^3$ respectively for people with CD4 count < 200, between 200-349, between 350-499, ≥ 500 cells/$mm^3$ and HIV- people (43c).

Overall, a weak production of IL-10 by $FoxP3^{+}$ Treg cells was observed (44c). However, this decline was more pronounced in ART-naive HIV-1 participants with CD4 count <200 cells/$mm^3$ [74 (51-82) cells/$mm^3$, $p<0.0001$], between 200-349 cells/$mm^3$ [76 (61.25-93) cells/$mm^3$, $p<0.001$] and between 350-499 cells/$mm^3$ [66 (61.5-92.25) cells/mm3, $p<0.001$] than in seronegative people [120 (101.8-132.8) cells/$mm^3$]. No difference in IL-10 production was observed between seronegative people and participants with helper $CD4^{+}$ T cell count ≥ 500 cells/$mm^3$ [101 (87-109) cells/$mm^3$].

Regarding IL-4 production from Treg cells, we observed a gradual reduction corresponding with HIV progression: 135 (114-190), 130.5 (108-154), 127 (95.75-

190.8) and 98 (81-135) cells/mm$^3$, respectively for ART naive HIV-1 infected participants with CD4 ≥500, between 350-499, between 200-349 and <200 cells/mm$^3$. However, a statistically significance was found only for participants with CD4 count <200 cells/mm$^3$ ($p<0.001$) when compared with HIV-1 negative people [196.5 (154.8-210.5) cells/mm$^3$; Fig.44e].

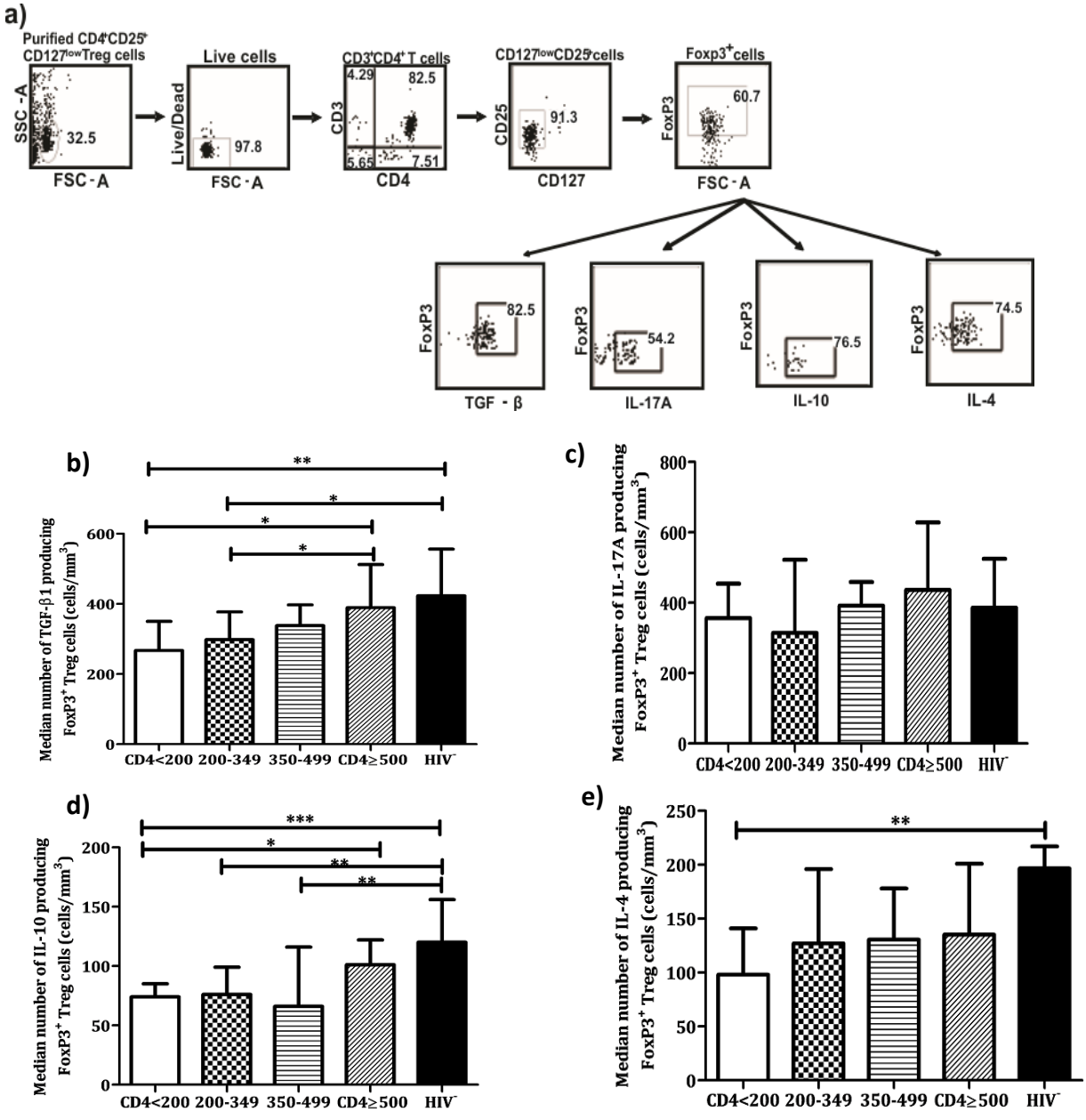



**Figure 43**:Single cytokine production profiles of Treg cells*. Dot plots showing expression of each cytokine on CD4$^+$CD25$^+$CD127$^{Low}$ FoxP3$^+$Tregs are shown in 44a. TGF-β, IL-17A, IL-10 and IL-4 expression on Treg cells are shown in 43b, c, d and e, respectively. The differences between groups were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison post-test.*

### III.1.5.2 Simultaneous multiple cytokine production profiles of Treg cells

Since multifunctional Treg cells could be associated with enhanced suppressive functions, we did an in-depth functional analysis of simultaneous multiple cytokine-producing Treg cells. Using a Boolean gate analysis (Fig.44 a), we were able to define 11 polyfunctional Treg cell subsets, each simultaneously expressing a combination of several cytokines including IL-4, TGF-β, IL-17A and IL-10 (Fig.44 b). Compared to seronegative participants, there was a significant reduction in the number of Treg cell subset simulataneously expressing four cytokines (IL-4$^+$TGF-β$^+$IL-17A$^+$ IL-10$^+$) in HIV$^+$ participants with CD4 count <200 cells/mm$^3$ [ 45 (31-51) cells/mm$^3$; p<0.001], between 200-349 cells/mm$^3$ [ 37 (33.5-44) cells/mm$^3$; p<0.0001] and between 350-499 cells/mm$^3$ [44 (37-73) cells/mm$^3$; p<0.05]. Interestingly, participants with no immunosuppression (CD4 count ≥ 500 cells/mm$^3$) had similar numbers of Treg cell simultaneously producing IL-4, TGF-β, IL-17A and IL-10 [56 (47-70) cells/mm$^3$] like seronegative individuals [77.5 (66-89) cells/mm$^3$]. Similar trends were also observed for Treg cells simultaneously producing three (IL-4$^+$TGF-β$^+$IL-17A$^+$, IL-4$^+$TGF-β$^+$IL-10$^+$, TGF-β$^+$IL-17A$^+$ IL-10$^+$) and two cytokines (IL-4$^+$TGF-β$^+$, TGF-β$^+$IL-17A$^+$, TGF-β$^+$IL-10$^+$). Conversely, the median number of double positive IL-4$^+$IL-17A$^+$ Treg cell subset was significantly lower in seronegative people compared with HIV$^+$ participants with CD4 counts <200 and between 350-499 cells/mm$^3$ (p<0.05, respectively). However, the numbers of triple (IL-4/IL-17A/IL-10) and double (IL-4/IL-10 or IL-17A/IL-10) cytokine secreting FoxP3$^+$Treg cells were comparable between HIV$^+$ and HIV$^-$ participants. Taking into consideration these intracellular cytokine production profiles , it appears that distinct single and multiples cytokine forming Treg cell subsets circulate in the peripheral blood of ART naive HIV-1 infected people

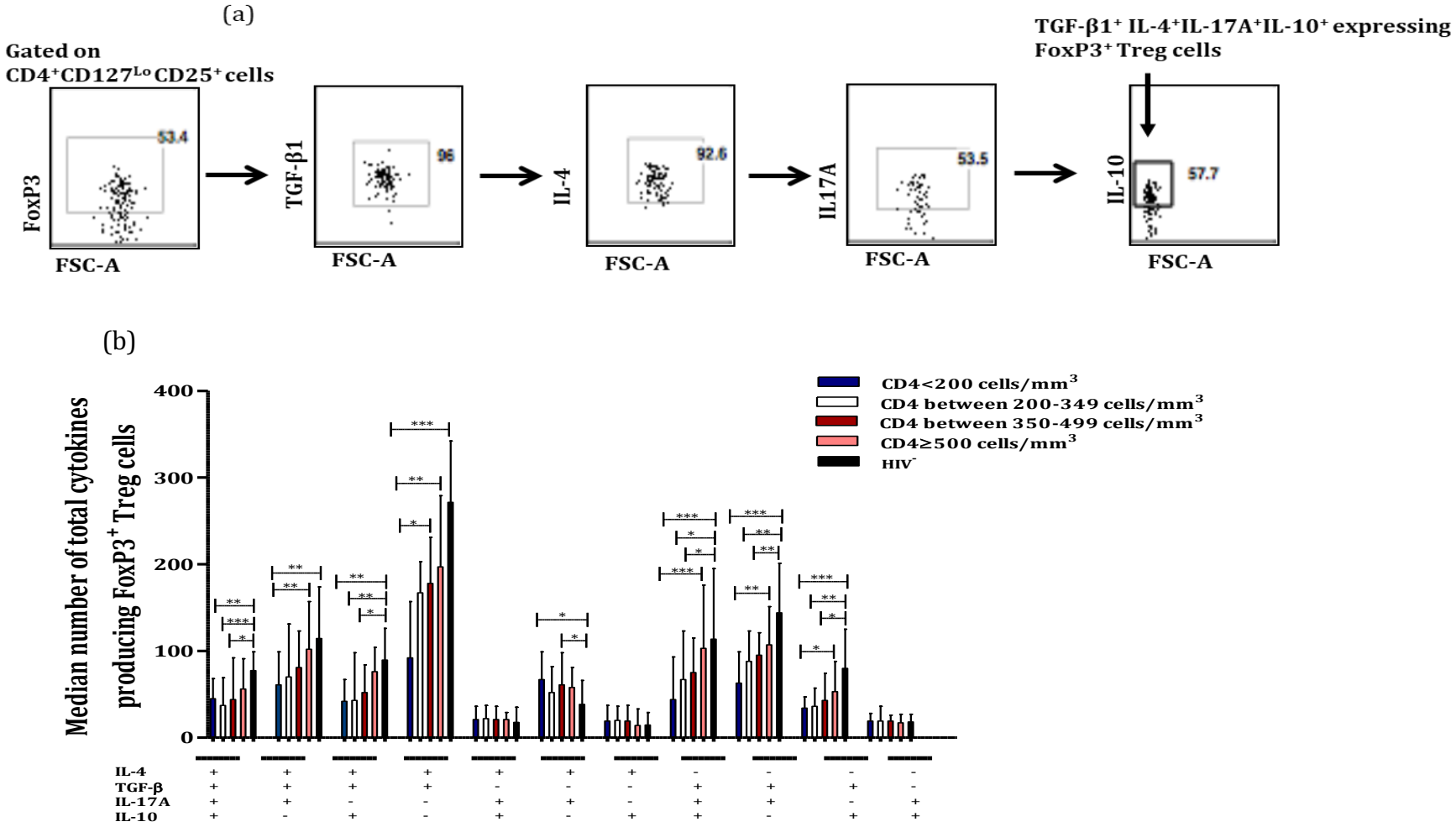



**Figure 44:** Simultaneous multiple cytokine production profile of Treg cells.. *(a) is the representative example of gating strategy used to identify TGF-β1, IL-4, IL-17A and IL-10 simultaneous cytokine producing FoxP3⁺ Treg cells. Boolean gate analysis allowed the identification of eleven functional FoxP3⁺ Treg cell subsets producing different cytokine combinations (b). Kruskal–Wallis analysis with Dunn's post-test comparison was applied to compare the median numbers of cytokine-coproducing Treg cells between HIV⁺ and HIV⁻ participants. Except for IL-4 and IL-17A coproducing Treg cells which were less represented in seronegative participants ($p<0.05$), a significant increase in the number of other major polyfunctional Treg cell subsets was observed in HIV⁻ and HIV⁺ participants with CD4 count≥500 cells/mm³ compared to HIV-1infected participants with CD4 count between 350-499 cells/mm³, 200-349 cells/mm³ and <200 cells/mm³.*

### III.1.6 Modulatory effect of Treg cells on autologous monocytes

Previous studies assessing the functional properties of Treg cells focused mainly on their ability to suppress HIV-1-specific effector functions of T cells. In this study, we thought that it is necessary to assess the capacity of Treg cells to modulate the functions of monocytes, a potent source of pro-inflammatory cytokines and chemokines production during immune cell-recruitment and activation.

