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Abstracts of the Seventh International Workshop on HIV Persistence during Therapy

8–11 December 2015, Miami, Florida, USA

Dear Colleagues

Welcome to the Seventh International Workshop on HIV Persistence during Therapy. Since our first workshop in 2003 in St Maarten, the issue of HIV persistence and reservoirs has become even more relevant, not only for the biologist but also for the clinician facing the problem of the long-term control of this persistent retroviral infection.

This biennial workshop is widely recognised as the reference workshop on HIV reservoirs and eradication strategies. Our main objective is to keep it driven by science and new data. To this end, the abstracts have undergone a rigorous selection procedure by the Scientific Committee.

This year, we will be spending much more time on new data rather than reviewing what is already known. Each session of the workshop will begin with an update given by an invited speaker followed by oral presentations of selected abstracts. There will be sufficient time to allow interactive discussions during each session and we encourage robust scientific discussion of the new data in a relaxed and friendly atmosphere.

Lastly, we thank all the participants who have chosen to present their work here: the excellence of the abstracts we have received undoubtedly guarantees an interesting and thought-provoking workshop.

We wish you all an enjoyable and fruitful workshop!

Alain Lafeuillade MD
On behalf of the Steering Committee

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Aims and objectives

The aim of this journal is to provide a specialist, open access forum and fast-track pathway to publish work in the rapidly developing field of virus eradication, particularly of HIV, HBV and HCV. The Journal has been set up especially for these and other viruses, including herpes and flu, in a context of new therapeutic strategies, as well as societal eradication of viral infections with preventive interventions.

Scope

The Journal not only publishes original research, but also provides an opportunity for opinions, reviews, case studies and comments on the published literature. It focuses on evidence-based medicine as the major thrust in the successful management of HIV and AIDS, HBV and HCV as well as includes relevant work for other viral infections. The Journal encompasses virological, immunological, epidemiological, modelling, pharmacological, pre-clinical and *in vitro*, as well as clinical, data including but not limited to drugs, immunotherapy and gene therapy. It will be an important source of information on the development of vaccine programmes and preventative measures aimed at virus eradication.

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Oral presentations

Session 1: *In vitro* and *in vivo* models of HIV persistence

OP 1.0

HIV persistence: to the periphery and beyond

J.V. Garcia Martinez

Division of Infectious Diseases/UNC CFAR, University of North Carolina at Chapel Hill, USA

Despite suppressive antiretroviral therapy (ART), HIV reservoirs persist in most patients. The latent reservoir consists of cells harboring transcriptionally silent provirus and the residual active reservoir is characterized by low level viral RNA production. The goal of eradication therapies is to eliminate these viral reservoirs from the body. However, our knowledge of the systemic nature of HIV reservoirs is incomplete. To provide a comprehensive characterization of the anatomical distribution of persistent HIV, we performed a systemic examination of the latent and residual active HIV reservoirs in bone marrow-liver-thymus (BLT) humanized mice undergoing ART. Our results demonstrate that the latent HIV reservoir is broadly disseminated during ART. Furthermore, each of the tissues examined exhibited low level vRNA production indicating that the residual active HIV reservoir is also systemic in nature. Our data demonstrate that HIV reservoirs are broadly disseminated *in vivo* and provide a quantitative framework for the *in vivo* efficacy evaluation of HIV eradication interventions designed to deplete HIV reservoirs.

OP 1.1

Clones of SIV-infected cells are present in spleen and lymph nodes in rhesus macaques

S.H. Hughes[1], A.L. Ferris[1], G.Q. Del Prete[2], B.F. Keele[2], X. Wu[3], J.D. Lifson[2]

[1]HIV Dynamics and Replication Program, National Cancer Institute, Frederick, MD, USA, [2]AIDS and Cancer Virus Program, Leidos Biomedical Research, USA, [3]Laboratory of Molecular Technology, Leidos Biomedical Research, USA

Background: We and others showed that HIV infected cells can clonally expand in patients, and that integrated viral DNA can cause the growth and persistence of infected cells. More recently, we showed that a clonally expanded cell carries an intact provirus and produces infectious HIV in a patient. Because there are limits on the samples that can be obtained from patients, we developed a model using SIV-infected rhesus macaques.

Methods: The methods of Maldarelli *et al.* (*Science* 2014; **345**: 179) were used to generate an integration site library from rhesus macaque PBMCs infected in culture with SIV. Six additional libraries were generated from two rhesus macaques (two lymph node and one spleen sample from each animal) that were infected with SIV for 4 weeks and then treated for 1 year with a fully suppressive cART regimen. Samples were taken during necropsy at the end of the treatment period.

Results: The distribution of the SIV integration sites in the large integration site library (~50,000 independent sites) prepared from rhesus macaque PBMC infected *in vitro* was quite similar to the distribution of HIV integration sites in human PBMCs. We obtained approximately 380 independent integration sites from the monkey tissue sample and identified 13 clones of expanded cells. Cells from two of the clones were present in both the spleen and lymph node.

Conclusions: Cells that are infected early gave rise to expanded clones in two macaques that were treated after 4 weeks of SIV infection; at least some clones were not tissue restricted. We looked for integration sites in genes in which the integration of an HIV provirus can provide the infected cells with a selective growth advantage; there was no evidence for the selection of cells that have integration sites in either BACH2 or MKL2. Our results establish an SIV/macaque model that can be used to study the clonal expansion of infected cells using samples that cannot be obtained from patients.

OP 1.2

Evaluation of HIV latency reversal using designed PKC modulators in humanized BLT mice

M.D. Marsden[1], X. Wu[1], B.A. Loy[2], B.A. DeChristopher[2], A.J. Schrier[2], C.M.R. Kitchen[1], E. Beans[2], D. Fournogerakis[2], C. Gauntlett[2], L. Heumann[2], R. Kramer[2], D. Murray[3], T.W. Chun[3], P.A. Wender[2], J.A. Zack[1]

[1]UCLA, Los Angeles, CA, USA, [2]Stanford University, Stanford, CA, USA, [3]National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

Background: Latently infected CD4+ T cells represent a key barrier preventing the cure of HIV. Latently infected CD4+ T cells represent a key barrier preventing the cure of HIV infection in patients treated with antiretroviral therapy (ART). One potential approach for eliminating this latent reservoir is to induce the virus to express proteins, which would make the host cell susceptible to viral cytopathic effects, immune effector mechanisms, and other therapeutic approaches targeting viral proteins. For this strategy to be successful, safe and effective methods for activating latent HIV expression are needed. We have designed and synthesized new, potent HIV latency-reversing compounds based on prostratin and bryostatin that function through protein kinase C (PKC). In the current study we aimed to test these *in vivo* using the humanized bone marrow-liver-thymus (BLT) mouse model of HIV latency.

Methods: Synthetic analogs of the natural PKC-activating compounds were designed and synthesized. Several of these were highly effective in reversing HIV from latency in cell line models and latently infected cells obtained *ex vivo* from ART-treated patients. Promising compounds were then evaluated for acute toxicity and bioactivity in immunocompetent mice. Based on these studies, one particularly potent and well-tolerated bryostatin analog was tested for its ability to reverse HIV from latency in HIV-infected humanized BLT mice treated with ART.

Results: Several tested prostratin and bryostatin analogs were more bioactive and less toxic in immunocompetent mice than the corresponding natural products. The bryostatin analog PKC modulator tested in ART-treated BLT mice was found to be capable of activating expression of latent HIV in peripheral blood and tissues following *in vivo* administration.

Conclusions: Designed, synthetically accessible PKC modulators are capable of inducing HIV from latency *in vitro* and *in vivo*, and might therefore be useful in efforts to eliminate reservoirs of virus that persist during ART.

OP 1.3

Shining the RNA-Seq microscope on models of HIV latency

C.H. Woelk[4]**, C.H. White[1,2]*, A. Bosque[3]*, B. Moesker[4], C.A. Spina[1,5], D.D. Richman[1,2,5], N. Beliakova-Bethell[1,5], V. Planelles[3]**

[1]VA San Diego Healthcare System, San Diego, CA, USA, [2]Department of Pathology, University of California San Diego, La Jolla, CA, USA, [3]School of Medicine, University of Utah, Salt Lake City, UT, USA, [4]Faculty of Medicine, University of Southampton, UK, [5]Department of Medicine, University of California San Diego, La Jolla, CA, USA

*First authors contributed equally to this abstract. **Senior authors contributed equally to this abstract.

Background: The paucity of latently infected cells in patients necessitates *in vitro* models for the study of HIV persistence. Transcriptomics can be used to assess model validity and to identify biomarkers of latency. Our primary objective was to use RNA-Seq to validate the central memory CD4 T cell (TCM) model of HIV latency that utilizes replication competent virus.

Methods: Naive CD4 T cells were isolated from the blood of four healthy donors and activated with αCD3/αCD28 beads in the presence of cytokines to induce differentiation into TCM. Cells were infected (or mock-infected) with HIV NL43 for 6 days and then cultured for 4 days in the presence of ART to induce a latent state. Both infected and uninfected conditions for each donor were subjected to reactivation. These four conditions (uninfected, UI; latently infected, LI; uninfected activated, UIA; and latently infected activated, LIA) from four donors resulted in 16 samples for RNA-Seq analysis using the Illumina HiSeq 2000 platform.

Results: To validate the TCM model, the LI and LIA conditions were compared to reveal the upregulation of well-known markers of activation (e.g. IL2), as well as a 7-fold increase in HIV transcription, with a shift from unspliced to multiply spliced transcripts. Therefore, this model was reflective of a latent state of HIV infection. The UI and LI conditions were then compared to identify 827 differentially expressed biomarkers of latency associated with processes such as p53 signaling and DNA damage. Comparison to microarray data published by the F. Romerio laboratory for their HIV latency model identified an overlap of 51 upregulated and 32 downregulated genes.

Conclusions: RNA-Seq analysis provides a powerful tool to validate models of HIV latency and screen for biomarkers. Future work will evaluate the ability of biomarkers to identify latently infected cells from HIV infected patients.

OP 1.4

In vivo suppression of SIV-mediated immune activation by a p38 MAPK inhibitor combined with ART

A. Aldovini[1]*, O. Chaudhary[1]*, V. Narayan[1]*, R. Veazey[2]

[1]*Boston Children's Hospital, Department of Medicine and Harvard Medical School, Department of Pediatrics, Boston, MA, USA,* [2]*Tulane Primate Research Center, Tulane University, Covington, LA, USA*

Background: Differences in immune activation have been identified as the single most significant difference between AIDS-susceptible and resistant species. p308 MAPK, reported t3o be activated in HIV and SIV infection, is key to induction of interferon-stimulated genes (ISG) and is associated *in vivo* with some of the pathology produced by HIV and SIV infection. As small molecule p38 MAPK inhibitors are available and are currently being tested in human trials for other inflammatory diseases, we evaluated the effects of treating SIV-infected macaques with a p38 inhibitor in conjunction with ART.

Methods: Rhesus macaques were infected with SIVmac251 and cART and p38 MAPK inhibitor treatment was initiated in one group of animals 6 weeks after infection. Additional groups included ART alone, p38 inhibitor alone, and naive controls. We evaluated differences in expression of surface and intracellular molecules linked to immune activation, intracellular inflammatory cytokine expression in cell subpopulations of blood, lymph node and intestinal tissue, plasma levels of inflammatory cytokines viral loads, preservation of central memory CD4+ T cells, anti-SIV immune responses, protein levels of selected ISG.

Results: ART reduced viremia to levels of 70–80 copies/mL. The p38 inhibitor did not further reduce the residual viremia in blood, did not negatively affect anti-SIV immune responses and had no side effects. By itself it had no significant effect on immune activation. When combined with ART, numerous markers of immune activation were significantly reduced compared to the ART alone group. CD38/HLA-DR an Ki67 percentages in blood, lymph node and rectal CD4+ and CD8+ T cells and percentages of IL-6, IL-8, IFN-γ and IFN-α producing cells were all significantly reduced. IRF7, pSTAT1 and IP-10 protein accumulation was also reduced in APC. Significant preservation of CD4+/IL22+ and CD4+/IL-17+ T-cells in PBMC, rectal and lymph node mononuclear cells and of central memory CD4+ T cells in blood was also observed.

Conclusions: we conclude that the p38 MAPK inhibitor used in this studies, which is already in clinical trials for other inflammatory diseases, significantly reduced immune activation during ART treatment and could be a desirable addition to antiretroviral therapy.

OP 1.5

Memory CD4+ T cell subsets show differential responses to HIV latency-reversing agents

D.A. Kulpa[1], S. Yuan[2], A. Talla[3], A.G. Bebein-Blackwell[4], J. Brehm[5], R. Barnard[6], M. Miller[6], D. Hazuda[6], N. Chomont[7], R.P. Sékaly[3]

[1]*Southern Research Institute, Frederick, MD, USA,* [2]*Janssen Pharmaceuticals, Springhouse, PA, USA,* [3]*Case Western Reserve University, Cleveland, OH, USA,* [4]*University of Georgia, Athens, GA, USA,* [5]*GlaxoSmithKline, Raleigh, NC, USA,* [6]*Merck, West Point, PA, USA,* [7]*Université de Montréal, Montreal, QC, Canada*

HIV persists in individuals on antiretroviral therapy in central memory (CM), transitional memory (TM) and effector memory (EM) CD4+ T cells. Development of "shock and kill" strategies to eradicate the persistent HIV reservoir will depend on the ability to predict the responsiveness of each subset to latency-reversing agents (LRAs) in this heterogeneous population. To examine the contribution of each subset to latency reactivation, we developed a primary cell based assay that models the latent reservoir in virally suppressed HIV-infected subjects. Our **l**atency **a**nd **r**eversion **a**ssay (LARA) is based on infection of minimally activated CD4+ T cells and employs TGF-β, an inducer of T cell quiescence, and IL-7, an inducer of T cell survival and homeostatic proliferation, to efficiently establish HIV latency in CM, TM, and EM subsets. These conditions allow for the simultaneous assessment of HIV latency reversion in all subsets in a single assay. Using LARA, we demonstrate that all memory CD4+ T cell subsets are capable of robust latency reversion following TCR engagement (αCD3/CD28). Other classes of compounds such as PKC agonists, HDAC inhibitors, and γc cytokines demonstrated a diverse capacity to reverse latency in the different memory subsets. When compared to the response achieved from maximal TCR stimulation, compounds such as disulfiram could reverse latency effectively in CM (~72% of αCD3/CD28 signal) or TM (~95%), but had reduced efficiency in EM (~30%), whereas IL-15 was more efficient in EM (~64%) and TM (~56%), while CM were minimally responsive (~13%). Together these data support the significance of the biological diversity of memory CD4+ T cell subsets in their ability to respond to LRAs. We present LARA as an optimized platform for measuring the activity of LRAs in CD4+ T cell memory subsets that are known to contribute to the latent HIV reservoir *in vivo*, which will result in a streamlined process to advance candidate LRAs for future clinical use.

OP 1.6

Quantifying the impact of autologous transplantation on viral reservoirs in a nonhuman primate model of HIV/AIDS

C.W. Peterson[1], C. Benne[2], P. Polacino[3], A. Baldessari[4], R.D. Murnane[4], S.L. Hu[3], R. Sekaly[2], H. Kiem[1,3]

[1]*Fred Hutchinson Cancer Research Center, Seattle, WA, USA,* [2]*Case Western Reserve University, Cleveland, OH, USA,* [3]*University of Washington, Seattle, WA, USA,* [4]*Washington National Primate Research Center, Seattle, WA, USA*

Background: Hematopoietic stem cell (HSC) gene therapy holds great promise in the cure of HIV infection, but the feasibility of this approach in stably suppressed patients remains unclear. We are evaluating gene therapy approaches to reduce or eliminate viral reservoirs in a pigtailed macaque model of drug-suppressed HIV infection.

Methods: Animals are challenged with HIV enveloped simian/human immunodeficiency virus (SHIV), and suppressed by three-drug combination antiretroviral therapy (cART). During autologous transplantation, myeloablative total body irradiation (TBI) is used to increase engraftment of subsequently infused HSCs, and to target latently infected cells. Immunological and viral reservoir assays are conducted in peripheral blood and tissues from animals transplanted with engineered and non-engineered HSCs, as well as untransplanted controls.

Results: TBI leads to a drastic reduction in circulating CD4+ T-cell counts. Consistent with past reports, SHIV viremia rebounds following cART withdrawal in animals that are transplanted without a protective gene therapy approach. Early viral reservoir analyses suggest that persistent reservoir sites are seeded in these animals, and are impacted by the transplant procedure. Flow cytometry data reveal differential expression of markers including PD-1, SLAM, and TIGIT in transplanted animals relative to untransplanted controls.

Conclusions: Although autologous transplantation is known to be insufficient for viral eradication, we demonstrate that this procedure does modulate viral reservoirs. TBI significantly reduces the number of CD4+ memory T-cells, but may enhance viral rebound if cART is withdrawn prior to complete hematologic recovery. Our preclinical model allows not only for the evaluation of novel gene therapy and gene editing approaches to impact the latent viral reservoir, but also combinatorial approaches, which may include therapeutic vaccination, immune modulators, and/or latency reactivating agents.

Session 2: Basic science of HIV latency

OP 2.0

HIV-1 transcriptional latency in resting CD4 T-cell

M. Benkirane

CNRS, Montpellier, France

Abstract not available at the time of printing

OP 2.1

Single-cell analysis identifies biomarkers for HIV permissiveness

A. Ciuffi[1]*, S. Quenneville[1]*, A. Rausell[1,2]*, S. Rato[1], M. Muñoz[1], A. Telenti[3]*

[1]*Institute of Microbiology, University of Lausanne, Switzerland,* [2]*Vital-IT Group, Swiss Institute of Bioinformatics, Lausanne, Switzerland,* [3]*J Craig Venter Institute, La Jolla, CA, USA*

Background: Cellular permissiveness to HIV infection is highly heterogeneous across individuals. Heterogeneity is also found across CD4+ T cells from the same individual, where only a fraction of cells gets infected. We used a single-cell RNA-Seq approach to investigate cellular heterogeneity and identify biomarkers of HIV permissiveness.

Methods: CD4+ T cells from healthy donors were activated by TCR-mediated stimulation for three days and tested for their permissiveness to HIV infection. Non-infected activated CD4+ T cells from a highly and a poorly susceptible individual were selected and used for single-cell RNA-Seq analysis using Fluidigm C1 technology.

Results: RNA-Seq profiles from 85 highly permissive and 81 poorly permissive single cells were successfully obtained, with ~25 million reads per single cell. Transcriptional heterogeneity translated in a continuum of intermediary cell states in both highly and poorly permissive donor cells, which was mainly driven by TCR-mediated cell activation. Genes whose expression was bimodal across cells, across both donors and that encoded proteins expressed at the cell surface were further investigated as candidate biomarkers of HIV permissiveness. Seven single biomarkers were tested for their ability to capture permissive cells, showing enrichment in HIV infection from 1.5- to 10-fold. The combination of multiple candidate biomarkers further selected for highly permissive cells, thus defining the "HIV-permissive cell". This held true in CD25-sorted subpopulations as wells as in naive or memory CD4+ T cell subsets.

Conclusions: Our data identified activation as a major determinant driving cellular heterogeneity in HIV permissiveness and further revealed the role of single candidate biomarkers in defining the HIV highly permissive cell. The single-cell-based approach developed in this study should help characterizing the heterogeneity of the HIV latent reservoir, as well as its response to reactivating agents.

OP 2.2

Cellular HIV RNA/DNA as biomarkers of inducible virion production

A. Cillo[1], F. Hong[1], A. Tsai[2], A. Irrinki[2], J. Kaur[2], J. Lalezari[3], D. Sloan[2], J.P. Murry[2], J. Mellors[1]

[1]*University of Pittsburgh, Pittsburgh, PA, USA,* [2]*Gilead Sciences, Foster City, CA, USA,* [3]*Quest Clinical Research, San Francisco, CA, USA*

Background: Simple biomarkers of the inducible HIV reservoir have not been identified. We investigated whether the frequency of infected cells in blood and their transcriptional activity is related to the inducible HIV reservoir from resting CD4+ T cells in persons on antiretroviral therapy (ART).

Methods: PBMC were isolated from leukapheresis product obtained from donors on suppressive ART for ≥1 year. Cellular unspliced HIV RNA (CA-RNA) and proviral HIV DNA (CA-DNA) in uncultured PBMC were quantified by qPCR. Resting CD4+ T (rCD4) cells were isolated from PBMC by negative selection, and were then activated with PMA/ionomycin in the presence of 300 nM efavirenz. On day 6 of culture, supernatants were collected, centrifuged (500×*g* for 5 min), and stored at –80°C. HIV RNA in supernatants was quantified by COBAS Roche TaqMan v2.0. Correlations between virion production and cellular HIV RNA/DNA were assessed using Spearman's correlation coefficient.

Results: A total of 22 donors were evaluated. Levels of HIV-1 RNA after treatment with PMA/ionomycin varied >1000 fold between donors; the median was 4406 copies/mL of culture supernatant, ranging from 38 to 42,756 copies/mL. The median level of CA-RNA was 38 (range: <1–355) copies per 10^6 PBMC, and the median level of CA-DNA was 286 (range: 7–2972) copies per 10^6 PBMC. CA-RNA and CA-DNA levels were strongly correlated with each other (rho=0.80, $P<0.001$), and both were strongly correlated with inducible virion production (rho=0.76, $P<0.001$ for CA-RNA; rho=0.75, $P<0.001$ for CA-DNA).