#### III.1.6.1 ART naive HIV-1 infection is associated with monocyte activation

Increased monocyte activation resulting from exposure to bacteria arising from intestinal damage have been shown to exacerbate morbidity and mortality in HIV-1 infected individuals. During this study, monocyte activation was measured by tracking surface upregulation of CD38 and HLA-DR on target cells through multiparametric flow cytometry analysis. Dot plots illustrating the gating strategy of surface expression of HLA-DR and CD38 on purified monocytes are shown in Fig. 45. Whereas 92 % (88-97) of monocytes from HIV$^+$ participants expressed HLA-DR, only 61% (50-76%) expressed CD38. Moreover, almost all CD38 expressing monocytes (97%; 94-99%) were also HLA-DR$^+$, but only 64% (57-74%) of HLA-DR expressing monocytes were CD38$^+$. Thus making CD38 a more reliable marker for tracking monocyte activation during this study.

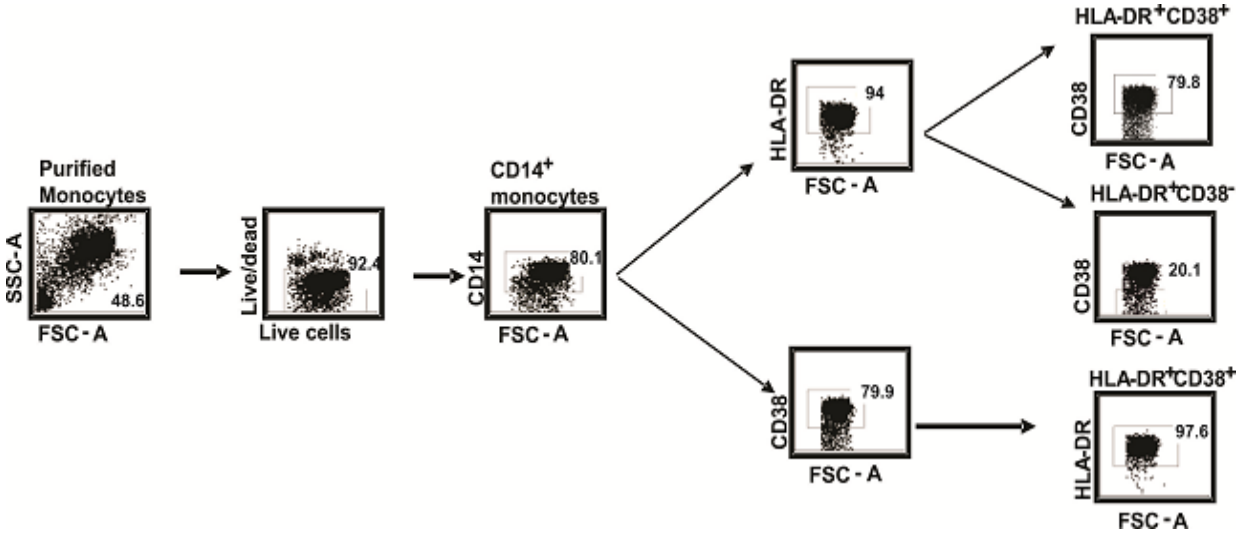



Figure 45: monocyte activation markers profile during ART naive HIV-1 infection. *Gating strategy for HLA-DR and CD38 expression on monocytes: following forward and side scatter selection, magnetically sorted monocytes were selected from live cells and then analyzed for CD14 expression. CD14$^+$ monocytes were more than 80% pure. Next, HLA-DR and CD38 expression was monitored on the purified Monocytes.*

### III.1.6.2 Tracking monocyte activation using surface expressed CD38

We further analyzed the association between monocyte activation, defined by the upregulation of the surface expression of HLA-DR or CD38 in MFI and HIV disease progression. Relative to HIVnegative people, the MFI of HLA-DR was significantly elevated in $HIV^+$ participants: 1703 (1281-1881), 1567 (1257-1902), 1530 (1278-1666) and 1452 (1324-1679), respectively for participants with CD4 count<200cells/$mm^3$ ($p<0.001$), between 200-349 cells/$mm^3$ ($p<0.001$), between 350-499 cells/$mm^3$ ($p<0.05$), and ≥500 cells/$mm^3$ ($p<0.0001$) as shown in Fig.46a. Additionally the MFI of HLA-DR on monocytes increased with plasmatic HIV-1 viral loads: 1392 (1285-1573), 1427 (1281-1670), 1688 (1315-1899), respectively for participants with plasmatic HIV-1 viral loads <3, between 3-4 and >4$Log_{10}$ copies/mL (Fig. 46c). However, HLA-DR expression on monocytes did not show any significant correlation with helper $CD4^+$ T cell counts ($r= -0.16$; $p=0.30$). A positive correlation was observed only with plasmatic HIV-1 viral loads ($r=0.34$; $p=0.02$) in HIV-1 infected people (Fig. 46b, d). The same trend was observed with respect to CD38 expression: significantly higher expression was observed in $HIV^+$ participants with CD4 counts < 200 cells/$mm^3$ [789 (627-991), $p<0.0001$)], between 200-349 cells/$mm^3$ [(719 (569.5-868.5), $p<0.0001$] and between 350-499 cells/$mm^3$ [593.5 (384.8-709.8) cells/$mm^3$] compared to seronegative participants (Fig. 46e). However, there was no significant difference in CD38 MFI between participants with CD4 count ≥ 500 cells/$mm^3$ [420 (300-550)] and seronegative participants [210 (186.3-255.3)]. CD38 expression correlated negatively with helper $CD4^+$ T cells ($r= -0.62$, $p< 0.0001$; Fig.46f) and positively with plasmatic HIV-1 viral loads ($r= 0.39$, $p=0.01$; Fig. 46h).

Although increased surface upregulation of both HLA-DR and CD38 markers was observed, we decided to track changes in monocytes activation using CD38 because it was reliable in predicting changes in monocytes activation relative to different clinical stages of HIV infection.

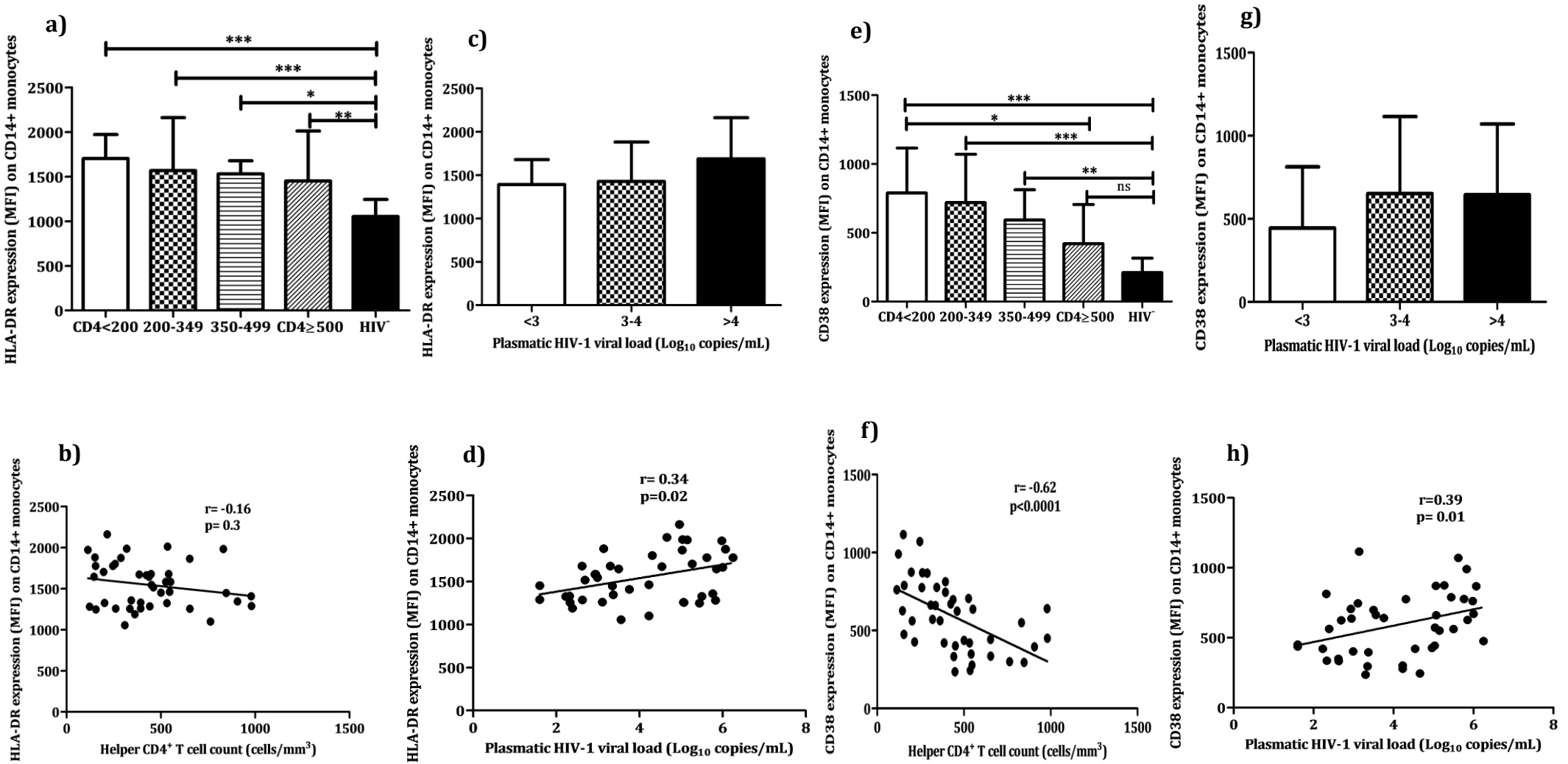



Figure 46: CD38 surface marker is more reliable than HLA-DR for monocyte activation. *Relative to seronegative controls, HIV infected individuals display increased levels of immune activation as evidenced by elevated expression of HLA-DR, CD38, using the Kruskal-Wallis test followed by Dunn's multiple comparison test (Fig. 46a, e). whereas HLA-DR expression was correlated only with plasmatic HIV-1 viral loads (r=0.34, p=0.02), CD38 expression correlated negatively with helper CD4+ T cells (r= -0.62, p< 0.0001; 46f) and positively with plasmatic HIV-1 viral loads (r= 0.39, p=0.01; 46h).*

### III.1.6.3 Suppressive effect of autologous Treg cells on poly-ICLC mediated monocytes activation .

Rapid progression of HIV infection has been linked to hyperactivation/inflammation of the immune system and its negative consequences. Due to the fact that HIV-1 driven monocytes activation escalates inflammation through the production of pro-inflammatory cytokines and chemokines we hypothesized that Treg cells can target monocytes to counter excessive inflammation. Thus in this study, we assessed in the context of ART naive HIV-1 infection, if Treg cells can modulate monocytes activation during stimulation with a TLR3 ligand ( poly-ICLC) following co-culture with autologous monocytes. Briefly monocytes were purified as described above and equilibrated to minimize variations among their numbers between participants. In order to mimic the situation of chronic HIV-1 infection, we used poly-ICLC which is a synthetic double stranded RNA and then analyzed CD38 expression in monocytes. As shown in Fig. 47a, a significant increase in CD38 surface expression was observed in poly-ICLC treated monocytes compared to monocytes minus the TLR3 ligand. The SI did not differ significantly between participants with helper $CD4^+$ T cell count ≥500 cells/$mm^3$ [1.8 (1.7-2.1)] and seronegative controls [2.83 (2.31-3.28); Fig. 47b]. However, significantly lower values of SI were reported for participants with CD4 counts <200 cells/$mm^3$ [1.4 (0.96-1.14); $p<0.0001$], between 200-349 cells/$mm^3$ [1.44 (1.11-1.64); $p<0.0001$] and between 350-499 cells/$mm^3$ [ 1.49 (1.38-1.79); $p<0.001$] compared to seronegative controls. Although no significant difference was noticed, the SI slightly decreased when HIV-1 plasmatic viral loads increased: 1.64 (1.42-2.05), 1.54 (1.29-1.94) and 1.49 (1.14-1.75), respectively for participants with viral loads <3, between 3-4 and >4$Log_{10}$ copies/mL (Fig. 47d). As shown in Fig. 47c, e, the SI correlated positively with the helper $CD4^+$ T cell counts ($r= 0.68$, $p<0.0001$) and negatively with HIV-1 plasmatic viral loads ($r= -0.64$, $p= 0.006$). The addition of autologous Treg cells to the culture medium resulted in a significant reduction in the monocytes surface expression of CD38 (suppression). Similar to the SI, the Treg cell suppression index (IS) decreased proportionately with the helper $CD4^+$ T cell counts (Fig. 47f), the lowest values being observed in participants with CD4 counts <200cells/$mm^3$ [0.26 (0.20-0.37)] and between 200-349 cells/$mm^3$ [0.29 (0.25-0.34)] compared to those with CD4 counts between 350-499 cells/$mm^3$ [0.44 (0.41-0.55)], ≥ 500cells/$mm^3$ [0.54 (0.43-0.65); $p<0.001$] and $HIV^-$ people [0.62 (0.58-0.69); $p<0.0001$]. Furthermore, the IS

decreased significantly when viral loads increased: 0.48 (0.43-0.59), 0.45 (0.24-0.61) and 0.35 (0.26-0.42), respectively for participants with viral loads <3, between 3-4 and > $4Log_{10}$ copies/mL ($p<0.05$; Fig. 47h). The IS equally correlated positively with the helper $CD4^+$ T cell count ($r= 0.78$, $p<0.0001$; Fig.47g) and negatively with HIV-1 plasmatic viral loads ($r= -0.49$, $p= 0.008$; Fig. 47i), demonstrating a strong correlation between SI and IS ($r= 0.52$, $p=0.0004$; Fig.47j). This demonstrates the potential of Treg cells to down modulate monocytes activation and at the same time indicates that this ability is associated with immunocompetence as measured by helper $CD4^+$ T cells counts.

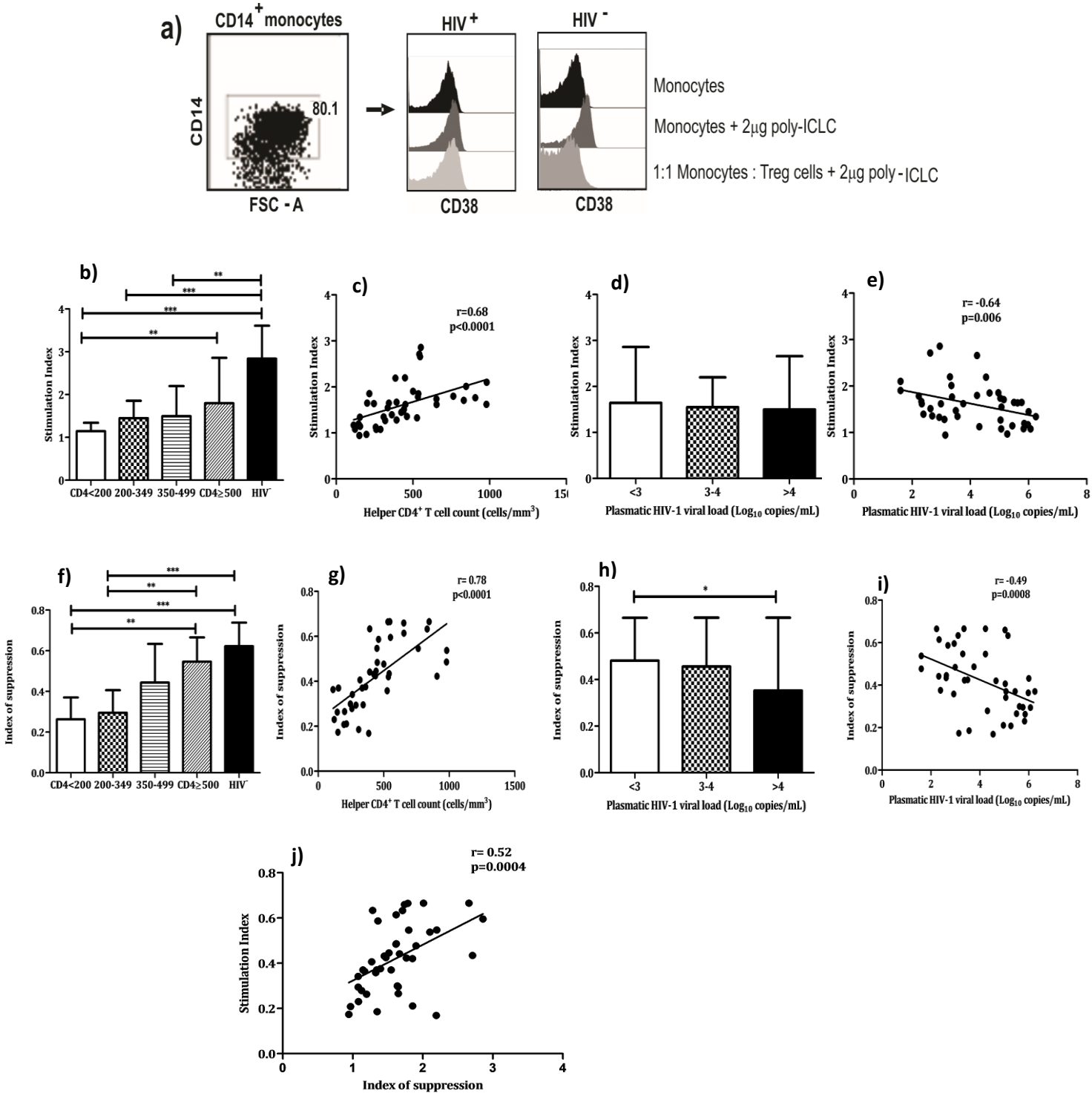



**Figure 47**: Suppressive effect of autologous Treg cell on poly-ICLC mediated monocytes activation. *As shown in 47a, a significant increase in CD38 surface expression was observed in poly-ICLC treated monocytes compared to monocytes alone. However, the addition of autologous Treg cells resulted into a significant suppression of CD38 surface expression. The SI and IS correlated positively with the helper CD4+ T cell count (b,c,f,g) and negatively with HIV-1 plasmatic viral loads (d,e,h,i), demonstrating a strong correlation between SI and IS (j). Median values were first compared using the Kruskal Wallis test, then followed by Dunn's multiple comparison post-test. Correlation coefficients and their significance were calculated by Spearman test.*

### III.1.6.4 Suppression of poly-ICLC mediated monocyte IL-6 production following *invitro* coculture with autologous Treg cells

HIV-related chronic inflammation is characterized by elevated levels of cytokines including IL-1β, IL-6 and TNF-α which have been associated with several negative disease outcomes. In this study, we investigated whether autologous Treg cells were capable of suppressing pro-inflammatory cytokine production by monocytes during activation with poly-ICLC. Monocytes were first stimulated with poly-ICLC and analyzed for IL-6 production using multi-parametric flow cytometry. As illustrated in Fig. 48a, poly-ICLC stimulated monocytes produced significant high levels of IL-6 relative to their unstimulated counterparts. This was revealed in stimulation index values, which were above one and correlated positively with helper $CD4^+$ T cell counts ($r = 0.59$, $p<0.0001$; Fig. 48b, c). Moreover, the stimulation index correlated negatively with plasmatic HIV-1 viral loads ($r= -0.31$, $p=0.04$; d, e). However, upon co-culture with autologous Treg cells, there was a significant reduction in IL-6 production suggesting an inhibitory effect of Treg cells upon poly-ICLC stimulated monocytes. The suppressive activity of Treg cells was greater in $HIV^-$ participants [0.71 (0.57-0.78)] and $HIV^+$ participants with CD4 counts ≥ 500 cells/$mm^3$ [0.62 (0.55-0.69)] than in $HIV^+$ participants with CD4 counts < 200 cells/$mm^3$ [0.25 (0.20-0.36), $p<0.0001$] and between 200-349 cells/$mm^3$ [0.32 (0.27-0.36), $p<0.0001$ and $p<0.001$, respectively]. Although, the suppressive index values of $HIV^+$ participants with CD4 counts between 350-499 cells/$mm^3$ were low [0.45 (0.39-0.52)], there was no significant diference with the values reported in $HIV^-$ and $HIV^+$ participants with CD4 ≥500 cells/$mm^3$ (Fig. 48f). In addition, the suppressive activity of Treg cells decreased when the plasmatic HIV-1 viral loads increased: 0. 55 (0.42-0.67), 0.44 (0.3-0.53), 0.35 (0.27-0.54), respectively for participants with viral loads <3, between 3-4 and >4 $Log_{10}$ RNA copies/mL (Fig. 48h). The index of suppression correlated positively with helper $CD4^+$ T cell counts ($r= 0.79$, $p< 0.0001$; Fig. 48g) and negatively with HIV-1 plasmatic viral loads ($r= -0.45$, $p= 0.002$; Fig. 48i). There was also a positive correlation between stimulation and suppression index ($r= 0.47$, $p= 0.001$; Fig.48j). Thus, Treg cells were able to dampen IL-6 production by polyICLC activated monocytes and this ability was dependent upon immune competence.