Conclusions: Inducible virion production from rCD4 cells *ex vivo* is strongly associated with the number of HIV-infected cells and the basal level of HIV-1 RNA transcription in PBMC. These results suggest simple measures of CA-RNA/DNA can be used to estimate the size of the inducible reservoir. These approaches could also be useful in assessing the effectiveness of interventions targeting the latent HIV reservoir. These approaches could also be useful in assessing the effectiveness of interventions targeting the latent HIV reservoir.

OP 2.3

The HIV-1 antisense transcript AST is an inducer of viral latency

F. Romerio[1], J.C. Zapata[1], F. Kashanchi[2], S. Iordanskyi[2]

[1]*Institute of Human Virology, Baltimore, MD, USA,* [2]*George Mason University, Manassas, VA, USA*

Background: Noncoding RNAs (ncRNAs) regulate gene expression by recruiting chromatin-modifying enzymes to the gene's promoter. EZH2, a histone methytransferase part of the Polycomb Repressor Complex 2 (PRC2), trimethylates lysine 27 in histone H3 at the proviral 5'LTR, which positions Nuc-0 and Nuc-1 and leads to latency. How are Nuc-0 and Nuc-1 precisely and invariably positioned at the 5'LTR irrespective of the site and orientation of HIV-1

integration in the host genome? What ncRNA recruits PRC2 to the 5'LTR? An attractive hypothesis is that HIV-1 encodes for its own ncRNA as an autonomous mechanism to recruit PRC2 to the 5'LTR, and to establish latency regardless of the surrounding chromatin context. Indeed, HIV-1 expresses an antisense transcript (AST) directed from a poorly defined promoter within the 3'LTR.

Methods: We developed a strand-specific quantitative RT-PCR assay to measure AST expression in infected cells. We generated cell lines stably transduced with AST, and tested the effect of AST on HIV-1 replication and latency. We performed ChIP assays to test whether AST affects the pattern of chromatin assembly at the 5'LTR. We performed RIP assays to test whether AST interacts with PRC2.

Results: AST is expressed in chronically infected cell lines, primary CD4+ T cells infected *in vitro*, and CD4+ T cells from patients under cART. Expression of AST suppressed HIV-1 replication, and promoted the establishment and maintenance of latency. This correlated with decreased levels of RNA Pol II, and increased levels of H3K27me3 and HDAC at the 5'LTR. In addition, we found direct interaction between AST and PRC2.

Conclusions: The 3'LTR directs the expression of an antisense transcript (AST), which recruits PRC2 to the 5'LTR, and promotes epigenetic modifications that lead to viral latency. Thus, AST is a virally encoded ncRNA that acts as an inducer of viral latency. These results could guide in designing new therapies aimed at stabilizing latency by exploiting AST.

OP 2.4

Integration site analysis of latently infected cell lines: evidence of ongoing replication

J. Symons[1], S.R. Lewin[1,5], A. Chopra[2], E. Malantinkova[3], W. De Spiegelaere[3], S. Leary[2], D. Cooper[2], L. Vandekerckhove[3], S. Mallal[2,4], P. Cameron[1,5]

[1]Peter Doherty Institute for Infection and Immunity, University of Melbourne, Australia, [2]Institute for Immunology and infectious diseases (IIID), Murdoch University, Perth, Australia, [3]HIV Translational Research Unit, Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University and Ghent University Hospital, Belgium, [4]Department of Infectious Diseases, Vanderbilt University, Nashville, TN, USA, [5]Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, Australia

Background: HIV cure is limited by persistence of long lived latently infected CD4+ T cells. Latently infected cell lines are widely used *in vitro* to study HIV latency. We identified and tested the stability of HIV integration sites in latently infected cell lines, using a newly developed high throughput method.

Method: To determine assay sensitivity/efficiency, genomic DNA of seven latent HIV cell lines (20 cells each) were isolated and mixed with genomic DNA from one million HIV negative PBMCs. Additionally, the latently infected cell lines ACH-2, U1 and J1.1 containing replication competent HIV and J-Lat 8.4, 9.2, 10.6 and 15.4 cell lines that contain a single replication deficient HIV were passed. DNA, isolated after passage 0, 2, 4, 6 and 8 was enzymatically cut to random sized fragments. These were end-repaired and a linker was ligated to the fragments. The fragments were subjected to LTR based nested PCR with barcoded nested primers and prepared for Miseq sequencing. Chromosomal alignment was determined using the Blat-UCSC Genome Browser (GRCH38/hg38).

Results: The efficiency was 35% and detected one HIV integration site in 50,000 uninfected PBMCs. J-Lat cell lines showed single integration sites. ACH-2, U1 and J1.1 demonstrated multiple distinct HIV integration sites per 150,000 cells (74, 42 and 93 respectively). J1.1, which is reported to have a single integrated copy per cell, demonstrated two major integration sites in equal frequency. ACH-2 cells, when passaged, demonstrated a 2-fold increase in unique HIV integration sites found across the human genome.

Conclusion: Cell lines latently infected with replication competent HIV demonstrated multiple unique HIV integration sites indicating these cell lines are not clonal. Furthermore, the increase and change in sites of HIV integration observed in ACH-2 cells over time is suggestive of low level virus replication. These findings have implications for the use of latently infected cell lines as models of HIV latency.

OP 2.5

Mixed effects of HDACi on host gene expression and their implications for HIV reactivation from latency

N. Beliakova-Bethell[1,2], A. Mukim[2], S. Deshmukh[2], C.H. White[3], C.H. Woelk[4], C.A. Spina[2,5]

[1]Department of Medicine, University of California San Diego, La Jolla, CA, USA, [2]VA San Diego Healthcare System, San Diego, CA, USA, [3]Graduate Program in Bioinformatics and Systems Biology, University of California San Diego, La Jolla, CA, USA, [4]Faculty of Medicine, University of Southampton, UK, [5]Department of Pathology, University of California San Diego, La Jolla, CA, USA

Background: Previously, we identified transcriptomic and proteomic effects of the HDACi SAHA, associated with promotion or inhibition of HIV reactivation. Here, we have further characterized these effects in primary CD4+ T cells of various maturation phenotypes and compared them with those induced by another HDACi, romidepsin (RMD).

Methods: A unique model was used to generate latent HIV infection in resting primary CD4+ T cells. Cells were treated for 24 h with SAHA (1 μM), RMD (15 nM), or their solvent dimethyl sulfoxide. Naïve (TN), central (TCM), and effector memory (TEM) cells were isolated by flow cytometry cell sorting. Differential gene expression analyses were performed using RNA-Seq and *EdgeR* package in Bioconductor R.

Results: Similar to SAHA, RMD induced gene expression changes that could be interpreted to promote or inhibit HIV reactivation. However, several RMD effects were consistent with the idea that it may be a more potent activator of HIV expression (downregulation of HIV transcriptional repressors *AES* and *ARID1B* to a greater extent, and upregulation of other repressors, *HMGA1* and *ASF1A,* to a lesser extent, than SAHA). Results from CD4+ T cell maturation subsets indicated that SAHA may be a more potent activator of HIV provirus from TN cells. For example, *AES* was downregulated to a greater extent in TN cells; while a gene encoding heat shock protein, required for proper folding of cyclin T1/Cdk9, was upregulated to a greater extent in TN, as compared to TCM and TEM cells.

Conclusions: These results may provide clues to the ineffectiveness of HDACi to clear the HIV reservoir in clinical trials. HDACi exhibit both inductive and inhibitory effects on HIV reactivation in CD4+ T cells. In addition, HDACi may be most active in TN cells, the minority component of the latent reservoir. Combination treatment strategies will be required to counteract inhibitory effects and ensure efficient viral reactivation from CD4+ T cells regardless of maturation state.

OP 2.6

HIV-1 latency is established preferentially in minimally activated and non-dividing cells during productive infection of primary CD4 T cells

C.A. Spina[1,2], P.C. Soto[1,2], V.H. Terry[1], M.K. Lewinski[3]

[1]Veterans Affairs San Diego Healthcare System, San Diego, CA, USA, [2]Department of Pathology, University of California San Diego, La Jolla, CA, USA, [3]Department of Medicine, University of California San Diego, La Jolla, CA, USA

Background: The primary barrier to HIV eradication is establishment of a stable reservoir of latently infected CD4 T cells very early in the course of infection that leads to life-long viral persistence. The mechanisms involved in establishment of this reservoir remain unclear. Three scenarios have been proposed: (1) activated proliferating cells become infected and revert back to quiescence; (2) activated cells become infected, while returning to a resting state; or (3) infection is established directly in resting cells.

Methods: To determine the phase of T cell activation at which acute infection is most likely to progress to latency, two approaches were used. First, cells were infected at different times, before or after stimulation by anti-CD3/CD28, and followed out to 14 days when

cells had returned to resting. Second, cell proliferation of acutely infected cultures was tracked using CFSE dye and cell subsets that had undergone varying degrees of proliferation were isolated by FACS, at the end of culture. Each recovered cell subset was analyzed for the quantity of integrated HIV DNA and replication-competent virus.

Results: Cells exposed to HIV, prior to stimulation, contained the highest levels of integrated provirus and replication competent virus after returning to quiescence. Cells infected, during the height of cell proliferation, retained the least HIV. Cells that did not divide or divided only a few times contained higher levels of integrated and replication-competent HIV than did cells, which had divided many times.

Conclusions: HIV latency is established very early within a heterogeneous population of CD4 T cells, which are undergoing varying degrees of cell activation in the presence of infectious virus. Minimally activated cells within this milieu are the most likely to develop persistent latent infection. These observations have important implications for the study of latency, and may aid in the design of strategies to purge this reservoir.

Session 3: Virology of HIV persistence

OP 3.0

The virology of HIV-1 persistence: integration, expansion, and expression

J. Coffin

Tufts University School of Medicine, Boston, USA

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OP 3.1

Developing and applying ultrasensitive p24 protein immunoassay for HIV latency

B.J. Howell, G. Wu, M. Swanson, M. Lu, D.J. Graham, J. Strizki, S. Wolkenberg, R.J.O. Barnard, W. Blair, D.J. Hazuda

Merck, West Point, PA, USA

Background: Novel approaches are being explored for eradicating HIV from tissue/cell reservoirs, and new assays with improved sensitivity are required to quantify reservoir size and examine the impact of latency-reversing agents on actively replicating virus following "flush-kill" strategies. Towards this goal, a highly sensitive digital immunoassay was optimized to quantify HIV p24 capsid protein in culture medium and cell lysates and can be used as a biomarker for evaluating viral protein production following perturbation of HIV latency state.

Methods: A commercial immunoassay was optimized to improve assay sensitivity and specificity and to quantify p24 in both cell lysates and culture medium. Viral supernatants from 18 genotypically diverse HIV isolates (including, HIV-2, HIV-1, subtypes A, B, C, D, E, G and group O) were serially diluted and evaluated. Quantification of p24 from CD4+ T cells isolated from ART suppressed HIV patients was determined following *ex vivo* treatment with latency-reversing agents, including PMA/Ionomycin and HDAC inhibitors. Kinetics of p24 expression were evaluated and confirmed.

Results: The optimized p24 immunoassay specifically recognized both recombinant p24 and uncleaved polyprotein p55 with equal affinity. The lowest limit of reliable quantitation in cell lysate and cultured medium was determined to be 14 fg/mL. p24 was detected and showed specificity and linear dilution across 18 clinical isolates. *Ex vivo* treatment of CD4+ T cells from ART suppressed patients with latency-reversing agents, including PMA/Ionomycin and HDAC inhibitors, showed robust, dose-dependent and time-dependent changes in p24 in cell lysates and culture medium.

Conclusions: The optimized, p24 immunoassay shows ~2000 fold increased sensitivity over gold-standard p24 assays and can quantify viral protein in cell lysates and culture medium from ART suppressed patient cells. HDACi treatment of resting CD4+ T cells from ART suppressed patients results in viral protein production in a dose- and time-dependent manner. The assay has potential to be used for rapid, sensitive detection of p24 in HIV latency studies.

OP 3.2

Detection and enrichment to near purity of rare HIV-1 infected cells by PrimeFlow RNA

F. Romerio, J.C. Zapata

Institute of Human Virology, Baltimore, MD, USA

Background: PrimeFlow RNA is a technique that detects RNA molecules by flow cytometry, generating gene expression data at the single cell level. Sets of 20–40 probe pairs hybridize to the target RNA. Signal amplification and detection is achieved by branched DNA (bDNA) via hybridization of adjacent probe pairs with pre-amplifiers, amplifiers and fluorochrome-conjugated label probes. This yields low background, high specificity and high signal-to-noise ratio. In PrimeFlow RNA, detection occurs through amplification of signal (up to 8,000×) rather than target sequence (PCR) for more consistent results. PrimeFlow RNA can be combined with detection of protein markers by traditional flow cytometry.

Methods: We developed three different probe sets for the detection of full length, single-spliced, or multiply spliced HIV-1 transcripts. We have used these probe sets (alone or in combination) for the detection and isolation of infected cells.

Results: All three HIV-1 specific probe sets detected productively infected cells, but not uninfected or latently infected cells. Through the combination of two HIV-1 specific probe sets labeled with different fluorochromes, we specifically detected infected cells mixed with uninfected cells at ratios as low as 10–100 infected cells per 10^6 uninfected cells. This technique allowed accurate enumeration of infected cells. In addition, we were able to sort the infected cells at purities >90% as assessed by qPCR of HIV-1 *vs.* GAPDH DNA copy numbers.

Conclusions: PrimeFlow RNA detects and accurately enumerates infected cells present in mixed populations at dilutions that are in the same range as those of latently infected cells in clinical samples. In addition, PrimeFlow RNA allows sorting these cells to near homogeneity. This technique may be useful to identify, enumerate and phenotype latently infected cells from cART-suppressed individuals after viral reactivation.

OP 3.3

Sustained HIV release by single persisting CD4+ T cells during latency disruption

J.M. Hataye, J.P. Casazza, D.R. Ambrozak, E. Boritz, T. Yamamoto, D.C. Douek, A.S. Perelson, R.A. Koup

National Institutes of Health, Vaccine Research Center, Bethesda, MD, USA

Background: While the dynamic details of HIV release from the source CD4+ T cells undergoing latency disruption are unknown, both multi-day sustained HIV release and instantaneous burst stochastic models of HIV replication have been proposed. Which model best represents latency disruption may have important implications for detecting replication-competent virus.

Methods: We used HIV *gag* RNA RT-PCR to quantify HIV release from isolated primary CD4+ T cells undergoing latency disruption. Limiting dilution viral inhibition cultures with efavirenz revealed released virus without new rounds of infection and were compared to respective outgrowth cultures. Stochastic models were implemented using the Gillespie algorithm with parameters derived from applying the deterministic model to experimental data.

Results: While many outgrowth wells accumulated high amounts of virus, many had declining virus that was nevertheless replication-competent, confirmed by virus transfer to new outgrowth wells. Culture wells with >77% probability of being seeded by a single virus-producing cell were analyzed for viral release. Half of cells releasing virus did so within 4 days. The accumulated virus for a well varied from less than 120 to 30,000 HIV *gag* RNA copies. Although a low amplitude

instantaneous burst pattern was observed, the vast majority of accumulated HIV RNA was attributed to a higher amplitude virus release sustained for 2–6 days. The half-life of virus in viral inhibition cultures was 3 days. Applying this result to the deterministic model, we estimated an average total sustained release per cell of 5500 HIV RNA copies.

Conclusions: Our results are consistent with a multi-day sustained virus release stochastic model that predicts replication-competent virus will be present in cultures that are negative for viral outgrowth, and further demonstrate that single virus-producing cells can survive and produce virus over several days without succumbing to viral cytopathic effects.

OP 3.4

Antiretroviral drug activity in macaque PrEP breakthrough infections has only a transient effect on cell-associated SHIV DNA reservoirs

J.G. García-Lerma, M.E. Cong, C.P. Pau, W. Heneine

Centers for Disease Control and Prevention, Atlanta, GA, USA

Background: Infection of macaques with simian HIV (SHIV) during concurrent pre-exposure prophylaxis (PrEP) with FTC/TDF is associated with reduced acute plasma viremias and limited virus diversity, thus providing a unique model to assess long-term effects of acute antiretroviral treatment. Here, we investigated the effect of PrEP on acute SHIV DNA dynamics in peripheral blood mononuclear cells (PBMCs), and on the size of the persistent virus reservoir in lymphoid tissues.

Methods: Cell-associated SHIV DNA levels were measured in PBMCs during acute infection in 8 macaques infected during concurrent PrEP with FTC/TDF combination or single-agent tenofovir alafenamide fumarate (TAF). Macaques continued treatment with 1–2 weekly drug doses to model suboptimal drug exposure during undiagnosed HIV infection in humans. Peak SHIV DNA and area under the curve values (AUC) over 5 or 20 weeks, as well as RNA levels, were compared to the values seen in untreated SHIV infections (n=10). SHIV DNA levels were also measured in lymphoid tissues collected from FTC/TDF or maraviroc PrEP breakthroughs after 1 year of infection.

Results: PrEP breakthrough infections had reduced plasma RNA viremias relative to untreated infections both at peak and during the first 20 weeks of infection (P<0.005). SHIV DNA levels in PBMCs were also reduced in PrEP breakthrough infections both at peak and at week 5 (P=0.022 and P=0.043, respectively), but not after 20 weeks of infection. At 1 year, SHIV DNA reservoirs in lymphoid tissues were similar in size among macaques that received PrEP with FTC/TDF (median=464 SHIV DNA copies/10^6 cells; range, 40–5,346), PrEP with maraviroc (median=1088 copies/10^6 cells; range, 554–10,090), or placebo (median = 952 copies/10^6 cells; range, undetectable–24,668) (P>0.05).

Conclusions: Antiviral drug activity due to PrEP limits acute SHIV replication but has only a transient effect on cell-associated DNA levels in PBMCs and lymphoid tissues. Our model suggests that suboptimal drug exposure in persons that are taking PrEP and become infected with HIV may not be sufficient to reduce the pool of HIV-infected cells, and that treatment intensification may be needed to sustain the virologic benefit from the PrEP regimen.

OP 3.5

In vivo expression of unspliced HIV RNA in expanded CD4+T-cell clones containing defective or replication-competent proviruses

M.F. Kearney[1], J. Spindler[1], M. Sobolewski[2], J.M. Coffin[3], J.W. Mellors[2]

[1]HIV Dynamics and Replication Program, Department of Medicine, National Cancer Institute, Frederick, MD, USA, [2]University of Pittsburgh, Pittsburgh, PA, USA, [3]Department of Molecular Biology and Microbiology, Tufts University, Boston, MA, USA

Background: Clonal expansion of HIV infected cells is an important mechanism for viral persistence during ART and cells that express unspliced HIV RNA (usRNA) may lead to rebound viremia when ART is interrupted. We investigated the proportion of proviral populations that express usRNA in ART-treated individuals and the fraction that can produce replication-competent virus.

Methods: PBMCs from five donors on ART for >5 years and with levels of viremia <20 copies/mL were analyzed for expression of usRNA using SGS of cell-associated RNA (CAR-SGS) and DNA (CAD-SGS). CAR-SGS was performed by extracting RNA from multiple PBMC aliquots per sample after diluting to an endpoint for HIV expressing cells. cDNA was synthesized from each RNA extraction and analyzed by gag-pol SGS. DNA was extracted from 1 PBMC aliquot to compare the genetics of CAR to CAD (CARD-SGS) and to quantify the number of pol-containing proviruses using real-time PCR. Identical CAD or CAR sequences across aliquots indicated the presence of clonally expanded populations. Infectious proviruses were detected by VOA performed on CD4+T-cells from the same ART-suppressed individuals and infectious proviruses were related to CAR-SGS via sequencing of gag-pol.

Results: Levels of pol-containing proviruses ranged from 103–874/10^6 PBMCs of which a median of 8% (4–12%) were found to express usRNA. All five donors had detectably expanded proviral clones (median >9 clonal populations per donor). A median of at least 13% (5–21%) of the expanded proviruses detected by CAD-SGS were found to express usRNA. However, of the 25 distinct infectious variants isolated by VOA and the 302 distinct CAR sequences identified, only one match was found (0.3%), suggesting that almost all clonal populations expressing usRNA are defective.

Conclusions: Although expanded proviral populations in individuals on ART often express usRNA, most appear to be defective. Rarely, they can be replication-competent and thus serve as a reservoir for HIV rebound.