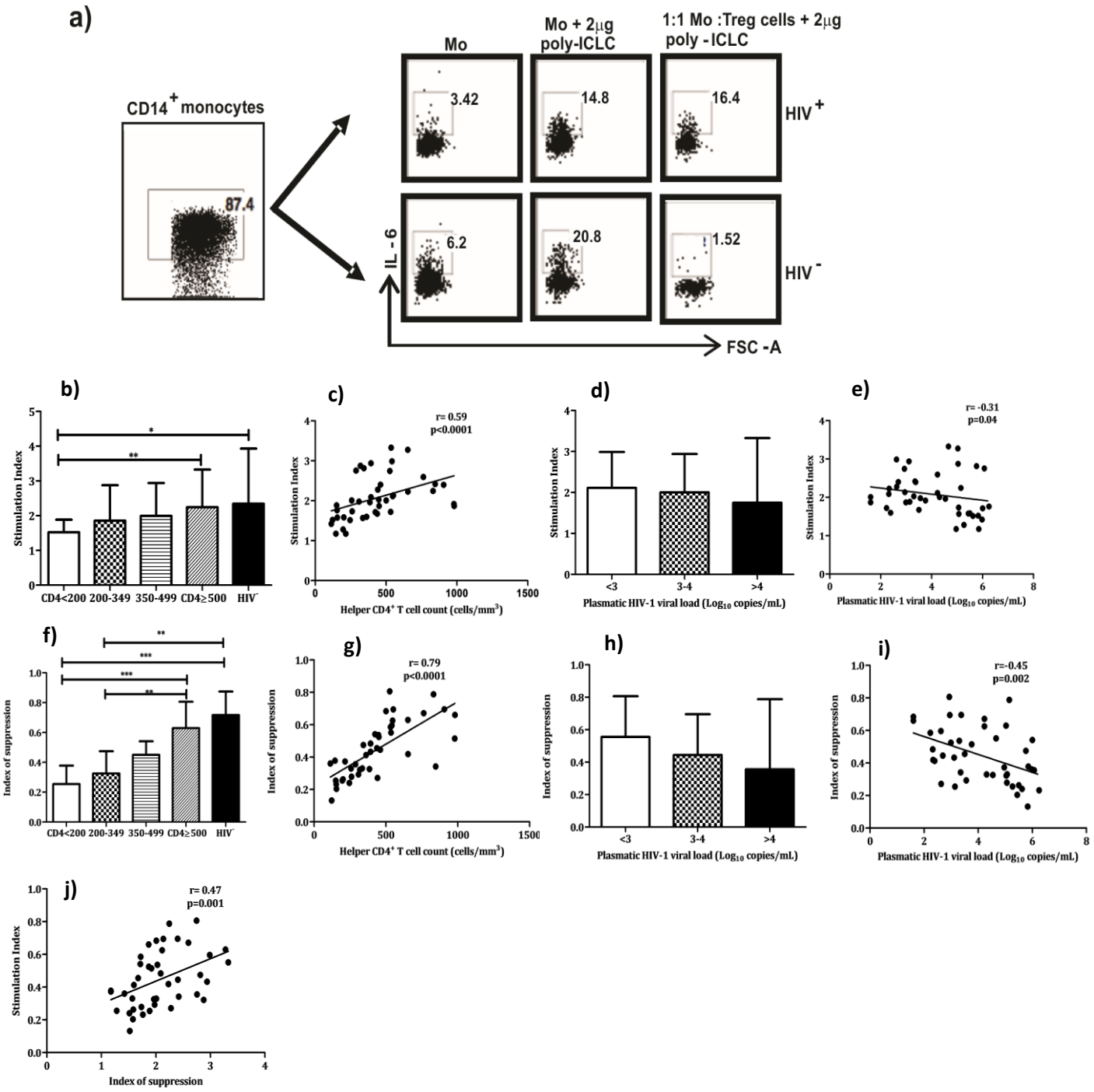



**Figure 48**: Suppression of poly-ICLC mediated monocyte IL-6 production following invitro coculture with autologous Treg cells. *In 48a, representative plots of IL-6 forming monocytes are shown for both HIV+ and HIV- participants. Monocytes stimulation with poly-ICLC resulted into increased IL-6 production relative to unstimulated monocytes. Upon co-culture with autologous Treg cells, there was a significant reduction in IL-6 production indicating the inhibitory effect of Treg cells on poly-ICLC stimulated monocytes. The SI and IS correlated positively with the helper CD4+ T cell count (b, c, f, g) and negatively with HIV-1 plasmatic viral loads (d, e, h, i), demonstrating a strong correlation between SI and IS (j). Median values were compared using the Kruskal Wallis test followed by Dunn's multiple comparisons post-test. Correlation coefficients and their significance were calculated using the Spearman test.*

### III.1.6.5 Suppression of poly-ICLC mediated monocyte TNF-α production following *in vitro* coculture with autologous Treg cells

Similarly, the suppressive activity of Treg cells on TNF-α production by poly-ICLC activated monocytes was also assessed as shown in Fig. 49a. As observed with IL-6, the stimulation of monocyte with poly-ICLC resulted to enhanced TNF-α production compared to monocytes cultured alone. Increase in TNF-α production correlated positively with helper $CD4^+$ T cell counts (r= 0.54, p= 0.0002; Fig. 49b, c) and negatively with HIV-1 plasmatic viral loads (r= -0.49, p= 0.001 Fig. 49d, e). Upon co-culture with autologous Treg cells, a significant reduction of TNF-α production was observed. This suppressive activity of Treg cells was significantly higher in $HIV^-$ participants [0.55 (0.51-0.72)] compared to $HIV^+$ participants with CD4 counts < 200 cells/$mm^3$ [0.25(0.22-0.35), p<0.0001] and between 200-349 cells/$mm^3$ [0.30 (0.23-0.42), p<0.001]. Interestingly, Treg cells from uninfected participants showed suppressive index values comparable to those of participants with CD4 counts between 350-499 cells/$mm^3$ [0.38 (0.34-0.53)] and ≥ 500 cells/$mm^3$ [0.54 (0.37-0.70); Fig. 49f]. In addition, the suppressive activity of Treg cells decreased significantly when the HIV-1 plasmatic viral loads increased: 0. 47 (0.39-0.64), 0.42 (0.32-0.54) and 0.33 (0.23-0.39), respectively for participants with viral loads <3, between 3-4 and >4 $Log_{10}$ RNA copies/mL (p<0.001; Fig. 49h). Furthermore, we found that the suppression index correlated positively with helper $CD4^+$ T cell counts (r= 0.67, p< 0.0001; Fig. 49g) and negatively with HIV-1 plasmatic viral loads (r= -0.44, p= 0.003; Fig. 49i). There was also a positive correlation between stimulation and suppression index (r= 0.51, p= 0.0006; Fig. 49j). Thus, Treg cells mediated suppression of TNF-α production by poly-ICIC activated monocytes was also dependent upon immune competence.

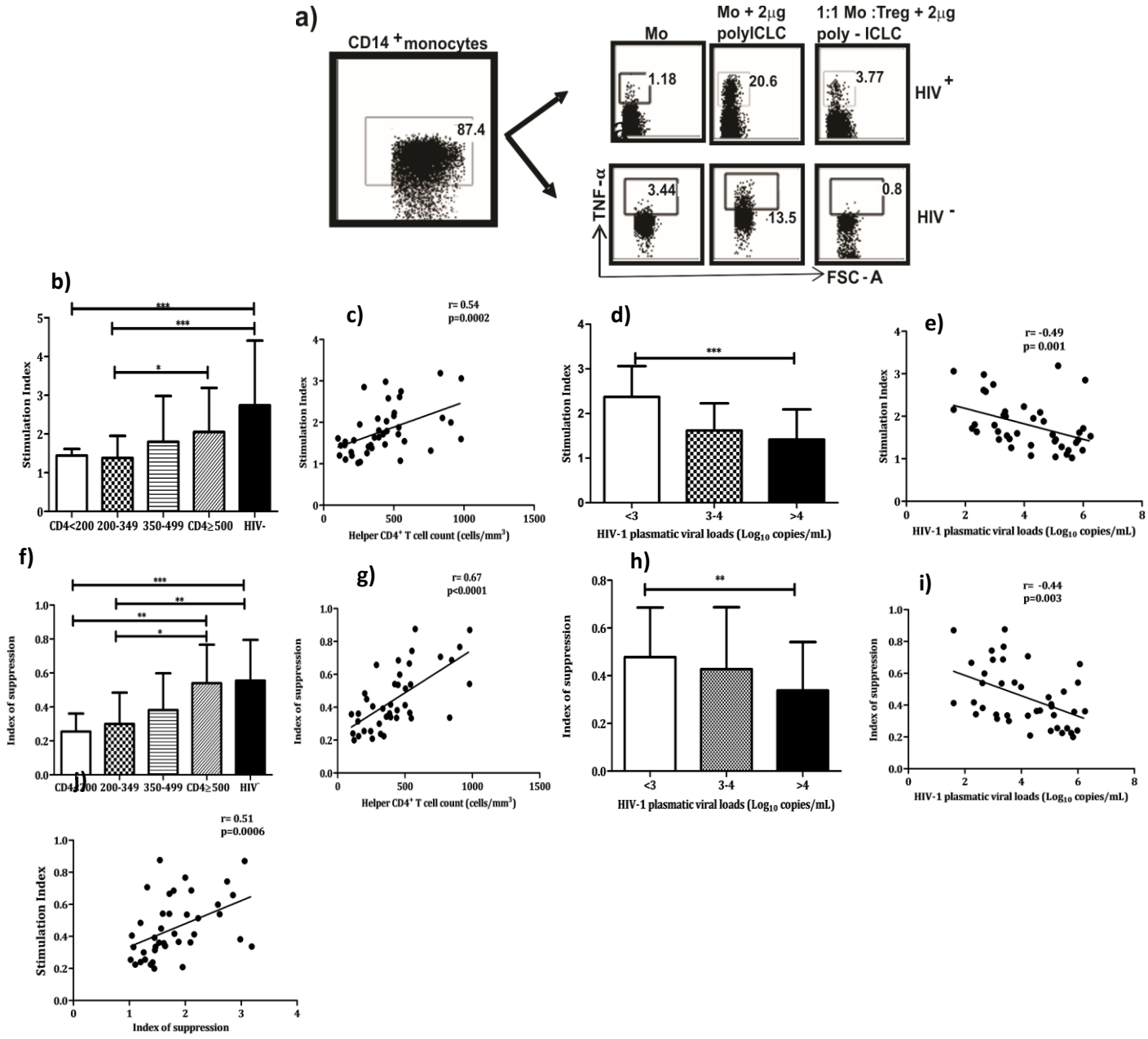



**Figure 49**: Suppression of poly-ICLC mediated monocyte TNF-α production following in vitro coculture with autologous Treg cells. *In 49a, representative plots of TNF-α forming monocytes are shown for both ART naive HIV-1 infected and uninfected participants. The addition of poly-ICLC in the culture medium enhanced the production of TNF-α by monocytes compared to monocytes cultured alone. This increase in TNF-α production correlated positively with helper CD4⁺ T cell counts (b, c) and negatively with HIV-1 plasmatic viral loads (d, e). Upon co-culture with autologous Treg cells, a significant reduction in TNF-α production was observed. This suppressive activity of Treg cells correlated positively with helper CD4⁺ T cell counts (f, g) and negatively with HIV-1 plasmatic viral loads (h, i). In addition, a positive correlation was also observed between the stimulation and the suppression index (j).*

## III.2 DISCUSSION

Chronic HIV-1 infection maintains the immune system in a persistent state of activation irrespective of antiretroviral therapy. However, during ART-naive HIV-1 infection, this inflammation is exacerbated by concurrent viral replication leading to a strong production of pro-inflammatory cytokines such as interleukin (IL)- 1β, IL-6 and tumour necrosis factor (TNF)-α and also the activation of the coagulation system. These cytokines are not only responsible for sustaining inflammation but equally maintain ongoing viral production leading to the loss of memory $CD4^+$ T cells in gut and damage of the thymus and lymphoid infrastructure (Schacker *et al.*, 2002, Naeger *et al.*, 2010). The loss of gut memory $CD4^+$ T cells is usually associated with damages to the intestinal epithelium which allow the increased translocation of microbial products into the circulatory system (Appay & Sauce, 2008). Circulating bacterial products drive persistent systemic activation of the immune system as a whole thereby initiating and maintain inflammation (Somsouk *et al.*, 2015, Nasi *et al.*, 2017). Direct or indirect consequences of inflammation during chronic HIV-1 infection include several important non-AIDS-related (NAR) complications such as metabolic disorders, neurocognitive decline, bone complication, pulmonary and cardiovascular diseases (Nasi *et al.*, 2017). Since persistent inflammation has been linked to increased risk of subsequent morbidity and mortality during chronic HIV infection (Sassé *et al.*, 2012, Deeks *et al.*, 2013), addressing its underlying causes could usher in immotherapeutic strategies for the long term management of HIV-1 infection. Thus optimizing strategies to treat the underlying causes of inflammation might become useful even in people under suppressive antiretroviral therapy. In steady state, Treg cells are expected to dampen excessive activation or diminished persistent inflammation thereby ensuring immune hemostasis (Angin *et al.*, 2012, Walter *et al.*, 2013). However, in the context of a challenging persistent infection such as HIV, it is not known how Treg cells function and also which subsets of Treg cells are involved. During this study, we have assessed the phenotypic and functional properties of Treg cells in the context of ART-naive HIV-1 infection in view of the eventual optimization of Treg cell for immunotherapeutic strategies against chronic inflammation.

Overall, 110 participants including 72 ART-naive $HIV^+$ and 38 $HIV^-$ participants were recruited for this study. The choice of ART-naive HIV-1 infection becomes expedient because it represents the most appropriate situation where the interaction

between HIV and the immune system can be explored without external confounding factors. The CIRCB AFRODEC cohort of ART-naive HIV-1 infected people has been in existence since 2012, permitting us to use historical samples and clinical data to measure virological and immunological parameters in association with purified Treg cell phenotypes. Compared to uninfected individuals serving as controls, there was a significant reduction ($p<0.0001$) of helper $CD4^+$ T cell counts as a consequence of ART-naive HIV-1 infection. As reported in previous studies (Gandhi *et al.*, 2002, Napravnik *et al.*, 2002, Touloumi *et al.*, 2004), HIV-1 infected females showed a lower plasmatic viral load ($p<0.05$) than males. However current data suggest that women might have a 1.6-fold higher risk of progressing to AIDS relative to their male counterparts (Addo & Altfeld, 2014). Differences in immunological responses to HIV-1, mediated through persistent higher production of IFN-α and interferon stimulated genes in females have been suggested as possible mechanisms (Chang *et al.*, 2013).