Session 4: Anatomic and non-CD4 cell reservoirs

OP 4.0

Tissue localization of human T cell responses

D. Farber

Columbia University Medical Center, New York, USA

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OP 4.1

Proliferation of perivascular macrophages in macaque models of lentiviral encephalitis: a potential mechanism for HIV/SIV persistence in the brain

W.K. Kim, A.R. Filipowicz, C.M. McGary, G.E. Holder, M.J. Kuroda

Eastern Virginia Medical School, Norfolk, VA, USA

In lentiviral encephalitis, the number of brain perivascular macrophages (PVM) is substantially increased. This may be due to recruitment of precursors from peripheral blood and/or *in situ* proliferation in the central nervous system (CNS). While the former possibility has been explored, research into the latter is lacking. We therefore investigated if macrophage proliferation occurs in the CNS during simian immunodeficiency virus (SIV) infection of adult macaques. Using immunohistochemistry and fluorescence microscopy, we examined the expression of proliferation markers including Ki-67 and incorporation of thymidine analogs by PVM in the brains of uninfected macaques and SIV-infected macaques with or without encephalitis. Double-label immunohistochemistry using antibodies against the pan-macrophage marker CD68 and Ki-67 showed that there was a significant increase in Ki-67+CD68+ cells in macaques with SIV encephalitis (SIVE) compared to uninfected controls and SIV-infected animals without encephalitis (SIVnoE). Multi-label immunofluorescence against CD163 (PVM marker) and Ki-67 with a nuclear counterstain confirmed that the vast majority of Ki-67+ nuclei were localized to CD163+ PVM in perivascular cuffs and lesions that characterize SIVE. The proliferative capacity of Ki-67+

PVM was confirmed by their nuclear incorporation of bromodeoxyuridine (BrdU) and 5-ethynyl-2′-deoxyuridine. Using combinations of the macrophage markers CD16, CD68, and CD163 with the proliferation markers BrdU and Ki-67 revealed that multinucleated giant cells (MNGC) also displayed a proliferative capacity. Examining the MNGC and SIVE lesions further, using double-label immunofluorescence with antibodies against Ki-67 and SIV Gag p28, showed that not only were the Ki-67+ cells productively infected, but that there was a significant positive correlation between the size of lesions and the number of Ki-67+ cells in these lesions. Altogether, this study shows that there are subpopulations of macrophages that both express proliferation markers and are SIV-infected in the brain, suggesting a mechanism of SIV persistence and accumulation in the CNS via PVM proliferation.

OP 4.2

The human lung is a site of productive HIV infection during long-term ART: novel tools to study ART-durable HIV reservoirs

D.W. Gludish[1], H.C. Mwandumba[2], K.C. Jambo[2], S.M. Amie[1], D.G. Russell[1]

[1]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA, [2]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, University of Malawi, College of Medicine, Blantyre, Malawi

Background: Tuberculosis is the leading cause of death among HIV-infected individuals, most notably in Sub-Saharan Africa. We developed novel tools to probe HIV biology in the lungs of Malawian adults to understand this susceptibility that persists even during ART. Our previous work found HIV mRNA in alveolar macrophages (AMs) of HIV patients. To determine if AMs produce infectious HIV, we sought a robust, rapid readout amenable to flow cytometry at point of care.

Methods: JC53 HeLa cells (express CD4, CCR5 and CXCR4) were modified to express GFP dependent on HIV Tat and Rev (TZM-GFP). In laboratory co-cultures with HIV-infected human monocyte-derived macrophages (HMDMs), we observed clear GFP induction in TZM-GFP. We showed by limiting dilution that TZM-GFP cells can report a single infected HMDM in 96-well co-culture, facilitating viral outgrowth assays to quantify cells that harbor infectious provirus. We then co-cultured TZM-GFP with bronchoalveolar lavage (BAL) cells from asymptomatic HIV-positive adults at the Queen Elizabeth Hospital, Blantyre, Malawi: GFP protein and HIV gag mRNA were assayed by fluorescent *in situ* hybridization (FISH) and flow cytometry.

Results: FISH analysis of BAL cells indicated that viral mRNA was found predominantly in AMs at HIV diagnosis. This AM signal was also found in aviremic individuals on ART, suggesting persistence of HIV in AMs despite successful therapy. Similarly, BAL cells from ART-treated and ART–naïve patients induced GFP in TZM-GFP co-cultures. The transfer of infectious virus was validated by HIV mRNA FISH.

Conclusions: (1) ART, though successful in clearing viremia, spares HIV-infected AMs. (2) BAL cells from ART-treated patients generate infectious virus, showing the lung is a site of HIV maintenance. (3) HIV in the lung is mainly found in AMs, a potential anatomical reservoir that remains understudied. This study reports on the lung as a site of viral persistence and productive infection in the face of effective ART.

OP 4.3

Lymphatic and cancer tissues are a potential reservoir of replicating virus in virally suppressed ART+ patients

R. Rose[1], S.L. Lamers[1], D.J. Nolan[1,2], M.S. McGrath[3]

[1]Bioinfoexperts, LLC, Thibodaux, LA, USA, [2]Department of Pathology, Immunology, and Laboratory Medicine, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA, [3]AIDS and Cancer Specimen Resource and the Department of Laboratory Medicine, Medicine and Pathology, University of California at San Francisco, CA, USA

Background: Antiretroviral therapy (ART) is effective in reducing plasma HIV loads to undetectable levels. However, HIV can rapidly rebound once therapy is removed. Furthermore, HIV-associated co-morbidities, particularly certain cancers, remain a significant co-morbidity even with effective ART. We hypothesized that HIV-infected macrophages in tumors may both protect HIV from ART and contribute to metastasis.

Methods: The ACSR provided 36 post mortem tissues from five HIV+/ART+ patients with no detectable viral load. All five (designated Pt1–5) died with a form of metastatic cancer. Tissues were assessed for HIV using digital droplet PCR (ddPCR). Single genome sequencing was used to generate *env-nef* DNA and RNA sequences. Maximum-likelihood phylogenies were inferred and statistical tests were performed to investigate viral evolutionary patterns and compartmentalization. An *in situ* RNA signal detection technique (RNAscope) combined with histological staining was used to visualize the cellular location of HIV in select tissues.

Results: All tissues were HIV+ by ddPCR. HIV *env-nef* DNA sequences were generated from 21/36 of the tissues, and a much smaller subset of these (n=5) contained both HIV RNA and DNA. Maximum-likelihood phylogenies and statistical testing showed little evidence of viral compartmentalization among tissues. Tree clades showed evidence of on-going evolution as well as clonal expansion. RNAscope for *Pt02* showed HIV RNA co-localizing with CD163+/CD68+ macrophages in tumor lymph node and, in the cerebellum, with infected cells surrounded by infiltrating macrophages.

Conclusions: Results suggest that cancer tissues may offer a privileged environment for persistent HIV replication within macrophages during ART. Two distinct patterns of virus evolution suggest different modes of replication/spread underlie persistence: ART-resistant HIV-infected macrophages with persistent evolution and migration of HIV infected cells that clonally expand.

OP 4.4

Immunological properties of testicular tissue as an anatomical reservoir in ART-treated HIV-infected adults

J.P. Routy[2,6],M.A. Jenabian[1], J. Brousseau[1], K. Vyboh[2], F.M. Ghazawi[1,3], P. Brassard[4], M. Bélanger[4], N. Chomont[5], P. Ancuta[5]

[1]Department of Biological Sciences and BioMed Research Centre, Université du Québec à Montréal (UQAM), Canada, [2]Chronic Viral Illness Service and Research Institute, McGill University Health Centre, Canada, [3]Faculty of Medicine, McGill University; [4]Metropolitan Centre for Plastic Surgery, Montréal, Canada, [5]Université de Montréal, Faculté de Médecine, Département of microbiologie, infectiologie et immunologie and Centre de recherche du CHUM, Montreal, Canada, [6]Division of Hematology, McGill University Health Centre, Canada

Background: As human testis is an immune privileged site that represents an anatomical viral reservoir, we assessed its immunological properties in HIV-infected adults on suppressive ART.

Methods: Testicular tissue and blood were collected from virally suppressed adults (n=6) on ART for at least 6 months prior to gender reassignment surgery and controls (n=10). T-cells were purified by CD3 microbeads from freshly isolated testicular interstitial cell suspensions. T-cell subsets, immune activation cell markers and mRNA expression were assessed by cytometry and RT-PCR.

Results: Lower CD4 T-cell proportion among total T-cells was found in testis *vs.* blood in both VIH+ and HIV- groups. A decrease in naive and an increase in effector-memory (EM) T-cell subsets were observed in testis *vs.* PBMCs of both groups. Importantly, up to 77-fold increases in CCR5 expression were observed on testicular T-cells when compared to PBMCs. Increased T-cell immune activation (CD38/HLA-DR) in testis was observed only in HIV+ group, while immunoregulatory soluble CD38 was lower in testis. Elevated Th2 and Th17 and lower Th1 cell expression was observed in testis. Global Treg population and FoxP3 expression were similar in testis and blood. Interestingly, higher frequencies of immunosuppressive

CD39+ Tregs were found in testis of both groups *vs.* blood. A massive increase in testicular CD73 expression, notably on memory CD8 T-cells *vs.* blood was also observed in both groups. A remarkable increase in both IDO-1 and IDO-2 immunosuppressive enzymes was observed in testis *vs.* blood.

Conclusion: We observed an increase in EM T-cell frequency, CCR5 expression and immunosuppressive enzymes CD39/CD73 and IDO-1/-2 in testis *vs.* blood regardless of HIV status. ART-treated adults had elevated levels of testicular T-cell immune activation compared to HIV- controls. These findings demonstrate the immune tolerance properties in testicular tissue representing a distinctive anatomical reservoir for HIV persistence.

OP 4.5

Persistence of HIV-infected alveolar macrophages after suppressive ART

F.F. Hong, H. Michael, S. Qin, L.A. Kingsley, D.D. McMahon, M.E. Fitzpatrick, A.M. Morris, J.W. Mellors

University of Pittsburgh, PA, USA

Background: No conclusive evidence is available for long-term persistence of HIV infection in macrophages during suppressive ART. We analyzed highly purified alveolar macrophages (AM) for HIV infection and transcriptional activity *in vivo*.

Methods: Two participants with HIV viremia (plasma HIV RNA >1,000 copies/mL) and seven on suppressive ART (<40 copies/mL) underwent bronchoscopy. Bronchoalveolar lavage (BAL) was separated into cellular and cell-free fractions by centrifugation. AM were purified from BAL cells by plastic adherence. Plasma and peripheral blood cells (PBMC) were collected. HIV RNA in the plasma and BAL supernatant, as well as cell-associated HIV DNA and RNA (CAD, CAR) in AM, total BAL cells and PBMCs were assayed by qPCR targeting *pol*. To detect contaminating or ingested CD4+ T cells, T-cell receptor (TCR) RNA was tested by PCR.

Results: Most participants (89%) were male. Median age was 50 yrs and CD4 count was 735. Donors on ART were suppressed for median of 3.6 yrs (range 2.4–12.3). In viremic participants, CAD and CAR were detected in AM (median 1.5 and 2.5 $\log_{10}$ copies/10^6 cells) and in PBMC (2.4 and 1.5). HIV RNA in BAL supernatant was low compared to plasma (median 0.9 and 4.4 $\log_{10}$ copies/mL). CAD was detected in AM from 6 of 7 participants on ART (median 1.7 $\log_{10}$ copies/10^6 cells) and CAR from 4 of 6 with detectable CAD (1.3). AM extracts were negative for TCR in 5 of 6 participants with samples available. By comparison, CAD was detected in PBMC from 6 of 6 individuals tested (median 2.5 $\log_{10}$ copies/10^6 cells) and CAR in 2 of 6 (1.9). HIV RNA in BAL supernatant was not detectable (<0.3 copies/mL) despite low-level plasma HIV RNA in 6 of 7 suppressed participants (median 7.7 copies/mL).

Conclusions: These results establish the persistence of HIV-infected alveolar macrophages in individuals on suppressive ART, which cannot be explained by contaminating or ingested CD4+T-cells. Studies are ongoing to characterize the proviruses in alveolar macrophages and their inducibility.

Session 5: Immunology of HIV persistence

OP 5.0

Immune control of HIV reservoirs and cure therapeutic strategies

B. Autran

Pitié Salpêtrière Hospital, Paris, France

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OP 5.1

Long-term spontaneous control of HIV-1 relates to low frequency of infected cells and inefficient viral reactivation

J.G. Prado[5]*, N. Noel[1–4], R. Peña[5], A. David[6], V. Avettand-Fenoel[7,8], I. Erkizia[5], E. Jimenez[5], C. Lecuroux[1,3], C. Rouzioux[7,8], F. Boufassa[9], G. Pancino[10], A. Venet[1], C. Van Lint[11], J. Martinez-Picado[5,12,13], O. Lambotte[1–4], A. Sáez-Cirión[6]* and for the ANRS CO21 Cohort

[1]Université Paris Sud , UMR-1184, France, [2]CEA, DSV/iMETI, Division of Immuno-Virology, IDMIT, France, [3]Inserm, U1184, Center for immunology of viral infections and autoimmune diseases, France, [4]APHP, Service de Médecine Interne & Immunologie Clinique, Hôpitaux Universitaires Paris Sud, le Kremlin Bicêtre, France, [5]AIDS Research Institute -IrsiCaixa-, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autônoma de Barcelona, Badalona, Spain,, [6]Institut Pasteur, Unité HIV Inflammation et Persistance, Paris, France, [7]APHP, Hôpital Necker, Service de Virologie, Paris, France, [8]Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, EA7327, Paris, France, [9]INSERM U1018, Faculté de Médecine Paris Sud, Le Kremlin-Bicêtre, France, [10]Institut Pasteur, Unité de Régulation des Infections Rétrovirales, Paris, France, [11]Service de Virologie Moléculaire, IBMM, Université Libre de Bruxelles (ULB), Gosselies, Belgique, [12]Universitat de Vic, Barcelona, Spain, [13]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain *equal contributors

Background: HIV-1 establishes reservoirs of infected cells that persist despite effective antiretroviral therapy (ART). A low frequency of HIV-1 infected cells has been associated with delayed viral rebound after ART interruption. Individuals who control HIV-1, the HIV-1 controllers (HICs) carry particularly low levels of infected cells. Here, we evaluate the role of the low number of HIV-1- infected cells in HICs to long-term spontaneous viral control.

Methods: We analysed 38 HICs and 12 patients on ART. We measured HIV-1 reactivation from resting CD4+ in the presence of: PHA/IL-2, anti-CD2/CD28, IL-7, prostatin, HMBA, 5-AzadC, chaetocin and SAHA and quantified HIV-1 RNA in the supernatants. We evaluated HIV-1 production in co-cultures of CD4+ with PBMC from seronegative donors and monitored viral outgrowth by p24 ELISA. We tested viral spread in those supernatants with p24>10 pg/mL. HIV-1 proviral DNA was quantified in resting CD4+ and total PBMCs. We evaluated CD8+ function in HICs by measuring CD8+ suppressive capacity against HIV-1 and IFN-γ production in response to optimal HIV-1 peptides.

Results: We found a lower number of HIV-1 reactivating conditions in HICs (4 HICs *vs.* 10 ARTs; P=0.01) that directly correlated with the proviral DNA levels in the resting CD4+ subset (Spearman 0.88; P<0.0001). Our data revealed a diminished HIV-1 production (9 HIC *vs.* 35 pg/mL ART, P=0.041) and lower frequency of spreading infections (3% HIC *vs.* 21.4% ART; P=0.045) in HICs. Viral production from CD4+ directly correlated to proviral DNA levels in PBMCs. Potent CD8+ responses with higher suppressive capacity and IFN-γ production were present in HICs whose CD4+ T cells produced virus.

Conclusions: Long-term spontaneous control of HIV-1 in HICs is sustained on the inefficient reactivation of viruses from a limited number of infected cells and the capacity of HICs to activate potent HIV-specific CD8+ responses to counteract viral reactivation events.

OP 5.2

Differential effects of HIV latency-reversing agents on T cell phenotype and function: implications for HIV cure

G. Clutton, N. Archin, Y. Xu, D.M. Margolis, N. Goonetilleke

University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Background: A number of drugs have been demonstrated to induce HIV RNA production from latently-infected cells. It is hoped that one

or more of these latency-reversing agents (LRAs) will be combined with a vaccine capable of eliciting cytotoxic T cells to clear reactivated cells. Therefore, we studied the effects of these drugs on T cells.

Methods: We examined the *in vitro* impact of clinically-relevant exposures to putative LRAs on T cell activation and function. Histone deacetylase inhibitors (HDACi) vorinostat (VOR), panobinostat (PAN) and romidepsin (ROMI) were compared with protein kinase C modulators ingenol 3,20 dibenzoate (ING), prostratin (PRO) and bryostatin-1 (BRYO).

Results: No effect of VOR was observed on T cell activation, whereas PAN and ROMI consistently increased expression of CD69 on CD4 and CD8 T cells. PKC modulators all induced increased expression of CD69 and MHC I on CD4 and CD8 T cells, which for CD69 was sustained over 72 hours. VOR and ROMI did not impact *ex vivo* antigen-specific CD8 T cell responses as measured by degranulation and cytokine production six hours after stimulation. PAN, however, produced a small but significant decrease in the frequency of antigen-specific CD8 T cell responses. PKC modulators all induced non-specific cytokine production, an effect that was more marked in HIV seropositive than in seronegative individuals, but did not significantly affect antigen-specific responses. Interestingly, ROM, PRO and BRYO inhibited antigen-dependent proliferation over a 5 day *in vitro* culture in both seropositive and seronegative participants. PRO and BRYO were toxic in these short term culture assays.

Conclusions: Even within the same class, LRAs vary in their effect on the phenotypic profile and functional capacity of T cells. Moreover, effects on T cell function may not be detectable until several days after dosing. These differences should be considered for dosing, designing and testing of HIV latency clearance strategies.

OP 5.3

CD8+ sensing relies on nanomolar levels of antigen presented upon HIV-1 reactivation

A. Ruiz[1], E. Jimenez[1], R. Peña[1], P. Goulder[2], B. Clotet[1], J.G. Prado[1]

[1]AIDS Research Institute IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain, [2]Department of Pediatrics, University of Oxford, UK

Background: Latency-reversing agents (LRAs) have being developed as strategy to reactivate silent provirus and eliminate HIV-1 reservoirs. However, the lack of cytopathic effects associated with LRA treatment precludes the elimination of reactivated cells and immune recognition is needed. The level of viral protein expression induced by LRAs upon reactivation for CD8 T-cell sensing is unknown, but it is key for recognition and killing of reactivated cells. Here, we aimed to define the level of HIV-1 reactivation needed for CD8 T-cell recognition.

Methods: We generated a resting-like latency model by infecting U937 cells with HIV-1GFP and culturing four days the GFP-negative cells with PIs. Resting-like cells were reactivated for 48 hours with PMA+ionomycin (PMA/IO) at different doses, panobinostat (PNB) at 30 nM or PNB+bryostatin at 10 nM. HIV-1 specific CD8 T-cells were added 20 hours after reactivation. We measured HIV-1 reactivation by intracellular p24 and CD8 T-cell activity by MIP1β/CD107a and IFN-γ staining. For comparison, we quantified CD8 T-cell activity in the presence of U937 cells pulsed with dilutions of cognate peptide.

Results: Our model produced a 2-fold with PMA/IO, a 1,3-fold with PNB and a 1,6-fold HIV-1 induction with PNB+bryostatin, compared to controls. Associated toxicity was low and did not affect CD8+ T-cells. Despite low levels of HIV-1 induction, we measured a specific increased in CD8+ T-cell activity after reactivation ranging from 4 to 7,2 % in MIP1β/CD107a expression and 2,6% to 7,4% in IFN-γ expression. By extrapolation of the data into a peptide-pulse standard curve, the threshold for CD8 T-cell activation in response to HIV-1 reactivation was 10^3 nM.

Conclusions: CD8 T-cell sensing is limited by a nanomolar threshold of antigen presented in the surface of reactivated cells. Our model could be a useful tool for screening of novel LRAs able to reactivate latently HIV-1 infected cells and to induce CD8+ T-cell recognition.

OP 5.4

Long-lived Th17 subsets contribute to HIV-1 persistence under ART

P. Ancuta[1,2]*, V.S. Wacleche[1,2], J.P. Goulet[3], A. Gosselin[2], P. Monteiro[1,2], H. Soudeyns[1,4], M.A. Jenabian[5], S.G. Deeks[6], N. Chomont[1,2], J.P. Routy[7,8]

[1]Université de Montréal, Faculté de Médecine, Département of microbiologie, infectiologie et immunologie, Montreal, QC, Canada, [2]Centre de recherche du CHUM, Montreal, QC, Canada, [3]Caprion, Montreal, QC, Canada, [4]Unité d'immunopathologie virale, Centre de recherche du CHU Sainte-Justine, Montreal, QC, Canada, [5]Département des sciences biologiques, Université du Québec, Montreal, QC, Canada, [6]Department of Medicine, University of California San Francisco, San Francisco, CA, USA, [7]Chronic Viral Illness Service and Research Institute, McGill University Health Centre, Montreal, QC, Canada, [8]Division of Hematology, McGill University Health Centre, Montreal, QC, Canada

The current paradigm is that Th17 cells are permissive to HIV-1 infection and subsequently depleted. Indeed, subsets of memory CD4+ T-cells with CCR6+CCR4+ (Th17 enriched) and CCR6+CXCR3+ (Th1Th17 enriched) phenotypes are HIV permissive and their frequency is not restored by ART. Whether fractions of Th17 cells are long-lived in humans and contribute to viral persistence under ART remains unknown. Here, we performed an in-depth transcriptional and functional characterization of two novel CCR6+ subsets, lacking (double negative, CCR6+DN) or co-expressing CXCR3 and CCR4 (double positive, CCR6+DP) and investigated their contribution to HIV persistence in ART-treated subjects. CCR6+DN and CCR6+DP shared with CXCR3+CCR6+/Th1Th17 and CCR4+CCR6+/Th17 multiple Th17-polarization markers and *C. albicans* antigenic specificity. All four subsets exhibited lineage commitment and plasticity when cultured under Th17 and Th1 conditions, respectively. In contrast to CCR6+DP and CXCR3+CCR6+/Th1Th17, fractions of CCR6+DN and CCR4+CCR6+/Th17 maintained their Th17-effector functions under Th1-polarization conditions, suggesting their stable Th17-lineage commitment. Despite these similarities, CCR6+DN expressed a unique transcriptional signature indicative of early Th17 development, lymph-node homing, follicular help, and self-renewal. Although all four CCR6+ subsets carried replication-competent integrated HIV-DNA, CCR6+DN distinguished from the other three subsets by preserved frequency/counts in ART-treated HIV-infected subjects versus uninfected controls. Longitudinal studies demonstrated that CCR6+DN cells were the most predominant CCR6+ subset in the blood before and after ART initiation. Finally, CCR6+DN were enriched within the memory CCR6+ fraction in lymph nodes versus blood. Together these results demonstrate the existence of at least four distinct stages of Th17 differentiation in humans and support the contribution of long-lived CCR6+DN cells to HIV-DNA persistence under ART.

OP 5.5

Uncovering mechanisms of HIV persistence in HIV controllers by HIV sequence analysis in CD4 T cell subsets

E. Boritz, S. Darko, F. Simonetti, D. Wells, X. Wu, L. Swaszek, G. Wolf, R. Hoh, A. Vostal, A. Ober, M. Hughes, D. Bunis, S. Migueles, J. Casazza, R. Koup, M. Connors, S. Moir, J. Martin, F. Maldarelli, S. Hughes, S. Deeks, D. Douek

Vaccine Research Center, NIAID, Bethesda, MD, USA

A small number of HIV-infected adults control HIV without antiretroviral therapy (ART). Characterizing how HIV persists in these individuals may elucidate barriers to HIV cure. We studied 14 HIV controllers (defined by plasma HIV-1 RNA<10^3 copies/mL off ART) as well as 6 non-controllers with higher virus loads. CD4 T cell subsets were sorted by FACS. Clonal HIV env sequence fragments were derived from cellular DNA or virion cDNA and used for phylogenetic analysis. T cell receptor beta chain genes and HIV integration sites were determined by deep sequencing. We found that whereas the peripheral blood CD4 T cell pool in non-controllers harbored

diverse HIV strains, blood CD4 T cells in HIV controllers harbored large clusters of identical sequences that were distinct from plasma viruses, suggesting clonal expansion of HIV-infected cells. Diversity of both HIV DNA sequences and T cell receptor beta chain genes in controllers was lower in effector memory (EM) than in central memory (CM) or transitional memory (TM) cells, suggesting an accumulation of clonally expanded HIV proviruses in the EM population. Integration site sequencing confirmed clonal expansion of HIV-infected cells in controllers. Some cells from some expanded, HIV-infected CD4 T cell clones in HIV controllers produced virions when stimulated *in vitro*. Unlike CD4 T cells in blood, lymph node (LN) CD4 T cells in HIV controllers harbored diverse virus strains closely related to plasma viruses. Among LN CD4 T cell subsets, T follicular helper cells most often harbored HIV DNA, but other subsets were also infected. We conclude that the predominant HIV reservoir in blood from HIV controllers is an oligoclonal population of expanded T cells, some of which may produce virus upon restimulation. By contrast, the lymphoid tissue in HIV controllers harbors diverse HIV strains that appear to be actively replicating. Both HIV populations may be barriers to cure, even in persons with potent antiviral defenses.

OP 5.6

The transcriptional program governed by RORγt favors HIV-1 replication in CCR4+CCR6+ Th17 cells

V. S. Wacleche[1,2], A. Cleret-Buhot[1,2], Y. Zhang[1,2], D. Planas[1,2], J.P. Goulet[3], P. Monteiro[1,2], J. Niessl[1,2], A. Gosselin[2], C. Tremblay[1,2], M.A. Jenabian[4], J.P. Routy[5,6,7], M. El-Far[2], N. Chomont[1,2], E.K. Haddad[8], R.P. Sekaly[9], P. Ancuta[1,2]

[1]Department of Microbiology, Infectiology and Immunology, Faculty of Medicine, Université de Montréal, QC, Canada, [2]CHUM-Research Centre, Montréal, QC, Canada, [3]Caprion, Montréal, QC, Canada, [4]Département des sciences biologiques, Université du Québec, Montreal, QC, Canada, [5]Chronic Viral Illness Service, McGill University Health Centre, Montreal, QC, Canada, [6]Research Institute, McGill University Health Centre, Montreal, QC, Canada, [7]Division of Hematology, McGill University Health Centre, Montreal, QC, Canada, [8]Drexel University, Division of infectious Diseases and HIV Medicine, Philadelphia, PA, USA, [9]Case Western Reserve University, Center for AIDS Research, Cleveland, OH, USA

HIV-1 infection is characterized by profound CD4+ T-cell destruction, with a marked Th17 dysfunction at mucosal level that is not restored in chronically infected subjects receiving antiretroviral therapy. In an effort to orient Th17-targeted reconstitution strategies, herein we investigated molecular mechanisms of HIV permissiveness in Th17 cells. Genome-wide transcriptional profiling in memory CD4+ T-cell subsets enriched in cells exhibiting Th17 (CCR4+CCR6+), Th1 (CXCR3+CCR6-), Th2 (CCR4+CCR6-), and Th1Th17 (CXCR3+CCR6+) features revealed the most remarkable transcriptional differences between Th17 and Th1 subsets. Accordingly, HIV-DNA integration was superior in Th17 versus Th1 upon exposure to both wild-type and VSV-G-pseudotyped HIV, indicative that post-entry mechanisms contribute to enhanced viral replication. Unique pathways enriched in Th17 versus Th1 included TCR signaling, T-helper differentiation, nuclear receptor transcription, and circadian repression of expression by REV-ERBα. Transcripts significantly enriched in Th17 versus Th1 were previously associated with the regulation of TCR signaling (ZAP-70, Lck, CD96), Th17 polarization (RORγt, ARNTL, PTPN13, RUNX1), and HIV replication (PPARG, PAK2, KLF2, ITGB7, PTEN, ATG16L1, Alix/AIP1/PDCD6IP, LGALS3, JAK1, TRIM8, MALT1, FOXO3, ARNTL/BMAL1, ABCB1/MDR1, TNFSF13B/BAFF, CDKN1B). Functional studies demonstrated increased Lck and ZAP-70 phosphorylation, proliferation potential, and NF-κB nuclear translocation and DNA-binding activity in Th17 versus Th1. Finally, RNA interference studies identified the Th17-specific transcription factor RORγt as a novel HIV dependency factor. Thus, the transcriptional program governed by RORγt includes molecules that regulate HIV replication at multiple post-entry steps, molecules that may represent potential targets for novel therapies aimed at protecting Th17 cells from infection and subsequent depletion.

OP 5.7

Latency-reversing agents and cellular activation affect antigen processing in primary CD4 T cells

J. Boucau, J. Madouasse, D. Wambua, M.J. Berberich, S. Le Gall

Ragon Institute of MGH, MIT and Harvard, Cambridge, MA, USA

Background: Strategies to purge HIV reservoirs commonly rely on reactivation of HIV provirus by latency-reversing agents (LRA) followed by death or immune clearance of reactivated CD4 T cells. LRAs such as HDAC inhibitors or PKC agonists showed variable efficacy *in vitro* and limited efficacy in clinical trials. While productive infection mostly occurs in activated CD4 T cells, reactivation occurs in memory resting cells receiving LRA and ART. How these parameters shape HIV antigen processing and presentation by CD4 T cells is unknown despite its critical role in immune recognition after reactivation.

Methods: We measured hydrolytic activities of cellular proteases involved in antigen processing in live primary CD4 T cells that were either resting, CD3/28-activated, LRA-treated, or in conditions used in the Lewin or Planelles latency models. To assess how changes in activities affect antigen processing we compared the degradation of HIV peptides in extracts of primary CD4 T cells treated as stated above. The degradation products were quantified by mass spectrometry and their antigenicity measured by killing assay with HIV-specific CTL.

Results: The peptidase activities of HDACi-treated primary CD4 T cells decreased by 20–60% compared to resting cells while PKCa-treated cells displayed enhanced activities comparable to activated cells. Memory-like CD4 T cells of the Lewin latency model showed activities level similar to resting CD4 T cells. CD4 T cells of the Planelles model displayed activities similar to activated cells. *In vitro* degradation of HIV epitope-containing peptides showed that resting primary CD4 T cells produced longer, more antigenic peptides and unique cleavage sites compared to activated cells, and that some LRAs caused discrete changes in degradation patterns.

Conclusions: These variations in peptidase activities and degradation patterns suggest that LRA-reactivated and productively infected CD4 T cells might process antigen differently, thus requiring different CTL responses for effective clearance.

Session 6: Pharmacology of HIV persistence

OP 6.0

A pharmacologic basis for HIV persistence

C. Fletcher

University of Nebraska Medical Center, Omaha, NE, USA

Abstract not available at the time of printing

OP 6.1

A subset of infectious proviruses persist and expand following activation *ex vivo*

J.K. Bui[1,2], E. Halvas[1], E. Fyne[1], M.D. Sobolewski[1], D. Koontz[1], M.F. Kearney[3], W. Shao[4], F.F. Hong[1], J.W. Mellors[1]

[1]University of Pittsburgh, Department of Medicine, Pittsburgh, PA, USA, [2]Howard Hughes Medical Institute, Medical Research Fellows Program, Bethesda, MD, USA, [3]National Cancer Institute (NCI), HIV Dynamics and Replication Program, Frederick, MD, USA, [4]Leidos, Advanced Biomedical Computing Center, Frederick, MD, USA

Background: The most effective latency-reversing agents for HIV-1 are also potent T-cell activators. Recent studies show that virus-producing cells can persist and expand *in vivo*. We hypothesized that

activation of HIV-infected CD4+ T-cells could lead to clonal expansion of proviruses rather than their elimination.

Methods: We established an *ex vivo* cell culture system involving stimulation of patient-derived CD4+ T cells with PMA/ionomycin (day 1–7), followed by rest (day 7–21), and then restimulation (day 21–28) in the presence of raltegravir and efavirenz. Cell-associated HIV-1 DNA (CAD) and virion RNA in the supernatant were quantified by qPCR. Single-genome sequencing (SGS) was performed to characterize proviruses and virion RNA. The replication-competence of proviruses in cultured cells was determined by the viral outgrowth assay (VOA) at multiple time points.

Results: Experiments were performed with purified CD4+ T-cells from five consecutive donors who had been suppressed on ART for ≥ 2 years (median = 13.4 years). In all experiments, HIV-1 RNA levels in supernatant increased following initial stimulation, decreased or remained stable during the rest period, and increased again with restimulation. Cell-associated HIV-1 DNA levels did not show a consistent pattern of change. SGS revealed outcomes of proviral populations including both elimination and expansion. Importantly, a subset of proviruses expanded and produced infectious virus continuously.

Conclusions: Reversal of HIV-1 latency by CD4+ T cell activation results in diverse outcomes for proviral populations, ranging from their apparent elimination to expansion of proviruses capable of infectious virus production. These findings underscore the complexity of eliminating HIV reservoirs with latency-reversal agents and highlight the need for new strategies to kill HIV-infected cells before they can clonally expand.

OP 6.2

Lymphoid tissue and blood CD4 T cells respond differently to latency-reversing agents: are we testing the right cells?

A. Gramatica, W.C. Greene, M. Montano

Gladstone Institute of Virology and Immunology and the University of California, San Francisco, CA, USA

Background: HIV latency is established early during acute infection and is primarily found within memory CD4 T cells residing in lymphoid tissue. However, due to their ease of acquisition, blood CD4 T cells are almost exclusively studied in latency-reversing experiments. Here, we compared CD4 T cells from blood and lymphoid tissue for their responses to various latency-reversing agents (LRAs).

Methods: A primary CD4 T-cell model of latency involving spinoculation of an infectious clone of NL4-3 expressing firefly luciferase from the native LTR was used to examine the responses of latently infected blood and tonsil cells to various LRAs. Luciferase activity was quantitated to assess reactivation of latent provirus.

Results: In our preliminary results, latently infected cells from lymphoid tissue displayed a more robust response than blood to several LRAs. CD4 T cells from lymphoid tissue had a slightly higher state of cellular activation as evidenced by increased expression of HLA-DR and CD69. In contrast, levels of CD25 were not higher. Tonsil cells displayed far greater responses to single agents, (e.g. ingenol B, JQ1 and HDACi). These responses were often observed within 24 hours of stimulation and often exceeded the response elicited by anti-CD3/CD28 antibodies. Synergistic activation was frequently observed when ingenol B was combined with HDACi or JQ1. In contrast, blood CD4 T cells responded only weakly to single agents. While combinations of ingenol B and HDACi or JQ1 modestly synergized, the responses were consistently weaker than the response elicited by anti-CD3/CD28 antibodies.

Conclusions: Lymphoid tissue CD4 T cells responded more strongly to a variety of single LRAs and displayed marked synergy when combinations of agents were used. These findings suggest that latency reversal may be more easily achieved in lymphoid tissues than in blood. Since the reservoir principally resides in lymphoid tissue, increased testing of such tissues should be encouraged.

OP 6.3

Role of drug transporters and metabolic enzymes in antiretroviral drug (ARV) disposition in testicular tissue: potential contribution to HIV-1 persistence

R. Bendayan, Y. Huang, S.K. Whyte, T. Hoque, M.A. Jenabian, K. Vyboh, N. Sheehan, P. Brassard, M. Belanger, N. Chomont, C.V. Fletcher, J.P. Routy

University of Toronto, Leslie Dan Faculty of Pharmacy, Toronto, USA

Background: Human immunodeficiency virus (HIV-1) is capable of persistent infection in the testis, where inadequate ARV penetration could be due in part to the functional expression of drug efflux transporters at the blood–testis barrier (BTB). This study aims to characterize expression and localization of drug transporters and metabolic enzymes in human testes and quantify ARV concentrations in HIV-infected testicular tissue. Since nuclear receptors can induce functional expression of drug transporters and metabolic enzymes in various tissues, we investigated the regulation of efflux transporters by these receptors.

Methods: mRNA and protein expression of the ATP-binding cassette (ABC) and solute carrier (SLC) transporters, phase I metabolic enzymes, and nuclear receptors were evaluated in testicular tissue obtained from uninfected (n=8) and HIV-1 infected (n=5) subjects on ARV therapy. Localization of transporters and metabolic enzymes was determined by confocal microscopy. ARV concentrations in testicular tissue or plasma were quantified by LCMS/MS. Nuclear receptor expression and their effect on efflux transporters following activation by ligands were investigated in TM4 Sertoli cells representing mice BTB.

Results: Expression and localization of ABC (P-gp, BCRP, MRPs) and SLC (OATP, OAT, OCT, CNT, ENT) transporters, as well as CYP3A4 and CYP2D6 were confirmed in human testes. HIV-protease inhibitors that are known substrates for ABC transporters displayed low testicular tissue penetration. Nuclear receptors, PXR, CAR, PPARs, were also expressed in human testicular tissue and TM4 Sertoli cells and mediated induction of P-gp and BCRP.

Conclusion: Our findings suggest that drug transporters, metabolic enzymes and nuclear receptors involved in ARV disposition are present in the testes, and could limit ARV tissue penetration; this could potentially contribute to persistent HIV-1 infection and formation of a viral sanctuary site. (Supported by CIHR).

Session 7: Drug discovery

OP 7.0

Abstract withdrawn

OP 7.1

Approaches to discover latency-reversing agents

D. Hazuda

Merck, Kenilworth, NJ, USA

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OP 7.2

Gene editing CCR5 in HIV subjects CD4 T cells

D. Ando

CMO Sangamo BioSciences, Richmond, VA, USA

Background: CCR5 modified autologous CD4 cells (SB-728-T) are safe and increase total CD4 counts. The cells traffic to lymphoid tissues and have a selective survival advantage during ART treatment interruption (TI). Additional studies in CCR5 Δ32 heterozygote HIV subjects showed VL reductions during TI correlated with circulating bi-allelic CCR5-modified CD4 cells supporting the importance of maximizing engraftment. Studies in immunologic non-responders show an increase of CD4 counts to >500 cells/μL and a 0.9 log decrease in proviral DNA measured in PBMC.

Low dose CTX has been successfully used to increase T cell engraftment. This study examines the effect of escalating doses of CTX on SB-728-T engraftment.

Methods: A dose escalation study of IV CTX, with doses ranging from 100 mg/m^2 to 2 g/m^2 (n=3–6/cohort), administered 1–3 days prior to SB-728-T (>90% CD4, <1% CD8) infusion was performed in 18 aviremic, ART treated HIV subjects with CD4 T cells ≥500/μL.

Results: CTX was generally well tolerated with low grade GI side-effects, managed with anti-emetics at doses up to 1 g/m^2. Grade 3 and 4 neutropenia requiring G-CSF developed at 1.5 and 2.0 g/m^2 CTX. On day 7, a dose-related increase in CD4 count and engraftment of bi-allelic CCR5 modified cells was observed with CTX doses up to 1 g/m^2 but did not increase at 2.0 and 1.5 g/m^2. By comparison, there was a progressive decline in CD8 cells with CTX dose escalation. Data is expressed as mean±SE.

A 1-log VL reduction from peak was seen in one subject each at 100 and 500 mg/m^2 of CTX while one subject each at the 1 and 1.5 g/m^2 dose level had a 2-log decline during a TI. At the conclusion of the study, three additional subjects were conditioned with 1 g/m^2 of CTX and administered CCR5 modified T cells containing 46.9±6.4% CD8 cells. CD8 count increased by 2236±967/μL (range 1029–4150/μL) with only modest increases in CD4 counts (733±233/μL; range 297–1096/μL) at 7 days in the three subjects. Two of the three subjects have had VL decreases to date during TI (<1000 copies/mL), suggesting an effect on viral control with the added CD8 T cells.

Conclusion: CTX conditioning is generally well tolerated and was associated with increased engraftment of CCR5-modified T cells at doses up to 1 g/m^2 in HIV subjects. CTX conditioning may be a useful strategy to maximize the engraftment and anti-viral effects of SB-728-T. The effects of co-administering CD8 cells with SB-728-T on VL will be presented.

OP 7.3

Cyanotriazoles activate latent HIV and strongly synergize with proteasome inhibitors *ex vivo* in resting CD4 T cells from suppressed HIV+ donors

J. Murry[1], A. Tsai[1], M. Graupe[1], G. Jones[1], M. Tsiang[1], A. Arvey[1], L. Li[1], G. Stepan[1], H. Yu[1], T. Cihlar[1], D. Sloan[1], J. Lalezari[2]

[1]Gilead Sciences, Foster City, USA, [2]Quest Clinical Research, San Francisco, CA, USA

Background: Current HIV therapies do not cure HIV because latent reservoirs persistent within infected CD4+ T cells. The induction of viral proteins in latently infected cells could facilitate their recognition and elimination by immune cells.

Methods: High throughput screening was used to identify novel compounds that induce viral expression in primary CD4 T cells infected *in vitro* with an HIV reporter virus. Active compounds were tested *ex vivo* for the induction of cell-associated HIV RNA transcripts and supernatant virions in resting CD4 T cells isolated from cART-suppressed HIV infected donors.

Results: We identified cyanotriazole-containing compounds that activate latent HIV. The most active hit, GS-46, induced 3.1-fold activation of virion production *ex vivo*, similar to the 3.6-fold activation induced by romidepsin (geometric mean, n=10). However, unlike HDAC inhibitors, GS-46 increased the production of polyadenylated HIV transcripts without affecting readthrough transcripts. The structure-activity relationship indicates that the cyanotriazole is required for activity, suggesting potential covalent modification of the target(s). Pairwise LRA combinations were tested and the most synergistic was the combination of cyanotriazoles with proteasome inhibitors (PIs), such as bortezomib. While a 24 hour pulse of 15 nM bortezomib alone induced 1.4-fold activation of HIV *ex vivo*, 40-fold activation was observed in combination with 3 uM GS-46 (geometric means, n=10). By comparison, PMA and ionomycin induced 63-fold induction. However, unlike mitogenic activation, the combination of GS-46 with bortezomib had minimal effects on cellular proliferation, T cell activation marker expression, or cytokine production.

Conclusions: Cyanotriazole-containing small molecules have been identified as a new class of LRAs. Cyanotriazoles strongly synergize with PIs to induce HIV latency reversal at levels similar to those seen with mitogenic stimulus, but without T cell activation.

OP 7.4

Triazol-1-ol analogues as novel therapeutic leads towards reactivating and eradicating latent HIV-1 by manipulating SUMOylation of STAT5

A. Bosque[1], A. Macedo[1], A. Spivak[2], C.L. Novis[1], L.J. Martins[1], M. Szaniawski[1], N.M. Archin[3], P.A. Luciw[4], D.M. Margolis[3], V. Planelles[1]

[1]Department of Pathology, University of Utah, Salt Lake City, UT 84132, USA, [2]Department of Medicine, University of Utah, Salt Lake City, UT 84132, USA, [3]Division of Infectious Diseases, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA, [4]Department of Pathology and Laboratory Medicine, School of Medicine, University of California at Davis, Davis, CA 95616, USA

Background: Reactivation of latent HIV *in vivo* for the purpose of purging the viral reservoir represents a formidable challenge. One major problem is to identify stimuli that will effectively reactivate latent proviruses without causing overt T-cell activation or proliferation. We have performed a medium-throughput screen of chemical libraries and have identified a family of compounds (triazol-1-ol analogues) that trigger viral reactivation.

Methods: We have characterized the ability of several triazol-1-ol analogues to i) reactivate latent virus; ii) reduce the pool of latently infected cells *in vitro*; iii) hamper the establishment of latency; iv) induce cell proliferation, activation and cytokine release in resting CD4 T cells; and finally v) we have characterized the mechanism of action of triazol-1-ol analogues involved in viral reactivation. To that end, we have used a primary cell model of HIV-1 latency that recapitulates the generation of latently infected cultured T_{CM} and uses a replication competent virus and ART.