To date CD4, CD127, CD25 and FoxP3 represent the most used markers for Treg cell identification (Presicce *et al.*, 2011, Horta *et al.*, 2013). We decided to purify Treg cells during this study because they represent a small fraction (5-10%) of $CD4^+$ T cells in steady state, which are further depeted during ART-naive HIV-1 infection, making it difficult to obtain an adequate amount for *in vito* studies with bulk PBMCs. In previous studies the combination of $CD25^+$ $CD127^{lo}$ expression on $CD3^+$ $CD4^+$ T cells has been used in place of $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ as an alternative phenotype to track and characterize Treg cells (Liu *et al.*, 2006, Seddiki *et al.*, 2006). Therefore, we used this approach to track FoxP3 expression in purified Treg cells from both ART-naive $HIV^+$ and $HIV^-$ people. Hence a positive correlation between $CD127^{lo}$ $CD25^+$ and $FoxP3^+$ was validated either through the staining of bulk PBMCs ($r = 0.94$; $P < 0.0001$; Fig. 30d), partially purified ($r = 0.86$; $P < 0.0001$; Fig. 30e) or fully purified Treg cells ($r = 0.71$; $P = 0.0002$; Fig. 30f). This allowed us to use $CD4^+$ $CD25^+$ $CD127^{lo}$ as an alternative to $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ for tracking and phenotypic characterization of Treg cells of ART-naive HIV-1 infected people during this study.

Previous studies demonstrated that Treg cells could be phenotypically and functionally divided into resting and activated subsets according to the surface expression of the maturation marker, CD45RA and the intracellular expression of the transcription factor, FoxP3. Both resting and activated Treg cells were shown to suppress proliferation of effector $CD4^+$ T cells in Vitro (Miyara *et al.*, 2009, Gaardbo *et*

*al.*, 2014). Herein, our study investigated the classification of Treg cells based on the expression of CD45RA, CD27, CD62L and CCR7 surface markers. We found a heterogeneous population of Treg cell subsets including naive ($CD45RA^+$ $CD27^+$ $CCR7^+$ $CD62L^+$), effector ($CD45RA^+CD27^-$ $CCR7^-$ $CD62L^-$), central memory ($CD45RA^-$ $CD27^+$ $CCR7^+$ $CD62L^+$) and effector memory ($CD45RA^-CD27^-$ $CCR7^-$ $CD62L^-$) phenotypes. A number of correlative experiments suggested that naive Treg cells exhibit low *in vivo* suppressive activity but, upon *in vitro* stimulation, they exhibit high proliferation and survival capacity leading to suppressive functions (Simonetta *et al.*, 2012). Naive Treg cells preferentially migrate to secondary lymphoid tissues where they acquire phenotypic features of effector or memory Treg cells following activation. These subsets can then upregulate expression of homing receptors such as CD62L and CCR7 which allow them to access non-lymphoid sites. According to their location, memory Treg cells are divided into central and effector memory Treg cells. Central or lymphoid memory cells can rapidly proliferate, expand and then serve as precursors of effector memory Treg cells, which can execute effector functions instantly (Rallón *et al.*, 2008, Campbell & Koch, 2011, Shen *et al.*, 2011). Whereas uninfected people displayed predominantly naive and central memory phenotypes ($P < 0.0001$), HIV-1 infected participants showed significantly elevated levels of effector and effector memory Treg cell subsets ($P < 0.0001$). On the other hand, Rallon *et al.* (2008) observed that naive Treg cells were less frequent in $HIV^+$ participants than in healthy controls. On the contrary, they found similar levels of effector and effector memory Treg cells in both $HIV^+$ and $HIV^-$ participants. To discriminate Treg cell subsets, these groups have used CD45RA and CD27 expression levels (Rallón *et al.*, 2008, Shen *et al.*, 2011). One major limitation of this strategy is that without taking into consideration the chemokine receptor CCR7 and the L-selectin receptor CD62L which are critical for T-cell homing into lymphoid tissues; it is impossible to clearly delineate the various Treg cell subsets. By including CCR7 and CD62L, for example, we were able to extend the surface markers used for delineating the four groups listed above to four compared to just CD45RA and CD27 previously reported by the groups mentioned above. By so doing we could also delineate certain populations (up to 16 Treg cell subsets, see Fig. 31b, c), which could be relevant in appreciating Treg cell phenotypes. Following an in-depth analysis of the levels of CD39, CD73, HLA-DR and CD38 on the aforementioned four Treg cell subsets, we found that effector and effector memory Treg cells in ART-naive HIV-infected participants

expressed significantly increased levels of CD39, CD73, HLA-DR and CD38 ($p<0.0001$). These markers have been associated with disease progression or immune activation (Schulze Zur Wiesch *et al.*, 2011, Xiao *et al.*, 2011) and have also been shown to be involved in the suppression of HIV-specific responses and/or in Treg cell survival in the inflammatory environment created by HIV infection (Presicce *et al.*, 2011). CD39 functions as a nucleoside triphosphate diphosphohydrolase-1, which hydrolyzes the pro-inflammatory ATP to AMP, which in turn is converted to anti-inflammatory adenosine by the ecto-5'-nucleotidase CD73, thereby dampening the general immune activation (Deaglio *et al.*, 2007, Schulze Zur Wiesch *et al.*, 2011). Significantly higher levels of CD39 expressing Treg cells were reported in long-term nonprogressors with low viral loads, suggesting the contribution of CD39 in the control of virus replication observed in these individuals (Schulze Zur Wiesch *et al.*, 2011). Recently, Simonetta & Bourgeois (2013) reported that all activated Treg cells as determined by increased expression of HLA-DR or CD38, co-express CD39 and are part of the effector compartment. In addition, these cells are able to suppress responder T-cell proliferation and cytokine secretion more efficiently and more rapidly than HLA-DR$^-$Treg cells. ART-naive HIV infection drives persistent immune activation, which is directly linked with CD4$^+$ T-cell depletion and disease progression (Sousa *et al.*, 2002, Chevalier & Weiss, 2013). In this context the role of Treg cells in limiting HIV-1- mediated persistent immune activation is conflicting because by limiting immune activation they also invariably would inhibit HIV-1-specific immune responses. Nevertheless, low immune activation has been associated with slower disease progression (Shaw *et al.*, 2013) but the general consensus remains that sustained immune activation accelerates disease progression irrespective of viral load (Chevalier & Weiss, 2013). On the contrary Gaardbo *et al.* (2014) recently suggested increased activation of Treg cells to be relevant in preserving CD4$^+$ T cells in long-term non-progressors and elite controllers. This is probably true in circumstances where there is no significant immunosuppression. However, the differences observed between Treg cell activation in elite controllers, long-term non-progressors and viral controllers may be because viral control is a transitory state. Viral controllers might ultimately become either long-term nonprogressors or progressors following prolonged interaction with the immune system.

To understand the features of Treg cells in freshly purified PBMCs, we assessed the relationship between both total Treg cell numbers and Treg cell frequencies (i.e.

Treg cell proportion in total $CD4^+$ T cells) with respect to helper $CD4^+$ T-cell count and HIV plasmatic viral load as markers of disease progression. As reported in previous studies (Kinter *et al.*, 2004, Rallón *et al.*, 2008, Chen *et al.*, 2011, Presicce *et al.*, 2011, Horta *et al.*, 2013), there was a proportionate reduction of Treg cell numbers vis-a-vis helper $CD4^+$ T-cell counts ($r = 0.44$, $P < 0.0001$). In contrast to plasmatic HIV load, which increased inversely with Treg cell numbers, here, significantly ($p<0.0001$) lower Treg cell numbers were observed in HIV-1 infected participants with helper CD4 T-cell counts < 200 cells/$mm^3$ [87 cells/$mm^3$ (62–173)], between 200- 349 cells/$mm^3$ [223 cells/$mm^3$ (163.5-323.5)], between 350–499 cells/$mm^3$ [298 cells/$mm^3$ (211-390)] and ≥500 cells/$mm^3$ [372 cells/$mm^3$ (240-496)] compared with uninfected controls [520.5 cells/$mm^3$ (456.8-571)]. This is probably due to exacerbated destruction of Treg cells alongside helper $CD4^+$ T cells during ART naive HIV-1 infection. Treg cells as a subset of $CD4^+$ T cells have previously been demonstrated to express chemokine receptors CCR5 and CXCR4, which are required for HIV-1 entry into host cells and are therefore potential targets for HIV-1 infection and destruction (Antons *et al.*, 2008, Cao *et al.*, 2009, Moreno-Fernandez *et al.*, 2009, Pion *et al.*, 2013, Lopez-Abente *et al.*, 2016). In line with these findings, it has been shown that the FoxP3 and CTLA-4 interfere with NF-kB activation and suppress the HIV-1 long terminal repeat -specific transcription, thus leading to a reduced virus replication capacity (Schulze Zur Wiesch *et al.*, 2011). Another probable scenario may be the recruitment of HIV-specific Treg cells to sites of HIV infection and replication, such as the mucosa and lymph nodes (Keynan & Rubinstein, 2008, Schulze Zur Wiesch *et al.*, 2011). However, when considered in terms of Treg cell frequencies within total $CD4^+$ T cells, there was a negative correlation between helper $CD4^+$ T-cell counts and Treg cell frequencies ($r = -0.82$, $P < 0.0001$; Fig. 38b) on the one hand and a positive correlation between plasmatic HIV-1 viral loads and Treg cell frequencies ($r = 0.5$; $P<0.0001$; Fig.40b) on the other hand in samples with advanced (CD4 count between 200-349 cells/$mm^3$) and severe (CD4 < 200 cells/$mm^3$) immuno-depression. This would imply that as HIV infection disease progresses the proportion of Treg cells within the total $CD4^+$ T-cell sub-population seemed to increase in the periphery. These elevated peripheral Treg cell frequencies can result from the reported high rate of conversion of conventional $CD4^+$ T cells into Treg cells during HIV-1 infection. Moreover, such Treg cells in the context of HIV-1 infection have also been shown to be significantly more proliferative than other memory $CD4^+$ T-cell subsets

(Schulze Zur Wiesch *et al.*, 2011, Chachage *et al.*, 2016). This coupled with the suggested low susceptibility of peripheral Treg cells to R5 viruses (Moreno-Fernandez *et al.*, 2009) could account for the apparent resistance of Treg cells to destruction by the HIV-1 in comparison with other $CD4^+$ T-cell subsets. In contrast to our data, Gaardbo *et al.* (2014) reported similar Treg cell numbers and frequencies in HIV-infected participants and healthy controls. The lack of difference between their study groups was probably due to similarity in their helper $CD4^+$ T-cell counts, which were all within the normal range (> 500 cells/$mm^3$). Hence, whereas their study population was mainly people with no detectable immunosuppression by CDC categorization the differences in Treg cell frequencies observed by our group were mainly in people with advanced and severe immunosuppression. Increased in Treg cell frequencies have generally been reported in severe and advanced immunosuppression which in essence could be an indication of Treg cell-mediated suppression against $CD4^+$ T cells (Simonetta & Bourgeois, 2013). This is in contrast to a reduction in Treg cell numbers, which probably suggests a decrease in their suppression capacity on other immune cells. This assertion is supported by the fact that efficacious ART is generally accompanied by a progressive decrease of Treg cell frequencies to normal levels (Bi *et al.*, 2009, Montes *et al.*, 2011, Schulze Zur Wiesch *et al.*, 2011, Zhuang *et al.*, 2012) and a proportionate increase of Treg cell counts with $CD4^+$ T-cell counts (Bi *et al.*, 2009, Montes *et al.*, 2011, Schulze Zur Wiesch *et al.*, 2011, Angin *et al.*, 2012). This implies that immunotherapeutic approaches targeting Treg cells during HIV-1 infection should aim at reducing Treg cell frequencies in people with advanced immunosuppression, which in effect can diminish their immunosuppressive effect on $CD4^+$ T cells. When considering Treg cell subsets, we found that the median numbers of naive and central memory Treg cells were not affected during HIV disease progression. In contrast, effector and effector memory Treg cell numbers correlated positively with helper $CD4^+$ T cell count ($r=0.61$, $p<0.0001$ and $r=0.74$, $p<0.0001$, respectively) and negatively with HIV-1 plasmatic viral loads ($r= -0.42$, $p=0.0009$ and $r= - 0.48$, $p<0.0001$, respectively), probably resulting to excessive immune activation and consequent helper $CD4^+$ $T^-$ cell depletion. Our findings suggest that effector and effector memory Treg cells are more susceptible to HIV infection than naive and central memory Treg cells. This is supported by data showing that effector Treg cells are more susceptible to R5 HIV-1 infection than naive Treg cells and their depletion appears to take place early during HIV infection and to persist during chronic phases of infection

(Simonetta *et al.*, 2012, Simonetta & Bourgeois, 2013). These data confirm that discriminating between naive, central memory, effector and effector memory Treg subsets provides additional information compared to total bulk Treg characterization.