Results: We have found that triazol-1-ol analogues reactivate latent HIV-1 in cultured T_{CM} in a dose dependent manner and in the absence of T cell proliferation, activation or cytokine release. Importantly, viral reactivation is followed by cell death of the reactivated cells and decreased in the pool of latently infected cells *in vitro*. Furthermore, these compounds also have the ability to hamper the establishment of latency in cultured T_{CM}. Analysis of the mechanism of action indicates that activation of latent HIV by triazol-1-ol analogues involves inhibition of SUMOylation of STAT5, a mechanism involved in downregulation of STAT5 activity.

Conclusions: These results demonstrate that triazol-1-ol analogues can be utilized to trigger activation of latent proviruses in primary cells. Key signaling elements controlling this pathway should be considered as novel therapeutic targets toward eradication.

Session 8: Practical issues in designing HIV cure trials

OP 8.0

Challenges in designing clinical trials in cure research

J. Eron

University of North Carolina, Chapel Hill, NC, USA

Abstract not available at the time of printing

OP 8.1

Real-time predictions of reservoir size and rebound time during antiretroviral therapy interruption trials for HIV

A.L. Hill[1], D.I.S. Rosenbloom[2], D.R. Kuritzkes[3], R.F. Siliciano[4], T.J. Henrich[5]

[1]Program for Evolutionary Dynamics, Harvard University, Cambridge, MA, USA, [2]Department of Biomedical Informatics, Columbia University Medical Center, New York, NY, USA, [3]Division of Infectious Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA, [4]Department of Medicine, Johns Hopkins University School of Medicine and Howard Hughes Medical Institute, Baltimore, MD, USA, [5]Division of Experimental Medicine, Department of Medicine, University of California, San Francisco, CA, USA

Background: Monitoring the efficacy of novel reservoir-reducing treatments for HIV is challenging. The limited ability to sample and quantify latent infection means that supervised antiretroviral therapy (ART) interruption studies are generally required.

Methods: We introduce a set of mathematical and statistical modeling tools to aid in the design and interpretation of ART-interruption trials. These tools combine previously developed models of reservoir dynamics and rebound with patient-derived data in a flexible Bayesian framework.

Results: We show how the likely size of the remaining reservoir can be updated in real-time as patients continue off treatment, by combining the output of laboratory assays with insights from dynamic models. We design an optimal schedule for viral load sampling during interruption, whereby the frequency of follow-up can be decreased as patients continue off ART without rebound. While this scheme can minimize costs when the chance of rebound between visits is low, we find that the reservoir will be almost completely reseeded before rebound is detected unless sampling occurs at least every two weeks and the most sensitive viral load assays are used. We use simulated data to predict the clinical trial size needed to estimate treatment effects in the face of highly variable patient outcomes and imperfect reservoir assays. Our findings suggest that large numbers of patients – between 40 and 150 – will be necessary to reliably estimate the reservoir-reducing potential of a new therapy and to compare this across interventions. As an example, we apply these methods to the two "Boston patients".

Conclusions: These tools can aid researchers in evaluating new potentially-curative strategies that target the latent reservoir.

OP 8.2

The importance of GPP implementation in HIV cure research: learning from prevention

J. Handibode, S. Hannah

AVAC, New York, NY, USA

Background: HIV prevention research has experienced community resistance to and strong community support for conduct of trials to advance the field. Where there is resistance, , delays in research timelines, and early closures have occurred. Recently, there has been an increase in HIV cure research, specifically safety and phase I/II clinical trials. As this research increases, so will the need for stakeholder engagement. The UNAIDS/AVAC Good Participatory Practice guidelines for biomedical HIV prevention trials (GPP) serve as a global reference for stakeholder engagement throughout the trial lifecycle. Adoption of GPP fosters ethical trial conduct and eases the trial process through early and broad stakeholder support. GPP has been implemented in a variety of ways for HIV prevention research. Lessons learned can serve as a framework for engagement in cure research moving forward.

Methods/Results: In South Africa, AVAC has conducted GPP trainings with several major HIV research institutions including Desmond Tutu HIV Foundation, Wits Reproductive Health and HIV Institute, and CAPRISA. In 2012 the FACTS 001 team used various GPP tools and strategies, including monitoring and evaluation tools and the GPP Blueprint, to standardize engagement at all sites. Recently South Africa has begun the process of developing a country-wide framework for stakeholder engagement rooted in GPP. This framework will be developed by community engagement managers at provincial levels who then coordinate with national stakeholders to achieve national adoption.

In Thailand, GPP has been rolled out with leading research organizations and is a key focus of the National Biomedical HIV Prevention Subcommittee of the Ministry of Public Health. Thailand has also formed a National CAB to provide civil society an independent platform to weigh in on the conduct of clinical trials in the country.

AVAC has organized a community of practice to gather and analyze best practices related to stakeholder engagement. These models and practices of GPP implementation may be used, replicated, or adapted for cure research.

Conclusion: Uptake and implementation of GPP within current and future HIV cure trials and programs will help site and trial leadership better establish and adjust community engagement goals that strategically align and complement the scientific research agenda.

OP 8.3

Emerging results of an extensive survey of potential participants' willingness to take risks in and donate to HIV cure research in the United States

J. Taylor[1], K. Dubé[1,4], D. Evans[2], L. Sylla[3], A. Burton[1], A. Skinner[4], S. Greene[4]

[1]Collaboratory of AIDS Researchers for Eradication (CARE) Community Advisory Board (CAB), USA, [2]Delaney AIDS Research Enterprise (DARE) CAB, USA, [3]DefeatHIV CAB, USA, [4]Health Policy and Management, University of North Carolina at Chapel Hill, NC, USA

Background: We assessed willingness to take risks and donate blood and tissues in HIV cure-related studies among adults living with HIV in the United States. We focused on attitudes toward perceived personal and societal risks and benefits of participation.

Methods: An extensive, online cross-sectional survey was conducted among 302 American adults (76.4% males; 22.2% females; 0.7% male-to-female transgenders) aged ≥ 18 years between September and October 2015. The sample was ethnically diverse (64.8% Caucasian, 16.6% African-American, 12.0% Hispanic, 1.3% Asian, 2.6% mixed) and 38 U.S. states were represented. Data collection and analyses are ongoing.

Results: Almost all respondents – 99.0% (95% CI: 97.9–100.0%) – were currently taking HIV medications and 96.3% (95% CI: 94.2–98.4%) were generally interested in HIV cure research. Eight percent reported participation in at least one clinical HIV cure-related study. Research types that respondents would most likely be willing to participate in were those that included: 1) blood draws (92.9%; 95% CI: 89.9–95.9%); 2) leukaphereses (74.3%; 95% CI: 69.3–79.3%); 3) therapeutic vaccinations (76.9%; 95% CI: 71.9–81.9%); or 4) phase II or III efficacy trials (74.2%; 95% CI: 69.0–79.4%). Studies that respondents would be less willing to participate in were: 1) stem cell transplants (54.0%; 95% CI: 48.1–59.9%) and 2) those involving latency-reversing agents (52.8%; 95% CI: 47.9–58.7%). Risks most likely to deter research participation included possible activation of proto-oncogenes (46.6%; 95% CI: 40.9–52.3%) and risks of developing resistance to ARVs (36.0%; 95% CI: 30.6–41.4%). The societal risk most likely to deter participation was transmitting HIV to others (26.3% 95% CI: 21.3–31.3%). Of the respondents, 26.7% (95% CI: 21.6–31.7%) would be very willing to interrupt treatment and 7.6% (95% CI: 4.6–10.6%) thought that a cure for HIV was presently available.

Conclusions: Data on willingness to participate are hypothetical and should not be used to predict enrollment rates; however, they can help anticipate practical issues affecting research implementation. The above results have implications for recruiting study participants and managing expectations around HIV cure science in the coming years. Given that some respondents thought a cure was currently available, heightened education efforts seem warranted.

Session 9: New therapeutic approaches 1

OP 9.0

Potential role for neutralizing antibodies in HIV-1 infection

J. Mascola

NIAID, Vaccine Research Center, Bethesda, MD, USA

Abstract not available at the time of printing

OP 9.1

Targeting HIV reservoir by DART molecules that recruit T cells to HIV Env expressing cells: comparison of HIV arms derived from broadly reactive neutralizing or non-neutralizing anti-Env antibodies

J.L. Nordstrom[1], D.D. Sloan[2], C.Y. Kao Lam[1], A. Irrinki[2], L. Liu[1], A. Tsai[2], C.S. Pace[2], J. Kaur[2], J.P. Murry[2], M. Balakrishnan[2], P.A. Moore[1], S. Johnson[1], T. Cihlar[2], S. Koenig[1]

[1]MacroGenics, 9640 Medical Center Drive, Rockville, MD, USA, [2]Gilead Sciences, 333 Lakeside Drive, Foster City, CA, USA

Background: HIV reservoirs and production of viral antigens are not eliminated in HIV-infected individuals treated with combination antiretroviral therapy (cART) and novel therapeutic strategies are needed. Dual-Affinity Re-Targeting (DART) molecules exhibit a distinct mechanism of action via binding the viral envelope (Env) antigen on the surface of HIV-infected cells and simultaneously engaging CD3 on cytotoxic T lymphocytes (CTLs).

Methods: HIVxCD3 DART proteins were made in which HIV arms were derived from diverse broadly reactive Env-specific antibodies with (bnAbs) or without (non-nAbs) virus neutralizing activity. They were evaluated for antigen binding by ELISA, cell surface antigen binding by flow cytometry, CD8-dependent killing of HIV- infected Env-expressing target cells, and effect on virus propagation.

Results: HIVxCD3 DART proteins derived from two bnAbs (PGT121, PGT145) and two non-nAbs (A32, 7B2), but not from two other bnAbs (VRC01, 10E8), mediated potent CTL-dependent killing of quiescent primary CD4 T cells infected with diverse HIV isolates. Similar redirected CTL activity was also observed with DART molecules structurally modified for *in vivo* half-life extension. In an *ex vivo* model using cells isolated from HIV-infected individuals on suppressive cART, combinations of the most potent DART molecules reduced HIV expression both in quiescent and activated peripheral blood mononuclear cell cultures. Importantly, DART molecules did not induce cell-to-cell virus spread in resting or activated CD4 T cell cultures and ones with HIV arms derived from bnAbs retained virus neutralizing activity.

Conclusion: HIVxCD3 DART molecules potently and selectively mediate the killing of HIV-infected cells. Furthermore, they perturb resting and activated viral reservoirs in cells isolated from individuals on antiviral therapy. The data provide support for further pursuit of HIVxCD3 DART molecules as a promising therapeutic strategy for targeting HIV reservoirs.

OP 9.2

CL572, a potent agonist of Toll-like receptor 2/7, as a novel latency-reversing agent

A. Macedo[1], C.M. Assis[1], C.L. Novis[1], A. Spivak[2], V. Planelles[1], A. Bosque[1]

[1]Department of Pathology, University of Utah, Salt Lake City, UT, USA, [2]Department of Medicine, University of Utah, Salt Lake City, UT, USA

Background: Successful therapies that target and reduce the latent HIV reservoir are a priority on the search of an HIV-1 cure. Previous findings in our laboratory demonstrated that Pam3CSK4 (a TLR1/2 ligand) is able to reactivate latent HIV-1 without global T-cell activation. So that, we decided to further study the potential application of TLR2 agonists as new latency -reversing agents (LRAs). CL572 is a synthetic multi-PRR agonist that has been recently shown to specifically activate human TLR2 and also TLR7. Our data indicate that CL572 can also efficiently reactivate latent HIV-1.

Methods: In order to perform our studies, we manipulated the tumoral cell line JLat 10.6 to increase TLR2 surface expression. We also tested the ability of CL572 to reactivate latent HIV-1 in a primary cell model of latency. Finally, we evaluated CD4+ T cells isolated from aviremic patients using the REVEAL assay (Rapid Ex Vivo Evaluation of Anti-Latency activity) that combines the ability to access viral reactivation, and to measure biomarkers of drug activity and toxicity in the same sample.

Results: We have observed that CL572 has activity in J-LAT cells expressing TLR2 and it retained activity at picomolar concentrations in this cell line. To confirm that CL572 is a more potent TLR-2 agonist that reactivates HIV-1 latency, we tested both Pam3CSK4 and CL572 in a primary cell model of latency. CL572 was less toxic than Pam3CSK4 in cultured $T_{CM.}$ Additionally, CL572 is a more potent agonist of HIV-1 latency than Pam3CSK4 with activity in the lower nanomolar range. Finally, the REVEAL assay indicated that CL572 is able to trigger viral reactivation without T-cell activation and apoptosis in primary CD4 T cells.

Conclusions: These results indicate that CL572 can be utilized to trigger activation of latent proviruses in primary cells *in vitro*. Further investigations need to be done in order to verify if the reactivation triggered by CL572 alone is sufficient to sensitize reactivated T cells to the cytopathic effects of HIV-1 or whether CL572 has to be combined with other eradication strategies.

OP 9.3

HIV conserved region vaccine in early cART-treated subjects (BCN01): impact on immunogenicity and the latent reservoir

S. Morón-López[1], B. Mothe[1–3], C. Manzardo[4], A. Sanchez-Bernabeu[1], P. Coll[1,2], M.C. Puertas[1], L. Dorrell[5], J.M. Miró[2], B. Clotet[1,2,3,6], C. Brander[1,3,6,7], J. Martinez-Picado[1,3,6,7], T. Hanke[8] for the BCN01 Study Group

[1]IrsiCaixa AIDS Research Institute-HIVACAT, Badalona, Spain, [2]Fundació Lluita contra la Sida, Hospital Universitari Germans Trias i Pujol, Badalona, Spain, [3]Universitat de Vic-Central de Catalunya (UVic-UCC), [4]Hospital Clinic-IDIBAPS, Barcelona, Spain, [5]John Radcliffe Hospital, Oxford, UK, [6]Universitat Autònoma de Barcelona (UAB), Barcelona, Spain, [7]ICREA, Barcelona, Spain, [8]Jenner Institute, University of Oxford, Oxford, UK

Background: Therapeutic T-cell vaccines targeting conserved regions of the HIV-1 proteome should enhance host immune control and contribute to reduce the latent viral reservoir.

Methods: BCN01 (NCT01712425) is a phase I, multicenter trial to evaluate the safety, immunogenicity and impact on the latent reservoir of ChAdV63 and MVA.HIVconsv vaccines in early-treated subjects (<6 m from HIV-1, n=24) who initiated cART 1 week after diagnosis. Patients received ChAdV63.HIVconsv (5×10^{10} vp, im) and MVA.HIVconsv (2×10^{8} pfu, im), 8 or 24 weeks after (Short *vs.* Long regimen), and were followed for 6 months. Immunogenicity to HIVconsv insert and HIV-1 proteome was assessed by IFNγ ELISPOT. 24 controls were included to compare HIV reservoir decay during 1st year of early-cART. Proviral DNA was quantified in CD4+ T cells by droplet digital PCR. Single-copy assay was performed to investigate viral reactivation after vaccinations.

Results: Responses to conserved regions increased in all subjects after vaccinations. HIVconsv-specific T-cells peaked 1 or 4 weeks after MVA booster vaccination (median [IQR]: 938[73–6,805] SFC/million PBMC, P=0.0001 compared to pre-vaccination). No significant differences in peak or longevity were observed between regimens. Levels and decay of proviral DNA after cART were not associated with vaccine-induced immunogenicity nor differed from non-vaccinated individuals. Viral reactivation was not observed during vaccinations.

Conclusions: Vaccination with ChAdV63 and MVA.HIVconsv was a safe strategy to shift pre-existing immune response towards conserved regions of HIV-1 in a cohort of early-treated individuals, without inducing viral reactivation. Reservoir decay during first year of early-cART was not impacted by HIVconsv vaccinations. This is the first therapeutic vaccine trial that demonstrates a manipulation of CTL immunodominance pattern towards unmutated regions of HIV-1 and may facilitate clearance of the viral reservoir after HIV latency reactivation strategies.

OP 9.4

Antiviral therapy by targeting nanoparticles to CD4+ cells for the delivery of SIV-specific RNA-guided Cas9 nucleases

L. Giavedoni[2], L. Smith[1,2], E. Carnes[3,4], C. Lino[4], V. Hodara[2], K. Reimann[5], L. Parodi[2]

[1]University of Texas Health Science Center, San Antonio, TX, USA, [2]Texas Biomedical Research Institute, San Antonio, TX, USA, [3]University of New Mexico, Albuquerque, NM, USA, [4]Sandia National Laboratories, Albuquerque, NM, USA, [5]University of Massachusetts Medical School, Boston, MA, USA

Background: Delivery of therapeutic materials to SIV-infected CD4+ T cells found deep within the body has proved a difficult task. Currently our group of collaborators is working on developing nanoparticles for the targeted delivery of vectors expressing RNA-guided nucleases. These materials will be loaded into mesoporous silica nanoparticles encased within supported lipid bilayers (protocells) conjugated with anti-CD4 antibodies to target delivery to SIV-infected cells *in vivo*.

Methods: We have developed SIV genome-specific RNA-guided nucleases targeting fifteen regions of the provirus. These guiding RNAs, which target highly conserved segments among the sequences of several SIVmac isolates, are designed to disrupt provirus via insertions and deletions in order to render virions non-infectious. *In vitro* assays in HEK293T cells co-transfected with a plasmid containing the SIVmac239 proviral genome and the individual RNA-guided nucleases were used to select the most effective constructs. Protocells internalization was tested by incorporating the fluorophore Dylite 633 inside particles coated with control antibody, or chimeric IgG_1 or IgG_4 anti-CD4 antibodies. Internalization was tested in uninfected or SIV-infected cell lines, or in rhesus white blood cells and PBMC.

Results: RNA-guided Cas9 nucleases targeting the LTR, the ribosome slip site (RSS) and the TAR regions showed a dramatic reduction in the levels of p27 antigen. Protocells conjugated with anti-CD4 IgG_4 entered 97.2% of CD4+ T-cells, compared to 70.4% with α-CD4 IgG_1. The same trend was seen when incubating the protocells with rhesus WBCs and CEM NK^r CCR5+ cells; SIV infection, which caused downregulation of CD4, did not affect the ability of α-CD4 IgG_4 to facilitate internalization of protocells.

Conclusions: Preliminary results show that RNA-guided Cas9 nucleases can eliminate viral replication. Protocells conjugated with α-CD4 IgG_4 show great promise for facilitating delivery of therapeutic materials into CD4+ T cells preferentially. These protocells will be loaded with vector encoded Cas9 to test whether the protocells can deliver effectively biomaterials to infected CD4+ T cell and reduce viral replication *in vivo*.

OP 9.5

Potent CTL responses to conserved element of HIV to improve therapeutic DNA vaccine efficacy

B.K. Felber[1], A. Valentin[1], J.I. Mullins[2], G.N. Pavlakis[1]

[1]National Cancer Institute, Frederick, MD, USA, [2]University of Washington, Seattle, WA, USA

Background: We reported that therapeutic DNA vaccination in ART-treated macaques provides durable control of viremia after ART release. To improve efficacy, DNA vaccines were developed to overcome HIV sequence diversity and to address potential competition with decoy epitopes and to focus the immune responses to epitopes associated with virus control.

Methods: We engineered DNA-based immunogens consisting of conserved elements (CE) selected on the basis of stringent conservation, functional importance, broad HLA-coverage, and association with viral control. Gag CE DNAs were designed to maximize cross-clade coverage. Macaques were immunized in prime-boost regimens using CE DNA followed by DNA encoding Gag.

Results: All CE DNA-vaccinated macaques developed robust CE specific responses with a significant fraction of cytotoxic T cells. Interestingly, CE responses were significantly boosted in both magnitude and breadth upon vaccination with DNA expressing intact Gag, indicating altering of immune hierarchy by this vaccine regimen. We also found that vaccination-induced T cell responses rapidly disseminate into secondary lymphoid organs and effector mucosal sites.

Conclusions: Combination of CE and full-length immunogens provides a novel strategy to increase the magnitude and breadth of cellular immunity targeting subdominant conserved viral epitopes. These novel vaccines induce broad CTL responses to vulnerable sites of the virus, while avoiding variable regions that divert T cell responses towards less protective epitopes aiming to control potential escape mutants. This DNA vaccine regimen is being pursued to test whether targeting highly conserved regions by therapeutic vaccination could contribute to the cure of HIV infection. This vaccine will be combined with heterodimeric IL-15, a cytokine which we developed for clinical use and which has therapeutic potential by promoting growth, mobilization and activation of lymphocytes to further strengthen the immune response.

OP 9.6

Immune response to sequences surrounding the 12 protease cleavage sites generated during ARV treatment improved CD4 counts of SIVmac251 infected rhesus monkeys

M. Luo[1,2], D. Tang[1], J. Pinto[3], M. Nykoluk[1], P. Lacap[1], J. Tuff[1], R. Capina[1], C. Czarnecki[1], J. Whitney[4], M. Alonso[3], T. Ball[2,5], G. Kobinger[2,6], P. Sandstrom[5], F. Plummer[2,7]

[1]National Microbiology Laboratory, HIV Host Genetics/NHRL, Winnipeg, Canada, [2]University of Manitoba, Medical Microbiology, Winnipeg, Canada, [3]University of Santiago de Compostela, Santiago de Compostela, Spain, [4]Harvard Medical School, Beth Israel Deconess Medical Center, Boston, USA, [5]National Microbiology Laboratory, NHRL, Winnipeg, Canada, [6]National Microbiology Laboratory, Special Pathogens, Winnipeg, Canada, [7]National Microbiology Laboratory, Winnipeg, Canada

Background: Effective therapeutic vaccines used in combination of ARV to treat HIV infected patients can reduce drug induced toxicity, help to re-constitute immune system and achieve a functional cure. We conducted a pilot study to test the therapeutic effect of a novel HIV vaccine targeting the 12 protease cleavage sites in combination of ARV.