We have demonstrated that the overall number of Treg cells decrease proportionately with helper $CD4^+$ T cells in ART naive HIV-1 infected participants. Interestingly, an abnormal increase in Treg cell frequencies within total $CD4^+$ T cells is observed in HIV-1 infected participants with severe and advanced immunosuppression. The question now is whether the discrepancies in Treg numbers and frequencies are synonymous to an impairment of their functions. To address this challenge, Treg cells were tested for their ability to modulate autologous monocytes activation as well as their production of pro-inflammatory cytokines during stimulation with poly-ICLC. We focused on monocytes because they contribute extensively to the chronic inflammatory process and tissue destruction via the production of pro-inflammatory cytokines such as TNF-α and IL-6. Elevated amounts of these cytokines have been associated with increased risk of cardiovascular diseases in HIV infected people (Hsue *et al.*, 2012, Brites-Alves *et al.*, 2018). Thus, down-modulation of excessive inflammatory cytokines produced by monocytes could contribute in diminishing immune activation, therefore improving and enhancing the life span of HIV-infected people. We first purified Treg cells in order to have a sufficient number of cells required for suppression assays in immunocompetent as well as immunodepressed HIV-1 infected participants. Similar numbers of Treg cells were used independently of the immunological or virological status of ART naive HIV-1 infected participants. We then used the same number of cells for all participants to avoid any bias during the immunosuppression analysis. Treg cells use several immunomodulatory mechanisms to maintain immune homeostasis and prevent autoimmunity. Amongst these, the secretion of suppressive cytokines has been shown in several models to be required for Treg cell function (Wan & Flavell, 2007b, Caridade *et al.*, 2013, Arce-Sillas *et al.*, 2016a). Based on this observation, we assessed the cytokine profile of Treg cells in the context of ART naive HIV-1 infection. We found that ART naive HIV-1 infected participants with no significant immune suppression showed significantly higher numbers of TGF-β and IL-10 producing $FoxP3^+$Treg cells compared to people with severe ($p<0.001$ and $p<0.0001$, respectively for TGF-β and IL-10) and advanced ($p<0.05$ for TGF-β) immune suppression. This elevated level of TGF-β and IL-10 expression was similar to that seen in uninfected individuals [373 (326-471)

cells/mm$^3$ versus 444 (321.3-533.5) cells/mm$^3$ for TGF-β and 101 (87-109) cells/mm$^3$ versus 151.5 (120.5-190.3) cells/mm$^3$ for IL-10]. Both TGF- β and IL-10 are immune suppressive cytokines preferentially expressed in Treg cells, which play an important role in maintaining the mucosal immune homeostasis (Sakaguchi *et al.*, 2009). IL-10 inhibits the maturation and activation of DCs by downregulating the MHC class II and costimulatory molecules on DCs (Rudensky & Campbell, 2006, Velavan & Ojurongbe, 2011, Gonzalez *et al.*, 2016). In addition, IL-10 limit effector T cell function as well as the recruitment of inflammatory myeloid cells such as monocytes to the site of inflammation via inhibition of IL-2. It also promotes the phagocytic activity, increasing the removal of cellular debris at the inflammation site (Walter *et al.*, 2013, Arce-Sillas *et al.*, 2016a). Previous studies have demonstrated that transfer of IL-10 producing FoxP3$^+$ Treg cells to colitic mice is sufficient to resolve the inflammatory response leading to restoration of normal intestinal architecture (Uhlig *et al.*, 2006). Like IL-10, TGF-β participates in Treg cell immunomodulatory activity by suppressing innate and adaptive immune cells (Sojka *et al.*, 2008, Velavan & Ojurongbe, 2011). One of the mechanisms by which TGF-β inhibits immune responses is through promoting the conversion of conventional CD4$^+$ T cells to FoxP3$^+$ Treg cells (Wan & Flavell, 2007a). In this study, we found increased production of both IL-10 and TGF-β in HIV$^+$ participants with no immunosuppression as well as in uninfected people and decreased production of these cytokines in participants with severe immunosuppression. Our data suggests that both IL-10 and TGF-β contribute in preventing and/or modulating sustained activation and inflammation. In addition to IL-10 and TGF-β, ART naive HIV-1 infected participants, especially those with severe immune suppression (CD4 <200 cells/mm$^3$) showed a significant reduction in IL-4 expression compared to uninfected participants ($p<0.05$). This observation suggests the role of IL-4 in the regulatory mechanisms of Treg cells. Like both IL-10 and TGF-β, IL-4 production is deeply affected by ART naive HIV-1 infection. Our results are supported by previous reports showing that IL-4 produced by FoxP3$^+$Treg cells was able to suppress LPS induced TNF-α and IL-6 production by monocytes and that this suppression was completely reversed when IL-4 was blocked (Tiemessen *et al.*, 2007, Roberts *et al.*, 2015). In addition to the aforementioned anti-inflammatory cytokines, we found that Foxp3$^+$Treg cells were able to produce IL-17A, a proinflammatory cytokine, which is typically produced by CD4$^+$ T helper 17 cells and which is believed to contribute to the expansion of the inflammatory response through cells recruitment and activation

at the sites of inflammation (Corthay, 2009, Valverde-Villegas *et al.*, 2015). A similar expression of IL-17A by Treg cells in human peripheral blood and lymphoid tissue was also reported in a study published by Voo *et al.* in 2009. They demonstrated that IL-17A producing Treg cells are generated from conventional Treg cells in the periphery during inflammatory responses and that they co-express FoxP3, CCR6 and RORγt, the Th17 lineage-specific transcription factor. Similarly, Walter *et al.* (2013) have demonstrated the capacity of Treg cells to produce several pro-inflammatory cytokines including IL-17A, IFN-γ and TNF-α upon interaction with activated monocytes. In these studies IL-17A producing Treg cells were shown to maintain their ability to suppress T cell proliferation and cytokine production. The production of IL-17A by Treg cells is thought to allow them to contribute to the antimicrobial innate immune defense while controlling inflammation at the same time, particularly at mucosal sites (Voo *et al.*, 2009, Zhang *et al.*, 2014). However, contradictory studies reported that IL-17A producing Treg cells rapidly lose their suppressive capacity upon strong activation in the presence of proinflammatory mediators such as IL-1$\beta$, IL-6, IL-7, IL-23 and TNF-α (Kitani & Xu, 2008, Valverde-Villegas *et al.*, 2015, Jung *et al.*, 2017). In the present study, we did not find a significant difference in the number of IL-17A producing Treg cells between HIV-1 infected and uninfected participants, while this sub-population is normally produced in the context of inflammation. One possible explanation is that participants recruited as healthy controls might present bacterial or parasitic infections that we could not rule out during the screening process. It is well known that cells that produce multiple cytokines, provide a more effective immune response to a pathogen than do cells that produce only a single cytokine. Based on this observation, we hypothesized that polyfunctional Treg cells could be associated with strong Treg responses. Using a Boolean gate analysis (Fig.44 b), we defined 11 polyfunctional Treg cell subsets. Compared to seronegative participants, there was a significant decrease in the number of quadruple (IL-4$^+$TGF-β$^+$IL-17A$^+$ IL-10$^+$), triple ((IL-4$^+$TGF-β$^+$IL-17A$^+$/ IL-4$^+$TGF-β$^+$IL-10$^+$/ TGF-β$^+$IL-17A$^+$ IL-10$^+$) and double (IL-4$^+$TGF-β$^+$/ TGF-β$^+$IL-17A$^+$/ TGF-β$^+$IL-10$^+$) positive Treg cell subsets in HIV$^+$ participants with CD4 count <500 cells/mm$^3$ ($p<0.0001$). Interestingly, participants with no immunosuppression (CD4 count ≥ 500 cells/mm$^3$) and uninfected participants had similar levels of polyfunctional Treg cells. Our findings may have physiological relevance since we demonstrate that Treg cells are able to produce more than one cytokine at a single cell basis and that this functional

property might be associated with immunocompetent status of individuals. These data provide additional information on the cytokine mediated regulation of Treg cells in the context of ART naive HIV-1 infection.

During chronic HIV-1 infection, sustained immune activation and inflammation are strong predictors of disease progression. Monocytes contribute extensively to the chronic inflammatory process and tissue destruction through the production of pro-inflammatory cytokines such as IL-1 beta IL-6 and TNFα (Sassé *et al.*, 2012, Deeks *et al.*, 2013, Walter *et al.*, 2013, Roberts *et al.*, 2015). Down-regulation of monocyte activation and functions by Treg cells may therefore contribute to slow down the disease progress during HIV-1 infection. In this study, we first analyzed the surface expression of HLA-DR and CD38 to assess the activation of purified monocytes. We found that 92 % (88-97) of monocytes from $HIV^+$ participants expressed HLA-DR while only 61% (50-76%) expressed CD38. Moreover, almost all CD38 expressing monocytes (97%; 94-99%) were also $HLA\text{-}DR^+$, but only 64% (57-74%) of HLA-DR expressing monocytes were $CD38^+$. This suggests that CD38 can be considered as the surrogate marker to evaluate monocytes activation, as HLA-DR is normally expressed by monocytes. We demonstrated that monocytes from ART naive HIV-1 infected participants displayed an activated phenotype, with high levels of CD38 compared with uninfected people ($p<0.0001$). Moreover, we found a direct correlation between HIV-1 disease progression and monocyte activation as CD38 expression correlated negatively with helper $CD4^+$ T cells ($r= -0.57$, $p< 0.0001$; Fig.46f) and positively with plasmatic HIV-1 viral loads ($r= 0.34$, $p=0.01$; 46h). This is consistent with the literature, which describes highly activated monocytes in HIV-1 infected people, especially in those with advanced HIV-1 disease progression (Mogensen *et al.*, 2010, Wilson *et al.*, 2014, Scully *et al.*, 2016). To mimic the situation of chronic HIV-1 infection, we used poly-ICLC which is a synthetic double stranded RNA and then analyzed CD38 expression in monocytes. We found a significant increase of CD38 expression in poly-ICLC treated monocytes compared to monocytes cultured alone. Interestingly, people with no infection and preserved CD4 counts displayed significantly elevated stimulation index (SI) relative to those with advanced HIV infection. This was also traduced by the positive correlation between the SI and the helper $CD4^+$ T cell counts ($r= 0.66$, $p<0.0001$) on one hand, and the negative correlation between the SI and the HIV-1 plasmatic viral loads ($r= -0.4$, $p= 0.002$) on the other hand. Following addition of autologous Treg cells in the culture medium, there was

a significant suppression of CD38 expression upon the activated monocytes. As observed with the SI, the index of Treg cell suppression (IS) correlated positively with the helper $CD4^+$ T cell count ($r= 0.76$, $p<0.0001$) and negatively with HIV-1 plasmatic viral loads ($r= -0.39$, $p= 0.003$). Of interest, there was a strong correlation between the IS and the SI ($r= 0.83$, $p<0.0001$). This demonstrates the potential of Treg cells to down modulate monocytes activation and at the same time indicates that this ability is associated with immunocompetence as measured by helper $CD4^+$ T cells counts. Our results are in agreement with a study published by Taams *et al.* (2005), who reported that Treg cells can modulate monocytes activation by challenging LPS receptor (TLR4/CD14 triggering) and hampering APC function. In accordance with our results in HIV-1 infected participants, Karlson *et al.* (2011) demonstrated that the *in vitro* suppressive capacity of peripheral Treg cells from chronically infected cynomolgus macaques is associated with preserved helper $CD4^+$ T-cell counts and lower T-cell activation(Karlsson *et al.*, 2011). Based on these observations we can speculate that suppression of immune activation in chronic HIV infection by Treg cells may be lost during disease progression and, thereby, contributes to exhaustion of the immune system (Karlsson *et al.*, 2011). With regard to pro-inflammatory cytokine production, we investigated whether autologous Treg cells were capable of suppressing IL-6 and TNF-α production by monocytes during activation with poly-ICLC. We observed a significant reduction in IL-6 and TNF-α production levels indicating the inhibitory effect of Treg cells upon poly-ICLC stimulated monocytes. This suppressive activity of Treg cells correlated positively with helper $CD4^+$ T cell counts ($r= 0.51$, $p< 0.0001$ for IL-6 and $r= 0.68$, $p< 0.0001$ for TNF-α) and negatively with HIV-1 plasmatic viral loads ($r= -0.33$, $p= 0.01$ for IL-6 and $r= -0.52$, $p< 0.0001$ for TNF-α). There was also a positive correlation between stimulation and suppression index for both IL-6 ($r= 0.40$, $p= 0.001$) and TNF-α ($r= 0.64$, $p< 0.0001$). This indicated that Treg cells mediated suppression of IL-6 and TNF-a production by poly-ICLC activated monocytes was also dependent upon immune competence. Similar results were obtained by Tiemessen *et al.* (2007) who worked with healthy individuals. They found that following coculture with Treg cells, LPS treated monocytes were significantly suppressed in their capacity to produce proinflammatory cytokines/chemokines (TNF-a IL-6, IL-1b, IL-8/CXCL8, and MIP-1a CCL3) compared with monocytes cultured alone. This suppressive activity of Treg cells required cell contact as well as soluble factors such as IL-10, IL-4, and IL-13. These cytokines were thought to inhibit NF-kB activation, which is required

for proinflammatory cytokine gene expression and which is involved in the regulation of surface markers such as CD40 and CD86.

# CONCLUSION, RECOMMENDATIONS AND PERSPECTIVES

# CONCLUSION

The current study aimed at characterizing the phenotypes and functions of Treg cells in ART naive HIV-1 infected participants. From the data obtained, we can draw the following conclusions:

- ❖ The total purification of Treg cells results in higher number of $CD3^{+}CD4^{+}CD25^{+}CD127^{Low}$ cells as compared to partially purified samples and bulk PBMCs. The combination of $CD25^{+}CD127^{Low}$ can provide a good alternative to FoxP3 for tracking Treg cells of ART-naive HIV-1 infected individuals.
- ❖ HIV-1 infection impacts the Treg cell phenotypes resulting mainly in effector and effector memory subsets. These subsets express increased levels of CD39, CD73, HLA-DR and CD38, markers involved in the suppression of HIV-specific responses and/or in Treg cell survival in the inflammatory environment created by HIV infection.
- ❖ Bulk Treg cell numbers decrease over HIV infection probably due to the susceptibility of Treg cells to HIV infection. This depletion of Treg cells in HIV-1 infected people may contribute to the excessive immune activation observed in participants with advanced immune suppression. Given the importance of Treg cells in regulating the immune-homeostasis, depletion would have a dramatic effect on monocyte activation. As stated previously, sustained immune activation is a hallmark of HIV disease progression, and depletion of Treg cell number may impact their function, thus contributing to disease progression. Interestingly, when considered in terms of Treg cell frequencies within total $CD4^{+}$ T cells, ART naive HIV-1 infected individuals with advanced immune system degradation show significantly higher percentages of Treg cells than those with preserved $CD4^{+}$ T cells. This is due to upregulation of FoxP3 expression by conventional $CD4^{+}$T cells following T cell receptor stimulation. Therefore, Treg cell frequencies may constitute a potential prognostic marker of disease progression. An in-depth analysis of Treg cell population has revealed that effector and effector memory Treg cell numbers correlate positively with helper $CD4^{+}$ T cell count and negatively with HIV-1 plasmatic viral load, while naive and central memory Treg cells are not affected during HIV disease progression. This suggests a high susceptibility of both effector and effector memory T cells to HIV infection compared to naive and central memory Treg cells.
- ❖ Treg cell suppression activity could be associated with increased production of IL-4, IL-10 and TGF-β since these cytokines were highly produced in HIV-

and HIV$^{+}$ participants with CD4 count≥500 cells/mm$^{3}$. Treg cells are able to produce more than one cytokine at a single cell basis.

❖ Regulatory T cells are capable of down modulating autologous monocytes activation as well as IL-6 and TNF-α production, following monocytes stimulation with a TLR3 ligand poly-ICLC. This capacity of Treg cells to control inflammation was observed in ART naive HIV-1 infected people with immunocompetent system (helper CD4$^{+}$ T cell count≥500 cells/mm$^{3}$). In contrast, this activity was impaired in people with advanced immune system degradation (helper CD4$^{+}$ T cell count<350 cells/mm$^{3}$).

Taken together, our findings show that Treg cell phenotypes, numbers and functions are affected by HIV-1 mediated immune depletion during ART naive HIV-1 infection. Considering monocytes and their mediators as major contributors to chronic inflammatory conditions, these findings provide support for the potential use of Treg cells as an immunotherapeutic target. Indeed, immunotherapeutic strategies geared toward restoring Treg cell phenotypes, numbers and functions could be beneficial in the long-term management of HIV-1 infection.

## RECOMMENDATIONS

We have demonstrated that alterations in the numbers and functions of Treg cells result in an increased immune system activation and inflammation during antiretroviral HIV-1 infection. With the advent of test and treat in Cameroon, there is need to assess the functional properties of Treg cells in people under antiretroviral therapy.Therefore, we recommend that Treg cells should be further optimized as an immunotherapeutic strategy for the management of persistent inflammation in HIV-1 infected people. For people with a strong depletion of Treg cells, we suggest a Treg cell-based immunotherapy, which consist of autologous Treg cell generation from stem cells.

## PERSPECTIVES

In order to universalize Treg cell immunotherapy, we think for our future work:

- To improve Treg cell purification and functional assay by sorting different Treg cell subsets
- To evaluate the role and function of T reg cells during antiretroviral therapy
- To study the transcriptomics of Treg cells

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## ANNEX 1 : INFORMED CONSENT

**Participant Code:.....Date of Enrolment: ..... Telephone:.....**

### I. Consent to the use, storage and sharing of your samples

We are asking whether you agree that your samples may be stored or used for subsequent experiments. Even if you do not allow us to keep your samples for other studies you can still be part of this study.

#### A- This research study

May we use and store your samples, which may contain DNA, for this study?

YES ☐ NO ☐

May we use and store your samples, which may contain DNA, for this study for the duration of the study?

YES ☐ NO ☐

If you answered ***"NO"*** to the question above, how long may we use and store your samples?

If you have said that we may use and store your samples, you may later change your mind. If you change your mind, contact the principal investigator and then your samples that have not been used in research will not be used.

#### B- Separate research studies

May we store, use and share your samples, which may contain DNA, for separate studies that may involve genetic tests, provided that your samples cannot be identified as having come from you or have been coded to protect your identity?

YES ☐ NO ☐

If you answered ***"YES"***:

Go to ***section I-C***

If you said ***"NO"***:

May we store, use and share your samples, which may contain DNA, for separate studies that do not involve genetic

tests?

YES ☐ NO ☐

If you answered ***"YES"***:

Write how long we may store, use and share your samples for separate studies that do not involve genetic tests:

If you answered ***"NO":***

Go to ***Section II***.

For as long as the researchers want to use them? YES ☐ NO ☐

Time limit: _____

If you have said that we may store, use and share your samples for separate studies that may or may not involve genetic tests, you may later change your mind.

If you change your mind, contact the person named in ***section III*** and then your samples that have not been used in research will not be used.

**C- Separate research studies that involve genetic tests**

May we store, use and share your samples for as long as the researchers want to use them for separate studies that may involve genetic tests?

YES ☐ NO ☐

If you answered ***"NO"*** to the question above, please write how long we may store, use and share your samples for separate studies that may involve genetic tests:

_____

_____

The researchers may want to contact you in the future to see if you would like to take part in separate studies involving genetic tests or to learn about information obtained in other studies. You can decide how much information you would like to be told by answering the questions below:

**a)** Would you like to take part in other studies involving genetic tests?

YES ☐ NO ☐

**b)** Would you like to receive information about the research purposes of other studies involving genetic tests?

YES ☐ NO ☐

**c)** Would you like to receive general information about research findings of a group of people who took part in other studies involving genetic tests?

YES ☐ NO ☐

**d)** Would you like to receive information about a genetic test on your samples that may help you or your family to make choices about health care?

YES ☐ NO ☐

In the future, a separate study that involves genetic tests may show that there is a link between genetic information and your disease or condition. This information may benefit you or your family. But, some people prefer not to know about their own genetic information. Knowing this information might make choices about health care, having children or other personal choices difficult. A genetic counselor or a personal doctor could help you decide whether to get information about genetic tests if and when genetic tests become available.

There may be some risks in getting this information.

The risks for genetic testing include:

- You may be upset to learn that you have a greater chance of having a disease or condition. Even if genetic tests show that you do not have a greater risk of disease, you may still be upset if you know that others in your family have that higher risk of disease. When genetic tests become available, you may want to have genetic counseling or a talk with your own doctor.
- You could face discrimination in your job, in getting a job or getting insurance if it were known that you have an increased genetic risk for a disease or condition. The researchers will keep your genetic test information confidential as allowed by the Cameroonian law.
- Genetic tests may show information that has nothing to do with the research study. For example, when parents and children are both tested, the tests may show if the parents are the biological parents of the child. If we learn this information from these tests, you will not be told.

If you decide now that you would like to take part in other studies involving genetic tests or would like to get genetic test information in the future, you may always change your mind later.

## II. Who do you call if a medical problem results from this research study?

If you believe that this study has led to a medical problem, you should call the researchers listed below right away. The researchers will ensure that you get appropriate medical care to solve the problem.

| | |
|---|---|
| *Nom:* **AMBADA GEORGIA** | *Name:* **Dr. GODWIN NCHINDA** |
| *Téléphone portable:* 675 887 250 | *Cell Phone Number:* 676 523 909 |
| *Téléphone fixe:* (237) 222 315 450 | *Phone Number:* (237) 222 315 450 |
| *Fax:* (237) 222 315 456 | *Fax Number:* (237) 222 315 456 |
| *Email*: ambadag@yahoo.fr | *Email*: nsehleseh@gmail.com |
| *Postal Adress* : Microbiology and Immunology Laboratory (CIRCB, Yaoundé) P.O. box: 3077 Messa (Yaoundé) | |

## III. Who do you contact if you have questions about the research study?

If you believe that this study has led to a medical problem, you should call the researcher listed below right away. The researchers will help you get the necessary and appropriate medical care to resolve the problem.

| | |
|---|---|
| *Name:* **AMBADA GEORGIA** | *Name:* **Dr. GODWIN NCHINDA** |
| *Cell Phone Number:* 675 887 250 | *Cell Phone Number:* 676 523 909 |
| *Email:* ambadag@yahoo.fr | *Email*: nsehleseh@gmail.com |
| *Téléphone fixe:* | (237) 222 315 450 |
| *Fax:* | (237) 222 315 456 |

If you have any concerns about your experience while taking part in this research study, you may contact the National Ethics Committee office at (237) 22 23 49 34/22 76 21 14**,** or The Scientific Direction of CIRCB at (237) 222 315 450.

## IV. How can we get in touch with you?

Please let us know how we can get in touch with you, by giving us your full name, mailing address, email address, telephone number and fax number:

Name: _____

Adress: _____

Phone number: _____

Fax number: _____

Email: _____

Please let us know of any changes to this information, so that we always have your correct contact information. Please send any changes to the following researchers:

| | |
|---|---|
| *Nom:* **AMBADA GEORGIA** | *Name:* **Dr. GODWIN NCHINDA** |
| *Téléphone portable:* 675 887 250 | *Cell Phone Number:* 676 523 909 |
| *Téléphone fixe:* (237) 222 315 450 | *Phone Number:* (237) 222 315 450 |
| *Fax:* (237) 222 315 456 | *Fax Number:* (237) 222 315 456 |
| *Email*: ambadag@yahoo.fr | *Email*: nsehleseh@gmail.com |

*Postal Adress* : Microbiology and Immunology Laboratory (CIRCB, Yaoundé)
P.O. box: 3077 Messa (Yaoundé)

**AGREEMENT TO PARTICIPATE (SIGNATURES REQUIRED)**

A copy of this consent form will be given to you. Please keep a copy of the form as it contains important information that you may wish to refer to during the research study and thereafter.