Methods: SIVmac251 infected rhesus monkeys were treated with a combination of FTC, PMPA and raltegravir for 49 days. Seven days after ARV initiation the monkeys in the treatment group received rVSVpcs (i.m.). Three additional therapeutic treatment with rVSVpcs (i.m.)/NANOpcs(i.m.), NANOpcs(i.m), and NANOpcs(i.m.) were carried out with 2-week intervals. ARV treatment was stopped after 49 days and viral load, CD4/CD8 counts, antibody and T cell response to PCS peptides and pooled Gag and Env peptide were analyzed.

Results: ARV treatment suppressed viral load of all macaques, but only the viral load of 6 out of 11 macaques was suppressed to non-detectable level during the treatment/ARV period. However, even with the short duration of ARV treatment and incomplete viral load suppression, the immune responses to PCS peptides were generated after 2–4 therapeutic treatments. The CD4 counts of PCS vaccine

treated macaques were significantly improved after 35 days and 49 days of ARV treatment(P=0.027 and 0.044), whereas there is no significant improvement in CD4 counts of monkeys only received ARV treatment despite the viral load suppression.

Conclusions: Our study showed that new immune response to PCS peptides can be generated even with incomplete viral load suppression after a short period ARV treatment. The combination of PCS vaccine treatment and ARV generated new immune response to PCS peptides, improved CD4 counts of SIVmac251 infected monkeys and can be used to improve patient care to achieve a functional cure.

OP 9.7

Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing

K. Khalili[1,2], R. Kaminski[1,2], Y. Chen[1,2], T. Fischer[1,2], E. Tedaldi[2,3],A. Napoli[1,2], Y. Zhang[1,2], J. Karn[4], W. Hu[1,2]

[1]Department of Neuroscience/Center for Neurovirology, Temple University School of Medicine, Philadelphia, PA 19140, USA, [2]Comprehensive NeuroAIDS Center, Temple University School of Medicine, Philadelphia, PA 19140, USA, [3]Department of Medicine, Temple HIV Program, Temple University School of Medicine, Philadelphia, PA 19140, USA, [4]Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106, USA

The ability of HIV-1 to permanently integrate into the genomes of its target CD4+ T-cells presents a strong challenge for an HIV-1 cure, and none of the currently available approaches have achieved eradication of latent host-integrated HIV-1 DNA from patient T-cells. We used RNA-guided CRISPR/Cas9 DNA cleavage to precisely remove the entire HIV-1 genome spanning between 5′ and 3′ LTRs of integrated HIV-1 proviral DNA copies from latently infected human CD4+ T-cell lines.

Our CRISPR/Cas9 strategy completely ablated viral gene reactivation. Comprehensive assessment of whole-genome sequencing of HIV-1 eradicated cells with ultra-deep coverage of >100x revealed many naturally-occurring insertion/deletion (InDel) mutations across the genome of control and HIV-1-eradicated cells, but none of these resulted from cleavage mediated by LTR-specific guide RNAs (gRNAs) aimed at host-integrated viral DNA. Furthermore, SURVEYOR assays ruled out any off-target effects that might compromise the integrity of the host genome, and we saw no side effects of HIV-1 genome removal, such as genotoxicity, dysregulation of the cell cycle, decreased cell viability or increased apoptosis. In addition, persistent co-expression of Cas9 and the specific targeting gRNAs in HIV-1-eradicated T-cells protected them against new infection by HIV-1. In primary CD4+ T-cell cultures, lentivirus-delivered CRISPR/Cas9 significantly diminished HIV-1 infection, evidenced by reduced viral copy numbers. Similarly, lentivirus-mediated delivery of Cas9 and HIV-1-targeting gRNAs *ex vivo* to cultured CD4+ T-cells obtained from HIV-1-infected patients undergoing antiretroviral therapy decreased viral replication by 68%–71%. Thus, gene editing using CRISPR/Cas9 may provide a new therapeutic path for eliminating HIV-1 DNA from CD4+ T-cells and potentially serve as a novel and effective platform toward curing AIDS.

Session 10: New therapeutic approaches 2

OP 10.0

The anti-inflammatory response and the HIV cure

R. Sekaly

Case Western Reserve University, Cleveland, USA

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OP 10.1

Emergence of treatment-resistant infectious HIV after genome-directed antiviral endonuclease therapy

K.R. Jerome[1,2,3], D. Stone[1], H.S. De Silva Feelixge[1], P. Roychoudhury[1], J.T. Schiffer[1,5]

[1]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, [2]Department of Laboratory Medicine, University of Washington, Seattle, WA, USA, [3]Department of Microbiology, University of Washington, Seattle, WA, USA, [4]Department of Biochemistry, University of Washington, Seattle, WA, USA, [5]Department of Medicine, University of Washington, Seattle, WA, USA

Background: Incurable chronic viral infections are a major cause of morbidity and mortality worldwide. One potential approach to cure persistent viral infections is via the use of targeted endonucleases. Nevertheless, a potential concern for endonuclease-based antiviral therapies is the emergence of treatment resistance. Here we demonstrate for the first time the emergence of an endonuclease-resistant infectious virus during endonuclease therapy.

Methods: We developed a series four of zinc finger nucleases (ZFNs) directed toward conserved sequences within the HIV *pol* gene, encoding protease, reverse transcriptase, or integrase. Activity of each ZFN against HIV sequences was confirmed in cell lines and primary human cells.

Results: While testing the activity of HIV *pol*-specific ZFNs alone or in combination with three prime repair exonuclease 2 (Trex2), we identified a treatment-resistant and infectious mutant virus (designated ZFN2 (+3)) that was derived from a ZFN-mediated disruption of reverse transcriptase (RT). Although gene disruption of HIV protease, RT and integrase could inhibit viral replication, a chance single amino acid insertion within the thumb domain of RT produced a virus that could actively replicate. The endonuclease-resistant virus could replicate in primary CD4+ T cells, but remained susceptible to treatment with antiretroviral RT inhibitors. When secondary ZFN-derived mutations were introduced into the mutant virus's RT or integrase domains, viral replication was abolished.

Conclusions: Our observations suggest that caution should be exercised using single endonuclease-based antiviral therapies. However, combination endonuclease therapies may prevent the emergence of resistance

OP 10.2

The effects of combination of ingenol-B and ART to SIV251 infected rhesus monkeys

G. dos Santos Goncalves[1], D. O'Connor[2], S. O'Connor[2], A. Tanuri[1]

[1]Universidade Federal do Rio de Janeiro, Brazil, [2]University of Wisconsin-Madison, USA

Ingenol-B is a PKC agonist that has showed its potential to reactivate latent virus *in vitro* in experimental conditions. It was previously described that ingenol-B when administered to SIV chronically infected rhesus increased the viral load, proviral load and virus diversity. To evaluate the effect of ingenol-B combined with ART we have infected 6 rhesus with SIV for 6 weeks, followed by 24 weeks of ART. Four of the six monkeys received two cycles of 4 weeks of ingenol-B during the ART (tested group) and 2 monkeys only ART (control). After the monkeys were submitted to a washout phase with no drugs. Comparing the level of viral load before ART and the washout, the control group shows similar level with a decrease not greater than 3 fold. The tested group shows a pronounced decrease in the viral load of at least 5 fold, with one animal going from 10^6 to 400 copies/mL, indicating a possible hole of ingenol-B on lowering the viral load. A deep sequencing analysis of the SIV env V1V2 region was done to access the viral diversity in each monkey. The control group had a higher diversity before ART then on the rebound during the washout. On the other hand, two of four monkeys on the tested group showed an increase in the viral diversity, one monkey showed similar diversity and one a slight decrease. Phylogenetic analysis of the viral subpopulations present on each monkey before ART and at the washout showed that the control monkeys present viral related subpopulations throughout the trial. While 3 out of 4 monkeys on the tested group show new subpopulations characterized by new branches

on the trees. This may indicate that ingenol-B is responsible for maintaining the viral diversity on plasma by reaching viral reservoir and reactivating viral variants that would not produce virions. By doing this, ingenol-B could facilitate the action of the immune system finding and destroying former HIV latent cells, and contribute to decrease viral reservoir in patients.

OP 10.3

Dendritic cell induced "kick" of latent HIV-1 *in vitro* during cART

R.B. Mailliard[1], J. Kristoff[1], D. Ratner, M. Ding, J.M. Zerbato[2], N.D. Sluis-Cremer[2], P. Gupta[1], C.R. Rinaldo[1]

[1]Department of Infectious Diseases and Microbiology, University of Pittsburgh, PA, USA, [2]Division of Infectious Diseases, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Background: Recently, increases in residual viremia were observed in cART-suppressed HIV infected individuals upon analytic treatment interruption after receiving a dendritic cell (DC)-based HIV vaccine, suggesting that DC may also play a role in HIV-1 latency reversal. Therefore, in this study we assessed the *in vitro* capacity of differentially programmed DC to drive latency reversal of replication-competent HIV-1 during cART.

Methods: Two models of HIV-1 latency reversal were tested: (1) Total resting CD4+ (rCD4) T cells from HIV-1 uninfected donors were treated with CCL19, infected with HIV-1_{xxLAI}, exposed to low-dose IL-2 with efavirenz to establish latency, and subsequently stimulated with DC. (2) DC derived from HIV-1 infected individuals on cART (viral RNA <20 copies/mL plasma) were co-cultured with autologous rCD4 T cells, in the absence or presence of efavirenz. The general role of antigen presentation was assessed using DC bound with either anti-CD3 (OKT-3) as an antigen surrogate, or the superantigen staphylococcal enterotoxin B (SEB). Three monocyte derived DC types were tested including immature DC (iDC), mature high IL-12p70 producing type-1 polarized DC (DC1), and mature IL-12p70 deficient DC (DC2). Cellular and culture supernatant viral RNA were quantified using qRT-PCR as well as a novel TZM-bl reporter cell based assay (TZA).

Results: All DC tested were found to induce replication competent virus from rCD4 T cells from HIV-1 infected participants on cART. However, DC1 consistently induced significantly greater levels of replication of HIV-1 RNA in rCD4 T cells than either iDC or DC2 in both models of HIV-1 latency reversal. Importantly, this DC-mediated effect was most pronounced in the presence of antigen.

Conclusions: While they are known to have a strong capacity to produce IL-12p70 and drive antigen specific CTL responses *in vitro*, this study demonstrates that DC1 are also superior to iDC and DC2 as a potential HIV-1 latency-reversal tool. Although the mechanisms of this effect have yet to be elucidated, the enhanced ability of DC1 to drive HIV-1 latency reversal was antigen dependent. Information resulting from these studies will be used towards the design of novel DC1-based vaccine strategies to facilitate the "kick" as well as the "kill" of the HIV-1 reservoir.

OP 10.4

Peripheral blood lymphocytes from patients with chronic myeloid leukemia on treatment with dasatinib are resistant to HIV-1 infection

M. Coiras[1], M. Bermejo[1], J. García-Pérez[1], J. Ambrosioni[2], J.M. Mirô[2], M. Plana[3], J. Alcamí[1]

[1]AIDS Immunopathology Unit, National Center of Microbiology, Instituto de Salud Carlos III, Madrid, Spain, [2]Infectious Diseases Service, AIDS Research Group, Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, Barcelona, Spain, [3]Retrovirology and Viral Immunopathology Laboratory, AIDS Research Group, Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, Barcelona, Spain

Background: Massive activation of infected CD4 cells during acute HIV-1 infection leads to neutralization of the antiviral effect of SAMHD1 through its phosphorylation. Dasatinib, a tyrosine kinase inhibitor used for chronic myeloid leukemia (CML), has been described to control HIV-1 replication through its negative effect on viral entry. We demonstrated that dasatinib actually interferes with SAMHD1 phosphorylation, preserving its antiviral activity against HIV-1.

Materials: PBMCs from healthy donors to determine the effect of dasatinib on SAMHD1 phosphorylation and HIV-1 fusion step. PBMCs from CML patients on treatment with dasatinib to analyze SAMHD1 phosphorylation and susceptibility to HIV-1 infection *ex vivo*.

Results: Dasatinib prevented SAMHD1 phosphorylation, preserving its antiviral activity and impairing HIV-1 retrotranscription and proviral integration. This was the major mechanism of action because Vpx, which degrades SAMHD1, impeded the inhibitory effect of dasatinib on HIV-1 replication. Fusion of BlaM-Vpr-containing HIV-1 viruses with activated PBMCs in the presence of dasatinib showed that dasatinib was not acting at fusion level. PBMCs from five CML patients on chronic treatment with dasatinib for more than two years showed reduced phosphorylation of SAMHD1 in response to T-cell activation *ex vivo*. These cells were resistant to HIV-1 infection *ex vivo*, showing very low proviral integration.

Conclusions: Dasatinib is the first compound currently used in clinic that has been described to preserve the function of the innate antiviral factor SAMHD1. The use of dasatinib with antiretroviral therapy during primary HIV-1 infection could control massive activation of CD4 cells, making them refractory to infection by interfering mostly with retrotranscription. This would reduce the reservoir size and preserve HIV-specific immune responses, creating a more favorable immunologic environment for future interventional studies aiming at HIV-1 eradication and cure.

OP 10.5

Restricted HIV-1 diversity and clonal expansion following cytoreductive chemotherapy

T.J. Henrich[1,2], E.P. Scully[3], K.S. Hobbs[1,2], E. Hanhauser[1,2], L.E. Hogan[1,2], C.D. Palmer[3], Y.P. Robles[1], K.S. Leadabrand[2], A.S. LaCasce[4], D.R. Kuritzkes[1]

[1]Brigham and Women's Hospital, Boston, MA, USA, [2]University of California San Francisco, San Francisco, CA, USA, [3]Ragon Institute of MGH, MIT and Harvard, USA, [4]Dana-Farber Cancer Center, Boston, MA, USA

Background: Cytoreductive chemotherapy does not lead to consistent changes in HIV-1 DNA or RNA. However, reductions in CD4 T cells during chemotherapy suggest that reservoirs decrease. Constriction and subsequent oligoclonal expansion of HIV diversity is a better measure of the reservoir response to HIV eradication strategies. We examined HIV sequence evolution in the context of immune correlates, and quantified reservoirs in cells responding to non-HIV stimuli.

Methods: Longitudinal, single-genome HIV envelope sequencing was performed in 10 individuals on ART receiving cancer chemotherapy. Changes in T cell subsets, activation/proliferation and HIV-specific immune responses were measured. HIV was quantified from CD4 T cells sorted on intracellular IL2 and/or INFγ staining after stimulation with αCD3/αCD28 or combined EBV/CMV lysates.

Results: Although CD4 T cell counts decreased by up to 75%, CD4 T cell HIV DNA did not change and RNA increased following completion of chemotherapy (P=0.203 and P=0.037). Despite temporary reductions in cell counts, no changes in the percentage of naive, central or effector memory CD4 and CD8 T cell subsets were observed. Markers of activation/proliferation decreased or remained stable in all populations, but HIV-specific T cell responses either increased or developed novel signatures following chemotherapy. Clonal expansion of HIV-1 envelope sequences following completion of chemotherapy was observed in three of six patients for whom data was obtained. CD4 T cells that responded to EBV/CMV lysates had higher HIV DNA levels compared to those that did not respond or responded to αCD3/αCD28 stimulation.

Conclusions: Despite the lack of changes in peripheral blood HIV DNA, our results suggest that cytoreductive therapy reduces HIV reservoirs in some patients manifesting as a constriction of HIV sequence diversity.

Our data also suggest that response to non-HIV antigens can lead to oligoconal expansion of the DNA reservoir.

OP 10.6

Dual-affinity re-targeting (DART) proteins overcome viral diversity to deplete the latent HIV-1 reservoir

J.M. Sung, J. Pickeral, M. Bednar, L. Liu, S.A. Stanfield-Oakley, J. Pollara, C. LaBranche, M.A. Moody, M. Bonsignori, C.Y. Kao Lam, S. Johnson, C. Garrido, N. Archin, J. Kuruc, M. Cohen, K. Soderberg, H.X. Liao, D. Montefiori, R. Swanstrom, S. Koenig, J. Nordstrom, B.F. Haynes, G. Ferrari, D. Margolis

University of North Carolina, Chapel Hill, NC, USA

Background: Dual-Affinity Re-Targeting (DART) proteins are bispecific, antibody derived molecules consisting of an HIV binding arm based on broadly binding antibodies targeting highly conserved regions of Env, coupled to a binding arm for CD3 T-cells. We reported that DARTs clear HIV-1 infected cells, including rare latently infected cells following exposure to vorinostat. We now report initial data on viral sequences in the single patient in whom the kinetics of viral clearance by DARTs was delayed.

Methods: A viral clearance assay to assess DART activity against targets infected with autologous reservoir virus. A latency clearance assay measured the ability of DARTs to clear patient resting CD4 T cells induced to express viral antigens either by stimulation with PHA, or with clinically relevant doses of vorinostat.

Results: Addition of DARTs to a viral clearance assay led to a significantly greater log fold reduction in autologous reservoir virus compared to co-culture with CD8 T cells alone (0.79 *vs.* 0.02, respectively, $P<0.05$). Addition of DARTs to a latency clearance assay with PHA-stimulated latently infected cells reduced viral recovery in all six out of six subjects. Viral recovery from vorinostat exposed resting CD4 T cells was also reduced in three out of four subjects. Importantly sequencing of outgrowth virus from cells of the one subject without an initial response to DARTs did not detect any mutations to the binding residues for DARTs, and prolonging the co-culture period led to complete ablation of viral recovery.

Conclusion: Viral clearance assays and sequencing demonstrate that DARTs are capable of redirecting T cells against cells infected with autologous reservoir virus and resting CD4 T cells emerging from latency. The kinetics of viral clearance require further study, but there is thus far no evidence of viral escape from DARTs. In combination with latency-reversing agents, DARTs may be potent tools to deplete the latent HIV-1 reservoir.

OP 10.7

Human galectin-9 is a potent mediator of HIV transcription and reactivation

M. Abdel-Mohsen[1,2], L. Chavez[1], G.M. Chew[3], X. Deng[1], A. Danesh[1,2], S. Keating[1], R. Hoh[2], S.G. Deeks[2], L.C. Ndhlovu[3], S.K. Pillai[1,2]

[1]Blood Systems Research Institute, San Francisco, CA, USA, [2]University of California, San Francisco, USA, [3]University of Hawaii, Hawaii Center for AIDS, John A. Burns School of Medicine, Honolulu, HI, USA

Background: Based on our recent finding that the host factor p21 regulates HIV transcription during antiretroviral therapy (ART), and published data demonstrating that the glycan-binding protein galectin-9 (Gal-9) regulates p21, we hypothesized that Gal-9 modulates HIV transcription.

Methods: Plasma Gal-9 levels were examined in relation to measures of the latent HIV reservoir in 72 HIV-infected ART-suppressed individuals. The ability of recombinant Gal-9 (rGal-9) to reactivate latent HIV was evaluated *in vitro* and *ex vivo*. Cell-surface deglycosylation was used to explore the requirement of glycans in viral reactivation by rGal-9. Effects of rGal-9 on the host transcriptome were evaluated using RNA-seq.

Results: Endogenous levels of Gal-9 were associated with HIV transcription ($P<0.02$) *in vivo* during ART. rGal-9 reactivated virus in J-Lat cells (15.1%) more potently than anti-CD3/CD28 stimulation (4.8%, $P<0.0001$). In CD4+ T cells from 13 HIV-infected ART-suppressed individuals, rGal-9 induced a mean 7.3-fold increase in intracellular HIV RNA levels ($P=0.002$). Induction was significantly higher than vorinostat ($P=0.02$, 3.2 fold). Cell surface N-linked oligosaccharides and O-linked hexasaccharides were essential for rGal-9-induced HIV reactivation, mediated by key transcription initiation, and chromatin remodeling factors (FDR<0.05). rGal-9 induced expression of APOBEC3G up to 29-fold *in vitro* and *ex vivo* (FDR<0.006), resulting in 6.7-fold reduction in infectivity of progeny virus.

Conclusions: rGal-9 potently reactivates latent HIV and induces APOBEC3G expression *in vitro* and *ex vivo*. rGal-9-induced virus will likely be rendered replication-incompetent as a result of APOBEC3G induction in the producer cell, ensuring that the reservoir will not be replenished when latency is reversed, even in the setting of suboptimal ART suppression. Our data suggest that galectin-9 and the glycosylation machinery should be explored as a foundation for novel HIV cure strategies.

Poster presentations

Session 1: *In vitro* and *in vivo* models of HIV persistence

PP 1.0

Characterization of humanized NSG mice to evaluate latency

S. Satheesan, L. Haitang, J. Burnett, J. Rossi

City of Hope, Duarte, CA, USA

Background: A current challenge to efforts to eliminate HIV-1 from infected individuals is the establishment of persistent long-lived HIV infected cells in blood and various anatomic compartments. The resting memory CD4+ T cells are the best characterized HIV-1 reservoir and they can be detected in blood, lymph node and gut.

Methods: We have utilized NOD/SCID/γc null (NSG) mice for our studies as NSG newborn mice can be successfully reconstituted with human lymphoid and myeloerythroid components following fetal-liver derived HSC injection. Our results demonstrate that humanized NSG mice support production of human cell types permissive to HIV-1 infection. We also assessed ability of these mice to sustain long-term infection by infecting them with R5-tropic HIV-1 and viral infection was assessed by qRT-PCR and CD4+ T cell levels in peripheral blood were quantified by flow cytometry.

Results: Our results show that R-5 tropic virus is capable of infecting humanized NSG mice as demonstrated by high levels of plasma viremia and that HIV-1 infection leads to CD4+ T cell depletion in peripheral blood, thus mimicking the key aspects of HIV-1 pathogenesis. The NSG mice with demonstrable HIV infection were treated for 6–10 weeks with combinatorial antiretroviral therapy composed of drugs that block new infections, but not drugs that inhibit the viral production of infected cells.

Conclusions: The treatment blocked emergence of viral RNA, as expected and plasma viremia was confirmed to be below detectable limits within 4 weeks following initiation of treatment in all animals. The persistence of HIV during antiretroviral treatment is due to the latently infected resting CD4+ T cell population in post integration phase of infection. After discontinuation of ART following 6 weeks of fully suppressive therapy, virus rebounded in all animals and viral RNA levels correlated with viremia during active infection and proviral DNA levels in various tissue compartments contributed to time to rebound.

PP 1.1

Ex vivo determination of stem cell transplantation graft-versus-HIV reservoir effects

L.E. Hogan, K.S. Hobbs, D.R. Kuritzkes, J. Ritz, T.J. Henrich

UCSF, San Francisco, CA, USA

Background: Allogeneic hematopoietic stem cell transplantation (HSCT) is one of the few strategies that substantially reduces HIV-1 reservoir size. Graft-versus-host (GVH) responses likely result in clearance of residual recipient cells harboring HIV. Beneficial GVH responses, which permit donor cells to clear tumor or residual host hematopoietic cells, may be mediated largely by the innate immune system. To investigate the role of NK cells and other lymphocytes in reactivating and eliminating latent HIV following HSCT, we designed a novel *ex vivo* assay to determine the activity of HLA-matched, post-HSCT donor effector cells on latently infected, pre-HSCT host CD4 T cells.

Methods: We adapted a latency model to enable infection of high numbers of CD4 T cells from individuals with hematopoietic malignancies prior to HSCT with an iGFP-gag HIV viral strain. The infected pre-HSCT CD4 T cells were then co-incubated with PBMC obtained from the same individuals 9–12 months after HSCT, and following full donor cell chimerism. We then determined lymphocyte activation, proliferation, viral reactivation and death over a 2 week period using flow cytometric analyses.

Results: We included samples from a total of 30 HIV-negative individuals who received either full myeloablative or reduced intensity HSCT. Up to 95% pre-HSCT CD4 T cells were infected with iGFP-HIV-1, with subsequent resting resulting in large numbers of latently infected cells. Flow cytometry was performed 0–13 days following lymphocyte mixing and co-culture. Of note, higher levels of non-proliferating HIV reactivated cells were found in the autogeneic setting compared to that of the allogeneic samples. Conversely, higher levels of proliferating HIV-infected cells were seen in the allogeneic samples, peaking at day 7. While expression of activation markers increased on NK, NKT and CD8 T cells, there were no differences found between the autogeneic and allogeneic groups. However, CD8 T cell activation was strongly correlated with HIV production (R^2=0.975).

Conclusions: Our findings suggest that lymphocytes, including NK and NKT cells, may play an important role in surveillance and clearance of residual HIV-infected cells following HSCT.

PP 1.2

NNRTIs reduce HIV-1 production from latently infected resting CD4+ T cells

J. Zerbato, N. Sluis-Cremer

Department of Medicine, Division of Infectious Diseases, University of Pittsburgh, Pittsburgh, PA, USA

Background: Clinical trials are investigating the potential for LRAs to reduce the size of the latent HIV-1 reservoir. In theory, LRAs "kick" HIV-1 out of latency which promotes the "kill" of the infected cell by viral cytopathic effects and/or the host's immune response. Importantly, this approach is always carried out with ART to prevent de novo infection by the LRA-induced HIV-1. Here, we assessed whether different ARVs impact the "kick and kill" strategy.

Methods: Latency reversal was evaluated in a primary cell model of latency. Two hrs prior to addition of anti-CD3/CD28 antibodies, cells were treated with a PI (atazanavir, darunavir), NRTI (lamivudine), NNRTI (rilpivirine, efavirenz) or INSTI (raltegravir). Controls included cells that were exposed to antibody only or to ARVs only. Seven days post antibody administration cell-associated DNA and extracellular virion-associated HIV-1 were quantified. T cell activation and viability were assessed by flow cytometry.

Results: NNRTIs were found to decrease HIV-1 production (by ~2-log fold-change) in resting CD4+ T cells exposed to anti-CD3/CD28 antibodies. This decrease in HIV-1 production was not due to toxicity, or the NNRTI impacting CD25, CD69 or HLA-DR expression in the absence or presence of anti-CD3/CD28 antibody. In contrast, none of the other ARVs, including PIs which target the late stages of HIV-1 replication, had a significant impact on virus production. Despite the decrease in virus production, HIV-1 DNA declined by ~85% in the NNRTI-treated cells, which suggests decay of the latent reservoir. We hypothesize that the NNRTIs enhance premature activation of HIV-1 PR that results in intracellular processing of Gag and Gag-Pol, decreased viral particle production, and cellular toxicity.

Conclusions: NNRTIs reduce HIV-1 production from latently infected cells. *Ex vivo* studies that use NNRTIs to prevent virus spread or cells from donors on NNRTI containing regimens should be cautiously interpreted.

PP 1.3

Towards achieving a state of reversible HIV-1 latency in primary monocyte-derived macrophages (MDM) by M1 polarization

G. Poli, F. Graziano, E. Vicenzi

San Raffaele University and Scientific Institute, Milan, Italy

Background: Whether myeloid cells are latently infected remains to be firmly established. In this regard, we have previously reported that short-term exposure of primary MDM to pro-inflammatory cytokines (IFN-γ plus TNF–α), i.e. "M1 polarization", partially prevented productive virus infection and reduced proviral transcription.

Methods: M1-polarized MDM were restimulated with M1 cytokines R5 HIV-1 ($M1^2$ protocol). Cell cultures were monitored for supernatant-associated RT activity, HIV-1 DNA load, APOBEC3G/3A expression, and for cell reactivation by co-culture with T cell blasts.

Results: We observed a significant, further reduction of virus replication down to near undetectable levels by RT activity over 30 days of culture. HIV-1 DNA levels were ca. 100- and 1,000-fold lower in M1-MDM and $M1^2$-MDM *vs.* control, unpolarized cells, respectively. APOBEC3A, but not APOBEC3G, was significantly upregulated by the $M1^2$ protocol protocol 15 days post-infection. No effect of T cell blast co-culture on control, infected MDM was observed, whereas significant levels of RT activity were induced in $M1^2$-MDM by this approach.

Conclusions: Stimulation of already infected M1-MDM with pro-inflammatory, M1-cytokines counterintuitively resulted in a further, significant inhibition of virus replication down to "near-latency" levels in terms of RT activity and viral DNA levels and upregulation of APOBEC3A expression. Recovery of virus production was achieved by cocultivation of $M1^2$-infected MDM with allogeneic T cell blasts, indicating the existence of a pool of infected cells carrying inducible proviruses. Additional experiments are ongoing to define the levels of integrated proviruses *vs.* total HIV-1 DNA by Alu-PCR, the reproducibility of the $M1^2$ protocol with a VSV-g pseudotyped single round virus, and the panel of stimuli capable of reactivating virus expression.

PP 1.4

Co-culture of T-cells with dendritic cells facilitates HIV latency in proliferating CD4+ T-cells: implications for the establishment and reversal of latency

N. Kumar, R.M. van der Sluis, T. Mota, V.A. Evans, S.R. Lewin, P.U. Cameron

University of Melbourne, Melbourne, Australia

The major barrier to HIV cure is latently infected resting CD4+ T-cells. Previously we showed that myeloid dendritic cells (mDC) and monocytes induce latency in non-proliferating CD4+ T-cells, and we now examined whether latency is established in proliferating CD4+ T-cells under similar conditions. Proliferation dye labeled resting CD4+ T-cells were cultured alone or with syngeneic mDC, plasmacytoid DC (pDC) or monocytes in the presence of staphylococcal enterotoxin B (SEB). After 24h, cultures were infected with CCR5-tropic enhanced green fluorescent protein (EGFP)-reporter HIV. Five-days post-infection, non-productively-infected, non-proliferating and proliferating T-cells were sorted and cultured until day 12 with IL-7, fusion inhibitor and integrase inhibitor. On day 5 and 12 post-infection, EGFP was quantified after activation with αCD3/αCD28±L8 as a marker of latency. Latency was detected in non-productively infected, proliferating T-cells co-cultured with mDC, pDC and monocytes (median(IQR), 4(0.5–15), 0.4(0.01–2), 1.7(0.01–10)%; *n*=15). Using ALU-LTR integrated HIV DNA was only detected in mDC and monocyte co-cultures (10% each; *n*=7). At day 12, latency was detected in proliferating T-cells co-cultured with mDC and monocyte, but not pDC where cell viability was below 20% (*n*=4). At day 5, proliferating CD4+ T-cells from mDC, pDC and monocyte co-cultures highly expressed CD25 (97, 86, 99% respectively, *n*=6), CD69 (39, 32, 46%; *n*=5), Tim-3 (23, 67, 48; *n*=4), and had low expression of PD-1 (0.1, 1.9, 3%; *n*=4) and Ki67 (7, 18, 11%; *n*=7). At day 5, measurement of T-cell receptor Vβ chains that were SEB specific (17, 3) and non-specific (13.1) showed no enrichment on non-proliferating or proliferating T-cells, suggesting that latency establishment is independent of cognate interaction. Our data shows that latently infected proliferating cells may be an important mechanism for the establishment of latency and should be included in future studies of HIV persistence.

PP 1.5

Reactivation of VOA-inducible and -uninducible HIV-1 proviruses using immune-compromised mice engrafted with human resting CD4+ T cells

Z. Yuan[1,2], G. Kang[1,2], W. Lu[1,2], Q. Li[1,2]

[1]Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE, USA, [2]School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA

Background: HIV-1 infection remains incurable by antiretroviral therapy (ART) due to virus latency.

To better elucidate the mechanisms of HIV-1 latency and develop strategies to purge latently infected cells, quantitative assays designed to measure the size of the integrated HIV-1 latent reservoirs and animal models employed to evaluate latency-reversing agents (LRAs) are critical. To that end, enhanced co-culture assays, including quantitative viral outgrowth assay (VOA), are frequently used. However, VOA can only reactivate a small fraction of intact proviruses.

Methods: To test the feasibility of studying HIV-1 latency and its reactivation using immune-compromised mice, we engrafted NSG mice with human resting CD4+ T cells from an HIV-1 aviremic individual on ART. After clonal expansion of resting CD4+ T cells *in vitro*, CD4+ T cells were split into equal portions for VOA and mouse engraftment. Each of cryopreserved cells corresponding to VOA positive wells (P46P), VOA negative wells (P46N) and uncultured resting CD4+ T cells (P46U) were thawed and intravenously injected into NSG mice respectively. Plasma viral load (pVL) and peripheral blood CD4+ T cells were quantified every other week using qRT-PCR and flow cytometry.

Results and Discussion: We found that NSG mice reactivated latent HIV-1 from VOA-inducible CD4+ T cells (P46P) and uncultured resting CD4+ T cells (P46U) at 4 weeks post cell injection (wpi) and VOA-uninducible CD4+ T cells (P46N) at 10 wpi. Engrafted CD4+ T cells proliferated considerably *in vivo* and peaked prior to provirus reactivation. After solely injection of the patient resting CD4+ T cells, this model can support consistent viremia for at least 14 weeks. Sequence analyses revealed that reactivated viruses from VOA-inducible and -uninducible proviruses are different, indicating they were reactivated from distinct proviruses.

Conclusions: Immune-compromised mice can support long-term engraftment of human resting CD4+ T cells and spontaneously reactivate VOA-inducible and -uninducible proviruses. This small animal model has the potential to be used to study the underlying mechanisms of HIV-1 latency and its reactivation. This model can also be used to evaluate LRAs to eliminate HIV-1 latent infection through "shock and kill".

Session 2: Basic science of HIV latency

PP 2.0

Estrogen potently blocks HIV re-emergence from latency and points to gender-specific differences in HIV reservoirs and novel cure strategies

J. Karn[1], B. Das[1], C. Dobrowolski[1], E. Scully[2], S.G. Deeks[3], M. Gandhi[3], R.E. Johnston[4]

[1]Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, OH, USA, [2]Brigham and Women's/Massachusetts General Hospital, Boston, MA, USA, [3]UCSF School of Medicine, San Francisco, CA, USA, [4]amfAR, The Foundation for AIDS Research, New York, NY, USA

Background: The estrogen receptor type 1 (ESR-1) recruits specific repressive complexes to the HIV LTR. This study was designed to test whether ESR-1 modulates gender specific differences in the control of HIV latency.

Methods: ESR-1 was identified an essential factor used to maintain HIV latency by unbiased shRNA library screens. Specific agonists and antagonists of ESR-1 were used to inhibit or promote HIV reactivation. Patient cells from 10 well-matched male and female donors were evaluated by a novel assay for induction of spliced env-mRNA based on next-generation sequencing (EDITS assay).

Results: Antagonists of ESR-1 (tamoxifen and fulvestrant) are weak proviral activators but sensitize latently infected cells to low doses of TNF-α (NF-κB inducer) and SAHA (HDACi). Blocking of co-activator 3 (SRC-3), an upstream modulator of ESR-1, by gossypol also induces latent proviruses. By contrast, ESR-1 agonists, (propylpyrazoletriol, diethylstilbestrol, β-estradiol) strongly suppress both TNF-α and SAHA reactivation of latent proviruses. Chromatin immunoprecipitation (ChIP) assays show ESR-1 accumulates on the latent proviral genome. In HAART-treated patient samples there was a small increase of spliced HIV env mRNA when resting memory cells were treated with fulvestrant or tamoxifen. Proviral reactivation by these ESR antagonists was synergistically increased by SAHA and IL-15. β-estradiol at concentrations in the physiological range led to dramatic reductions in proviral reactivation. Importantly, females showed higher levels of inhibition in response to β-estradiol and higher reactivity in response to ESR-1 modulators than males.

Conclusions: ESR-1 is a pharmacologically attractive target that can be exploited in the design of therapeutic strategies aimed at eradication of the latent reservoir. Clinically useful drugs targeting ESR-1 can be used to either promote the re-activation of latent proviruses (antagonists) or limit their responses (agonists). The profound effects of β-estradiol on HIV reservoir reactivation suggests there may be gender specific differences in HIV reservoirs and highlights the need to tailor latency reactivation strategies for both men and women.

PP 2.1

HIV-1 silencing mediated by TRIM22 inhibition of Sp1 binding to the promoter

E. Vicenzi[1], F. Turrini[1], S.S. Marelli[1], A. Kajaste-Rudnitski[1], C. Van Lint[2], A.T. Das[3], B. Berkout[3], G. Poli[1]

[1]San Raffaele Scientific Institute, Milan, Italy, [2]University of Bruxelles, Gosselies, Belgium, [3]Center for Infection and Immunity Amsterdam, Academic Medical Center, University of Amsterdam, the Netherlands

Background: We have previously described that IFN-inducible TRIM22 is a suppressor of basal and PMA-dependent transcription acting independently of NF-kB and Tat/TAR. We have here investigated whether TRIM22 could interfere with such Sp1-driven transcriptional activation of HIV-1 LTR.

Methods: 293T cells, lacking of endogenous TRIM22, were co-transfected with a TRIM22-expressing plasmid together with reporter vectors driven by the HIV-1 promoter containing either wild-type or mutated Sp1 binding sites or lacking of either one or two sites; reporter expression was assessed 48 hours post-transfection. Endogenous TRIM22 was knocked-down (KD) in SupT1 cells that were subsequently infected with HIV-1 molecular clones engineered to be dependent on an incorporated Tet-On gene expression system for activation of transcription while being independent of Tat/TAR interaction. Virus replication was monitored up to 32 days post-infection. Cell extracts from TRIM22-transfected 293T was subjected to i) immunoprecipitation, ii) Western blotting iii), DNA pull-down and iv) Chromatin Immunoprecipitation (ChIP).

Results: TRIM22 overexpression suppressed Sp1-driven transcription of HIV-1, as its inhibitory activity was lost in the absence of Sp1 binding sites. In contrast, TRIM22 KD increased the replication of infectious clones that were exclusively dependent upon Sp1 binding to the promoter. Furthermore, immunoprecipitation experiments showed that TRIM22 did not interact with Sp1 and did not directly bind to the HIV-1 LTR, however TRIM22 expression drastically prevented Sp1 binding to the HIV-1 LTR.

Conclusions: TRIM22 inhibits Sp1-dependent transcription by preventing Sp1 binding to the HIV-1 LTR likely through the recruitment of factors that remain to be defined. Our findings bear relevance for the discovery of novel factors that mediate HIV-1 transcriptional silencing.

PP 2.2

Viral counteractions against CTIP2 in HIV-1 permissive cells

F. Forouzanfar, S. Ali, V. Le Douce, M. El Maasarrani, A. Aït-Amar, A. Janossy, E. Candolfi, F. Margottin-Goguet, C. Van Lint, C. Schwartz, O. Rohr

University of Strasbourg, EA7292 DHPI, Institute of Parasitology and Tropical Pathology of Strasbourg, France

Background: Latently infected cells constitute major blocks to an HIV-1 eradication and a functional cure of the patients. We have previously reported that the cellular co-factor CTIP2 plays a key role in the establishment and persistence of HIV latency in microglial cells, the main reservoirs of the virus in the brain. By recruiting large enzymatic complexes at the viral promoter, CTIP2 silences HIV-1 gene transcription and disfavors the viral reactivation from the reservoirs. However, nothing is known on how can HIV-1 counteracts the effects of CTIP2 in permissively infected cells. Usurping the host ubiquitination machinery to target undesirable host proteins is a common strategy utilized by retroviruses. Here, we tend to postulate that HIV-1 Vpr may target CTIP2 by Cul4A-DDB1-DCAF1 complex to counteract its effects on HIV-1 replication.

Methods: Investigations were performed at the biochemical, molecular and cellular levels.

Results: We demonstrated that interferon treatments induce expression of CTIP2 at the mRNA and the protein levels suggesting that this factor may be part of the cellular response to viral infections. We observed that replication of WT- but not Vpr-deleted HIV-1 reduced CTIP2 expression in productively infected cells. Vpr expression was correlated with low levels of CTIP2 and increased levels HIV-1 gene transcription. In addition, co-immunoprecipitation experiments showed that CTIP2 interacts with DDB1, DCAF1 and HIV-1 Vpr in order to induce the degradation of CTIP2 by proteasome. Finally, the abrogation of Vpr binding to the DCAF1-CUL4-DDB1 complex prevented CTIP2 degradation.

Conclusions: Our results suggest that Vpr engages the ubiquitination machinery to induce CTIP2 degradation. By degrading CTIP2, HIV-1 counteracts CTIP2-mediated silencing of its expression and thus favors viral replication.

PP 2.3

The unique enrichment of histone modifications and its relationship with HIV-1 latency in some chromosomes

K.C. Kim[1], H. Lim[1], J. Park[2], T.Y. Roh[2,3], C. Kang[1], B.S. Choi[1]

[1]Division of AIDS, Center for Immunology and Pathology, Korea National Institute of Health, Chung-buk, Republic of Korea, [2]Department of Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea, [3]Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang, Republic of Korea

Background: Histone modifications such as acetylation and methylation play a central epigenetic role in the organization of chromatin domains and the up/downregulation of gene expression. Although many studies have reported that the epigenetic mechanism is strongly involved in the maintenance of HIV-1 transcriptional latency, the epigenetic control of viral replication and how HIV-1 latency maintains are not fully understood.

Methods: We used the high-throughput parallel DNA sequencing (ChIP-seq) approach to investigate the effect of histone modifications, H3K4me3 and H3K9ac, on HIV-1 latency. ChIP-seq outputs from

CD4+ T cell line (A3.01) and HIV-1 latently infected cells (ACH2, J1.1, and NCHA1) were aligned to hg18 using bowtie and analyzed with SICER, CEAS, HOMER, WebGestalt, and PCViz.

Results: Chromosomes 16, 17, 19 and 22 were significantly enriched for histone modifications in both decreased and increased islands. 38 decreased islands from 126 H3K4me3 islands and 302 H3K9ac islands common at specific chromosomes in HIV-1 latent cells and 41 increased islands from 130 H3K4me3 islands and 164 H3K9ac islands were selected for functional annotation. In Gene Ontology analysis, 38 genes were involved in regulation of biological process, regulation of cellular process, biological regulation and purinergic receptor signaling pathway. 41 genes were in nucleic acid binding, calcium activated cation channel activity, DNA binding and zinc ion binding. By pathway commons analysis in WebGestalt, 38 genes were strongly involved in p63 transcription factor network and 41 genes were in RNA polymerase III transcription termination pathway. TOP2A, ITGB4, TRAF4, SEC14L2, NFIC and NFIX were selected as candidates for HIV latency. Additionally, TRAF4 was found to directly interact with NFIX.