***I have read this consent form, and my questions have been answered. I hereby voluntarily consent to take part in this research study.***

Name of study participant :_____

_____

Signature of study participant Date (To be Filled in by study participant)

_____ _____

***I have explained the research protocol and this consent form to the participant and have answered the participant's questions about this research study and/or the consent process.***

Name of the person discussing consent:_____

Signature of the Person Date (To be filled in by person discussing consent)

_____

_____

## ANNEXE 2 : CONSENTEMENT ECLAIRÉ

**Code du participant: _____**

**Date d'enregistrement: _____ /_____ /_____**

**Téléphone: _____**

### I. Consentement pour l'utilisation, la conservation et partage de vos échantillons.

Nous souhaitons avoir votre avis sur l'utilisation de vos échantillons pour des expériences ultérieures. Si vous n'approuvez pas le fait que vos échantillons soient conservés et utilisés pour d'autres études, vous ferez toujours partie de la présente étude.

**A- Pour cette étude :**

Pourrions-nous utiliser et conserver vos échantillons qui contiennent l'ADN ?

OUI ☐ NON ☐

Pourrions-nous utiliser et conserver vos échantillons qui contiennent l'ADN pour toute la durée de cette étude ?

OUI ☐ NON ☐

Si vous répondez ***"NON"*** à la question ci-dessus, combien de temps pourrions-nous utiliser et conserver vos échantillons ? _____

_____

Si vous acceptez que nous utilisions et conservions vos échantillons, et que par la suite vous changiez d'idée, contactez la personne mentionnée à la ***section III***. Vos échantillons n'ayant pas encore été utilisés pour la recherche ne le seront pas.

**B- Pour d'autres études**

Pouvons-nous utiliser, garder et partager vos échantillons qui peuvent contenir de l'ADN pour des études différentes pouvant inclure des tests génétiques, étant donné que vos échantillons sont codés et que personne ne saura qu'ils proviennent de vous ?

OUI ☐ NON ☐

Si vous répondez ***"Oui"*** :

Allez à la ***section I-C***.

Si vous répondez ***"NON"*** :

Pouvons-nous garder, utiliser ou partager vos échantillons pouvant

contenir l'ADN pour des études différentes n'impliquant pas de tests génétiques ?

OUI ☐ NON ☐

Si vous répondez ***"OUI"*** :

Précisez pendant combien de temps nous pourrons garder, utiliser ou partager vos échantillons pour une autre étude n'impliquant pas de tests génétiques :

Si vous répondez ***"NON"*** :

Allez à la ***Section II***

Aussi longtemps que les chercheurs voudront les utiliser ? OUI ☐ NON ☐

Temps limité : _____

Si vous acceptez que nous puissions garder, utiliser et partager vos échantillons pour une autre étude qui peut ou ne pas impliquer des tests génétiques, vous pourrez changer d'idée plus tard.

Si vous le faites, contacter la personne indiquée dans la ***section III*** et vos échantillons qui n'ont pas été utilisés pour la recherche ne le seront pas.

**C- Pour d'autres études impliquant des tests génétiques**

Pouvons-nous garder, utiliser et partager vos échantillons aussi longtemps que les chercheurs le désireront pour d'autres études pouvant impliquer des tests génétiques?

OUI ☐ NON ☐

Si vous avez répondu ***"NON"*** à la question ci-dessus :

Précisez pendant combien de temps nous pouvons garder, utiliser et partager vos échantillons pour d'autres études pouvant impliquer des tests génétiques : _____

_____

Les chercheurs pourraient avoir besoin de vous contacter dans le futur pour savoir si vous seriez intéressé à prendre part à d'autres études impliquant des tests génétiques ou pour s'informer des résultats obtenus d'autres études.
Vous pouvez décider de quelle quantité d'informations partager, en répondant aux questions ci-dessous :

**a)** Voudriez-vous prendre part à d'autres études impliquant des tests génétiques ?

OUI ☐ NON ☐

**b)** Voudriez-vous recevoir des informations relatives aux objectifs d'autres études impliquant des tests génétiques ?

OUI ☐ NON ☐

**c)** Voudriez-vous recevoir des informations générales relatives aux résultats obtenus des études impliquant des tests génétiques effectués sur d'autres groupes de personnes ?

OUI ☐ NON ☐

**d)** Voudriez-vous recevoir des informations relatives aux tests génétiques effectués sur vos échantillons qui peuvent vous aider ou votre famille à prendre des décisions concernant votre santé?

OUI ☐ NON ☐

Dans l'avenir, une étude distincte impliquant des tests génétiques peut montrer qu'il existe une relation entre l'information génétique et votre maladie ou état de santé. Cette information peut être utile à vous ou à votre famille. Mais certaines personnes n'aiment pas avoir des informations génétiques sur elles.

La connaissance de ces informations permet d'opérer des choix sur les soins de santé, la procréation, ou d'autres aspects personnels difficiles. Un conseiller génétique ou un docteur privé peut vous aider à faire le choix. Il peut y avoir un risque à avoir ces informations.

Ces risques sont :

- Vous pouvez être mécontent de savoir que vous êtes plus susceptible à une maladie. Même si vous n'êtes pas prédisposé à une maladie, vous pouvez être mécontent de savoir qu'une autre personne de votre famille l'est. Quand les tests génétiques seront disponibles, vous pourrez en discuter avec un conseiller génétique ou votre médecin.
- Vous pouvez être sujet de discrimination à votre lieu de service, pour avoir un travail ou pour être assuré. Les chercheurs garderont votre information génétique confidentielle comme exigé par la loi camerounaise.

- Les tests génétiques peuvent révéler des informations qui ne sont pas relatives à notre étude. Par exemple, quand les parents et les enfants sont tous testés, les tests peuvent révéler que les parents sont biologiques ou non. Si nous avons de pareilles informations, elles ne vous seront pas révélées.

Si vous décidez maintenant de prendre part à d'autres études impliquant des tests génétiques et voudriez avoir des informations sur les tests génétiques dans l'avenir, vous pouvez toujours changer d'idée plus tard.

## II. Qui pouvez-vous appeler si vous avez un problème médical suite à cette étude ?

Si vous pensez que cette étude vous a provoqué un problème médical, vous devez appeler immédiatement les chercheurs suivants qui vous aideront à recevoir l'aide appropriée.

Leurs contacts sont :

| | |
|---|---|
| *Nom:* **AMBADA GEORGIA** | *Name:* **Dr. GODWIN NCHINDA** |
| *Téléphone portable:* 675 887 250 | *Cell Phone Number:* 676 523 909 |
| *Email: ambadag@yahoo.fr* | *Email*: *nsehleseh@gmail.com* |
| *Téléphone fixe:* | (237) 222 315 450 |
| *Fax:* | (237) 222 315 456 |

## III. Qui pouvez-vous contacter si vous avez une question relative à cette étude ?

Posez autant de questions que vous voulez à propos de cette recherche ou de ce consentement. Si vous acceptez de prendre part à cette étude et que vous avez des questions à poser plus tard, n'hésitez pas à contacter la personne suivante :

| | |
|---|---|
| *Nom:* | **AMBADA GEORGIA** |
| *Téléphone portable:* | 675 887 250 |
| *Téléphone fixe:* | (237) 222 315 450 |
| *Fax:* | (237) 222 315 456 |
| *Email*: | *ambadag@yahoo.fr* |

Si vous avez une inquiétude à propos de votre expérience en prenant part à cette étude, vous pouvez appeler le secrétariat du Comité National d'Éthique au numéro de téléphone **(237) 22 23 49 34/22 76 21 14**, ou la direction du CIRCB au numéro ***(237)* 222 315 450**.

**IV. Comment est-ce que nous pouvons vous contacter ?**

S'il vous plaît, dites-nous comment vous contacter en nous donnant votre nom, votre numéro de téléphone portable, numéro de téléphone fixe, numéro de fax ou adresse e-mail.

Nom: _____

Adresse:_____

Téléphone Portable:_____

Téléphone fixe: _____

Fax:_____

Email: _____

Informez-nous quand vous changez d'adresse, pour nous permettre de rester en contact avec vous. Envoyez tout changement survenu dans votre adresse aux chercheurs ci-après :


*Nom:* **AMBADA GEORGIA**

*Téléphone portable:* 675 887 250

*Téléphone fixe:* (237) 222 315 450

*Fax:* (237) 222 315 456

*Email*: ambadag@yahoo.fr

*Name:* **Dr. GODWIN NCHINDA**

*Cell Phone Number:* 676 523 909

*Phone Number:* (237) 222 315 450

*Fax Number:* (237) 222 315 456

*Email*: nsehleseh@gmail.com

*Adresse Postale* : Laboratoire d'Immunologie et Microbiologie (CIRCB, Yaoundé)
B.P. 3077 Messa (Yaoundé)

**ACCORD DE PARTICIPATION (SIGNATURES REQUISES)**

Un exemplaire de ce formulaire de consentement éclairé vous sera remis. Gardez-le précieusement, il contient des informations importantes qui pourront servir tout au long de cette étude et même plus tard.

***J'ai lu ce formulaire de consentement éclairé, et j'ai obtenu des réponses à toutes mes questions. J'accepte librement de participer à cette étude.***

Nom du participant :_____

_____

***J'ai expliqué le protocole de cette étude ainsi que ce formulaire de consentement éclairé au participant. J'ai également répondu à toutes les questions posées par le participant au sujet de cette étude.***

Signature du participant Date (*à remplir par le participant*)

_____ _____

Signature de l'investigateur principal Date

_____

## ANNEX 3 : QUESTIONNAIRE

**Title of project:** "***Functional and phenotypic features of regulatory T (Treg) cells in antiretroviral naive HIV-1 infected people".***

**General information**

Referring doctor:_____

Phone number:_____

Structure:_____

Place of enrolment: _____Date: ____ /_____/ _____

**Personal participant information**

- Patient's code: _____ Phone number: _____
- Name: _____
- Surname: _____
- Sex: ☐ M ☐ F
- Date of birth (dd/mm/yy): _____
- Status: MARIED ☐ WIDOWED ☐ CONCUBINAGE ☐ SINGLE ☐
- Home address (City): _____

**Clinical information**

**HIV serology:** POSITIVE ☐ NEGATIVE ☐

Date of diagnosis: _____

**CD4 and Viral load values at the time of diagnosis:** DONE ☐ NOT DONE ☐

HIV viral load (cp/ml) _____ (Log) _____ Date _____ /_____/ _____

CD4 cell count (cell/mm$^3$) _____(%) _____ Date _____ /_____/ _____

**Last CD4 and viral load values**

HIV viral load (cp/ml) _____ (Log) _____ Date _____ /_____/ _____

CD4 cell count (cell/mm$^3$) _____(%) _____ Date _____ /_____/ _____

Status of therapy: ☐ NAIVE ☐ on HAART

**Infectious diseases**: YES ☐ NO ☐

Precise: _____

PTME: YES ☐ NO ☐

If *"YES"*, precise the period _____

## ANNEXE 4 : QUESTIONNAIRE

**Titre du projet : "*Caractérisation phénotypique et fonctionnelle des cellules T régulatrices chez les personnes infectées par le VIH de type 1 et naïves aux antirétroviraux*".**

**Informations générales**

Nom du clinicien: _____

Téléphone: _____

Structure:_____

Lieu d'enrôlement:_____Date: _____ /_____/ _____

**Informations personnelles sur le participant**

- Code du participant: _____ Téléphone:_____
- Nom: _____
- Prénom:_____
- Date naissance (jj/mm/aa): _____
- Sexe: M ☐ F ☐
- Ville/Quartier de provenance:_____ /_____
- Statut matrimonial: MARIÉ ☐ VEUF ☐ CONCUBINAGE ☐ CÉLIBATAIRE ☐

**Historique de l'infection par le VIH**

Statut sérologique : POSITIF ☐ NÉGATIF ☐ Date de diagnostic : _____

**Suivi biologique**

- CD4 au moment du diagnostic : NON FAIT ☐ FAIT ☐
- Valeur des CD4 (cellules/mm$^3$) :_____ (%) _____ Date _____ /_____/ _____
- CV au moment du diagnostic : NON FAIT ☐ FAIT ☐
- Valeur CV (copies/ml) :_____ (Log) _____ Date _____ /_____/ _____

**Dernier suivi biologique**

- CD4 (cellules/mm$^3$) _____ (%) _____ Date _____ /_____/ _____
- CV (copies/ml)_____(Log) _____ Date _____ /_____/ _____

Traitement ARV : OUI ☐ NON ☐

Infections opportunistes récentes : OUI ☐ NON ☐

Si *"OUI"*, préciser :_____

PTME : OUI ☐ NON ☐

Si *"OUI"*, préciser la période :_____