Conclusions: The unique enrichment of histone modifications and its cross-talk in specific chromosomes might play a crucial role in the establishment and maintenance of HIV-1 latency.

PP 2.4

Exosomes from HIV-1 infected cells stimulate production of pro-inflammatory cytokines through TAR RNA.

G.C. Sampey[1], M. Saifuddin[1], A. Schwab[1], R. Barclay[1], S. Punya[1], M.C. Chung[1], R.M. Hakami[1], M. Asad Zadeh[1], B. Lepene[2], Z.A. Klase[3], N. El-Hage[4], M. Young[5], S. Iordanskiy[1], F. Kashanchi[1]

[1]Laboratory of Molecular Virology, George Mason University, Manassas, VA, USA, [2]Ceres Nanosciences, Manassas, VA, USA, [3]Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA, [4]Department of Immunology, Herbert Wertheim College of Medicine, Miami, FL, USA, [5]Department of Medicine, Women's Intra-Agency HIV Study, Georgetown University, Washington DC, USA

Background: The primary objective of the current study was to elucidate the mechanism(s) by which exosomes derived from HIV-1 infected cells control uninfected cells and assist in viral spread *in vitro* and *in vivo*. We hypothesized that unique viral RNA present in the exosomes of infected cells alter recipient cells impacting regulation of gene expression and establishment of inflammatory response.

Methods: Exosomes were isolated from culture supernatants and sera using differential ultracentrifugation or by nanotrap particle capture. Levels of the TAR RNA were quantified from purified exosomes by RT-qPCR. Functionality of TAR RNA on primary macrophages was determined using a cytokine array to determine alterations in cytokine production. Lastly, the mechanism(s) by which TAR activates naïve recipient cells was determined through various biomolecular techniques.

Results: We have demonstrated that exosomes isolated from culture supernatants of cell lines, primary latently infected cells and PBMC infected with HIV contained abundant levels of TAR RNA. Moreover, we found that the level of these exosomes can increase from donor cells under cART treatment. Functionally, we observed that TAR RNA can activate uninfected macrophages and increase cytokine production. Additionally, we show that TAR activates the TLR3 and IKK/NF-κB pathway in recipient uninfected cells.

Conclusions: As we have shown that viral RNA can be detected in exosomes from culture supernatants and patient sera, it strongly suggesting that the RNAs are in a protected environment and that exosomes originating from infected cells can exert functional influences on naïve bystander cells. Collectively our data indicates that infected cells under cART still secret TAR RNA containing exosomes and that these exosomes activate naïve recipient cells resulting in unwanted proinflammatory signals.

PP 2.5

Ingenol derivatives are potent reactivators of latent HIV

V. Planelles, A.M. Spivak, E.T. Larragoite, C.L. Novis, L.J. Martins A. Bosque

Division of Microbiology and Immunology, Department of Pathology, University of Utah, Salt Lake City, UT, USA

Background: HIV patients must indefinitely remain on ART due to the existence of a latent HIV reservoir. The "shock and kill" strategy has been proposed as a method to reactivate the latent HIV reservoir with latency-reversing agents (LRAs). The purpose of this research is to evaluate the abilities of ingenol and ingenol derivatives (ingenol 3,20-dibenzoate, ingenol mebutate, and ingenol-3-hexanoate (ingenol B)) to reactivate latent HIV.

Methods: HIV-1 latency was modeled in primary T cells using a replication competent virus (HIV-1_{NL4-3}). Ingenol and ingenol derivatives (10 uM, 1 uM, 100 nM, and 10 nM) were tested for their abilities to reactivate latent HIV. Additionally, iIngenol derivatives were tested in cells from aviremic patients via the Rapid *Ex Vivo* Evaluation of Anti-Latency (REVEAL) assay, in which virion-bound HIV-1 genomic RNA was quantified.

Results: All ingenol derivatives successfully reactivated latent HIV at comparable levels to αCD3/αCD28. Optimal reactivation and viability was seen with the addition of a 100 nM concentration of Ingenol derivatives. The ingenol core itself was devoid of activity. Additionally, ingenol 3,20-dibenzoate increased viral mRNA copies to a greater extent than panobinostat and other HDAC inhibitors *ex vivo*. All ingenol derivatives were found to induce release of select cytokines in a donor-dependent fashion, and to lack cytotoxicity in primary cells.

Conclusions: Ingenol derivatives are promising LRAs and our lab is pursuing novel methods to chemically engineer a library of ingenol derivatives with combinatorial substitutions modifying activity and specificity.

PP 2.6

LEDGF/p75 and Iws1 participate both cooperatively and independently to distinct steps of HIV transcription

S. Emiliani[1–3], A. Abdouni[1–3], M. Hamoudi[1–3], M. Naughtin[1–3], A. Gérard[1–3], E. Ségéral[1–3]

[1]INSERM, U1016, Institut Cochin, Paris, France, [2]CNRS, UMR8104, Paris, France, [3]Université Paris Descartes, Sorbonne Paris Cité, Paris, France

In human immunodeficiency virus (HIV)-infected individuals on antiretroviral therapy (ART), the persistence of the virus in latently infected cells is thought to be the barrier for cure of infection. The main latent reservoir is constituted of resting memory CD4+ T cells containing integrated proviruses that are transcriptionally silent. HIV utilizes the chromatin-associated protein LEDGF/p75 as a docking factor to selectively integrate its genome within active coding regions of the cell chromosome. Although the majority of integration events leads to viral gene expression, rapid silencing of integrated proviruses also frequently arises during infection of activated CD4+ T cells.

We have recently identified a novel complex involved in HIV latency that contains LEDGF/p75 together with transcription factors Iws1 and Spt6. Using biochemical approaches; we characterized the interaction interfaces between LEDGF/p75 and Iws1. We showed that in activated CD4+T cells, both LEDGF/p75 and Iws1 contributed to the maintenance of HIV latency. ChIP experiments revealed that LEDGF/p75 and Iws1 colocalized with RNAPII at the viral promoter in latently infected cells. Upon PMA stimulation of HIV transcription, Iws1 and RNAPII levels strongly increased within the HIV genome, whereas LEDGF/p75 distribution was not affected. In addition to HIV, we found that LEDGF/p75 and Iws1 are also recruited at the cellular c-Myc gene in Jurkat cells. LEDGF/p75 accumulates within the gene body

of c-Myc with a pattern similar to that observed for Iws1 and H3K36me3.

Thus, our results suggest that LEDGF/p75 and Iws1 both cooperatively and independently to distinct steps of chromatin transcription regulation.

PP 2.7

Constraints on the dynamics of HIV-1 lifecycle elucidated by treatment with an integrase inhibitor reveal a subset of cells with very slow integration

R.M. Ribeiro, F. Cardozo, A.S. Perelson

Los Alamos National Laboratory, Los Alamos, USA

Background: Mathematical models of HIV antiretroviral therapy have shed light into the biology of the virus. We compared viral kinetics under treatment with and without integrase inhibitors to gain new insights into this step of the lifecycle, which is crucial in defining latency and the potential to avoid new rounds of replication under shock-and-kill.

Methods: We developed a model of HIV infection explicitly accounting for proviral integration to analyze therapy containing integrase inhibitors. We validated the model with data from 28 HIV-1 infected participants treated with raltegravir (RAL) monotherapy for 9 days and compared these dynamics with data from nine HIV-1-infected individuals treated with a highly active quad-regimen including lopinavir-ritonavir, efavirenz, lamivudine, and tenofovir DF. Plasma HIV-1 RNA was measured frequently and we fitted the data using mixed-effect models.

Results: The model predicts two phases of viral decay early on (phase 1a and 1b), as we have shown recently for combination therapy (RAL and reverse transcriptase inhibitors). Phase 1a corresponds to loss of productively infected cells (half-life of ~19h) and phase 1b reflects the loss of infected cells with non-integrated provirus. When we analyzed the decay with the quad regimen, we also found a two-phase decay early on. However, here the slope of the second phase was slower than phase 1b with RAL. The model predicts that this second phase slope is due to loss of a small subset of cells with very slow integration, up to 100-fold slower than in activated short-lived infected cells.

Conclusions: Analyses of HIV viral dynamics under treatment allows us to analyze possible sources of infected cells. We speculate that the cells with very slow integration could be resting cells, which are a potential source of latently infected cells. In any case, the time window for the immune system to kill these infected, but not productive cells, is much longer than previously thought.

PP 2.8

The steroid receptor coactivators are targets for reactivation of HIV latency

B.C. Nikolai[1], Q. Feng[1], R.B. York[1], C.E. Foulds[1], E.B. Siwak[2], D.M. Lonard[1], A.P. Rice[2], B.W. O'Malley[1]

[1]Department of Molecular and Cell Biology, Baylor College of Medicine, Houston, TX, USA, [2]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA

Antiretroviral therapy (ART) is effective for restricting the onset of AIDS in HIV-infected individuals. Unfortunately, withdrawal results in viral rebound and HIV patients must maintain life-long therapy of the drug cocktail. Recent evidence suggests potential for pharmacological eradication of HIV, yet proviral transcriptional events involving host chromatin present a challenge that is relatively understudied. Modulation of the latter has therapeutic implications for controlled induction of proviral expression in latent cell populations resistant to ART, with potential for "shock-and-kill" eradication. Here, we reveal pharmacological targeting of the steroid receptor coactivator (SRC) family of transcriptional coactivators can potently activate HIV, and SRC-3 is an obligate host factor for efficient proviral transcription. A screen designed to alter SRC-3 intrinsic activity using nearly 360,000 compounds identified several pharmacological agents capable of inducing LTR-reporter based transcription. To confirm these results in a T-cell model of HIV latency, we employed the 2D10/d2GFP Jurkat latency (J-Lat) model to test proviral activation upon treatment with compounds targeting SRC-3. Two SRC-3 activating compounds double the number of GFP positive cells at micromolar concentrations, while lead molecule MCB613 displays at least 5-fold activation in the nanomolar (nM) range. The SRC-3 activating compound MCB613 is synergistic with known HIV activating compounds SAHA and JQ1 in 2D10 cells. MCB613 directly binds to SRC-3 and promotes secondary recruitment of histone acetyltransferases CBP and p300 *in vitro*. Moreover, chemical derivatives of MCB613 activate HIV d2GFP reporter activity at low nM ranges and have no toxicity in primary T cells and PBMCs. Collectively, these results underscore the importance of host coregulators in HIV transcription and identify novel targets for preclinical studies seeking to activate latent HIV reservoirs.

Session 3: Clinical virology of HIV persistence

PP 3.0

Allogeneic stem cell transplantation in HIV-1 infected individuals

A.M.J. Wensing[1], J.L. Diez Martin[2], G. Hutter[3], J. Kuball[1], M. Nijhuis[1], A. Saez-Cirion[4]; V. Rocha[5], J. Schulze zur Wiesch[6], J. Martinez-Picado[7,8]

[1]University Medical Center Utrecht, Utrecht, the Netherlands, [2]Hospital Gral. Univ. Gregorio Marañon, Madrid, Spain, [3]Cellex, Dresden, Germany, [4]Pasteur Institute, Paris, France, [5]Oxford University Hospital, UK, [6]University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [7]Hospital AIDS Research Institute irsiCaixa, Badalona, Spain, [8]ICREA, Barcelona, Spain

To date, the only and most compelling evidence of a medical intervention that has been able to cure HIV-1 infection (the "Berlin patient"), involved an allogeneic stem cell transplant (SCT) from a donor who was homozygous for CCR5Δ32. Although this high-risk procedure is only indicated for certain hematological malignancies, the strategy raised tremendous scientific potential to fully understand the bases of such success. Unfortunately no other successful long-term outcome of allogeneic SCT has been published since the Berlin patient. This may at least partly be explained by the relatively rare frequency of the homozygous CCR5Δ32 genotype coupled with the need for stringent HLA matching. Alternative strategies that allow for less stringent matching such as double cord transplantation, or single cord blood transplantation followed by the co-infusion of CD34+ cells from a third party HLA-mismatched donor may increase the chances of selecting a CCR5Δ32 donor. Detailed analysis of these cases should provide insight as to whether additional factors such as conditioning regimen, total body irradiation and graft versus host disease contributed to the eradication of the potentially infectious viral reservoir in addition to the lack of a functional CCR5 receptor.

During the last year EpiStem consortium has generated a prospective observational cohort of 15 cases of allogeneic SCT in HIV-1-infected individuals with life-threatening hematological malignancies around Europe. Five cases had a CCR5Δ32 donor and 10 had a CCR5wt donor. In three cases the donor cells came from a cord blood unit and in 12 cases from an adult donor. So far three patients have successfully passed the 6 months follow-up after transplantation, and six patients have died after transplantation, despite achieving full donor cell chimerism in some cases. Preliminary analysis of virological and immunological data from blood and tissue samples shows a systematic reduction of HIV-1 reservoirs to very low levels. Moreover, cord blood units in multiple European blood banks and adult donors have been genotyped for CCR5 to generate a registry of CCR5Δ32 available donors.

EpiStem aims to continue recruiting new cases, and systematically monitor them for extensive periods of time to explore the potential clues to understand viral reservoirs reduction and potential cases of HIV-1 eradication among these patients.

PP 3.1

Genetic and functional characterization of HIV-1 *Nef* gene from North Indian HIV-1 infected patients

J. Singh, R. Kapoor, I. Ronsard, V.G. Ramachandran, A.C. Banerjea

National Institute of Immunology, New Dehli, India

Background: Designing an ideal vaccine against HIV-1 has been difficult due to enormous genetic variability as a result of high replication rate and lack of proof reading activity of reverse transcriptase leading to emergence of genetic variants and recombinants. Genetic studies reveal that HIV-1 *Nef* gene shows extensive genetic diversity. Functional studies have been carried out mostly with Nef derived from subtype B pNL4-3 virus. The rationale of this study was to characterize genetic variations that are present in the *nef* gene from HIV-1 infected individuals from North-India and determine their functional implications.

Methods: Genomic DNA was isolated from PBMCs of HIV-1 infected patients and *nef* gene was PCR amplified with specific primers followed by cloning, sequencing and sequence analyses using bioinformatics tools for predicting HIV-1 subtypes, recombination events and conservation of domains. The unique representative variants were then characterized with respect to their ability to downregulate CD4 and MHC-1 expressed on cell surface.

Results: Phylogenetic analysis of nef variants revealed sequence similarity with consensus subtype B and B/C recombinants. Bootscan analysis of some of our variants showed homology to B/C recombinant and some to wild type nef B. High amino acid variations was observed among our most of the variants. dN/dS ratio revealed 80% purifying selection and 20% diversifying selection implying the importance of variable mutations of Nef variants. There were some variants that possessed mutations in the functional domains of Nef responsible for its CD4 and MHC-1 activity.

Conclusions: We observed enhanced biological activities in some of our variants may be the result of amino acid substitutions in their functional domains. In summary, the CD4 and MHC-1 downregulation activity of Nef must be used by virus to its maximum advantage.

PP 3.2

The synergistic effect of PKA activator and HDAC inhibitor to reactivate HIV-1 provirus from latently infected cells

B.S. Choi, H. Lim, K.C. Kim, Y. Shin, C.H. Yoon, C. Kang

Division of AIDS, Center for Immunology and Pathology, Korea National Institute of Health, Chung-buk, Republic of Korea

Background: Protein kinase A (PKA) activator cAMP as well as epigenetic modifying agent HDACi is known to induce HIV-1 reactivation from the latently infected cells. However, it remains incompletely understood if the combination of both agents results in synergistic effect.

Methods: ACH2 and NCHA1 cells, an HIV-1 latently infected cell line derived from A3.01 cells, were used. PKA activator (8-Bromo-cAMP, dibutyryl-cAMP) and epigenetic modifying agents HDACi (vorinostat, trichostatin A) were used to stimulate HIV-1 latently infected cells. Cellular toxicity was determined by cell viability assay. HIV-1 reactivation was measured by HIV-1 p24 level in the culture supernatant by ELISA and the intracellular HIV-1 p24 level by flow cytometer.

Results: Single treatment of 500 μM of dibutyryl-cAMP, 1 μM of vorinostat, or 1 μM of trichostatin A efficiently reactivated HIV-1 provirus in both ACH2 and NCHA1 cells without any cytotoxicity. When dibutyryl-cAMP was treated with vorinostat or trichostatin A, the level of HIV-1 p24 in the supernatant were 5.6-fold (+ vorinostat) or 3-fold (+ trichostatin A) higher in ACH2 cells, and 23-fold (+ vorinostat) or 16-fold (+ trichostatin A) higher in NCHA1 cells compared to those treated with dibutyryl-cAMP alone. In addition, intracellular p24 levels were also dramatically increased in both co-treated HIV-1 latently infected cell lines compared to those of dibutyryl-cAMP single treatment. The phosphorylation of CREB by activated PKA and CREB-CBP interaction were found to be associated with the observed HIV-1 reactivation from the latently infected cells. These data demonstrate that combined approaches of PKA activator and HDACi result in synergistic HIV-1 reactivation from the latently infected cells.

Conclusions: The combined treatment of PKA activator and HDAC inhibitor is effective in reactivating HIV-1 from the latently infected cells and it may provide a promising therapeutic approach for reducing the level of HIV-1 reservoir.

PP 3.3

Mild cognitive impairment in a clinically latent HIV-1 patient population

K.N. Devlin[1], T. Giovannetti[1], W. Dampier[2–4], V. Pirrone[2,3], M.R. Nonnemacher[2,3], E. Schell[1], C. Lamberson[1], J.R. Kurczewski[1], J.M. Jacobson[5,6], D.J. Libon[7], B. Wigdahl[2,3,5]

[1]Department of Psychology, Temple University, Philadelphia, PA, USA, [2]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [3]Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [4]School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA, [5]Center for Clinical and Translational Medicine, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [6]Division of Infectious Diseases and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA, [7]Department of Neurology, Drexel University College of Medicine, Philadelphia, PA, USA

Background: Given increased longevity of patients with HIV infection, there is interest in the intersection of HIV and age-associated cognitive disorders. Mild cognitive impairment (MCI) is associated with increased dementia risk and is defined as cognitive decline greater than typical aging. We used modified Jak *et al.* (2009) criteria to identify the prevalence and neuropsychological (NP) profile of MCI among HIV-1-infected patients on antiretroviral therapy (ART).

Methods: 171 HIV-1-infected patients (89% black; 67% male) completed NP tests of verbal fluency, working memory, executive function, episodic memory, and psychomotor speed. Scores adjusted for age, race, gender, and education were subjected to principal component analysis (PCA).

Results: A 3-factor structure was obtained (66% of variance) consisting of motor (23%), memory (22%), and executive tests (21%). Three groups were constructed: 43 patients (25%) meeting criteria for MCI, defined as a score at least 1 SD below normative values on at least two PCA-derived scales; 52 (30%) who scored below cutoff on only one PCA-derived scale; and 76 (44%) who scored above cutoff on all scales. Within-group t-tests indicated that the MCI group demonstrated poorest performance on memory tests ($P<0.001$). The groups did not differ in age (52±8), education (12±2), years seropositive (19±8), ART duration (12±7), or CD4 count (653±365). However, the MCI group reported greater functional difficulties than the other groups ($P<0.01$).

Conclusions: Nearly a quarter of our sample met criteria for MCI. Patients with MCI did not differ from patients without MCI in demographics or CD4 count; however, they reported greater functional difficulties. Poor memory typified the NP profile in our MCI group. This NP profile along with greater functional deficits in this group suggests possible links between our HIV-1-infected patients with MCI and increased risk for dementia. Longitudinal assessment is needed to determine this outcome.

PP 3.4

Towards HIV reservoir measurements in ART-treated patients: integrated DNA quantification and HIV-1 clone expansion in a Japanese cohort

K. Stanoeva[1], A. Fukuda[1], A. König[2], Y. Satou[1,*], S. Matsushita[1,*]

[1]Center for AIDS Research, Kumamoto University, Kumamoto, Japan, [2]Faculty of Statistics, Technical University Dortmund, Germany

*contributed equally

Background: Despite successes of ART, persistence of HIV reservoir and its measurement remain unsolved issues. Understanding the integrated viral DNA and the mechanisms of persistence are important for the future HIV eradication. Therefore, our aim was to evaluate the integrated HIV proviral DNA(reservoir in PBMC, proviral load-PVL) in a cohort of Japanese patients on ART.

Methods: A retrospective study of PBMC samples collected from around 100 patients of Kumamoto University hospital in the period 1996–2015 was performed. Patients were subdivided into groups according to current ART and its total length. PBMC DNA samples were run in a qPCR and percentage of integrated proviral DNA was calculated. Ten patients with long-term stable ART were selected and integration sites analysis via LM-PCR and NGS (Illumina) was performed at two time points: early therapy and recently. Integration sites and expansion of the HIV-1 clone were traced under *in vivo* conditions of suppressive long-term ART.

Results: Levels of integrated proviral DNA were low (mean PVL for the 10 patients: 0.016%) and remained relatively stable over the years under ART and with similar trends when comparing patients receiving different ART protocols. Integration site analysis was performed with PBMC DNA samples at a recent time point *vs.* upon early ART initiation point and the result showed stable levels of clone expansion with similar trends among the ART groups.

Conclusions: Our data identifies some of the patterns of reservoir persistence in PBMC and its evolution under successful long-term ART. Despite suppressive ART the levels of integrated DNA remain stable and the expansion of the HIV-1 clone successfully continues. The latter might suggest more precise therapy, better clinical monitoring tools or both are needed for routine evaluation and management of the HIV reservoir.

PP 3.5

Significance of a novel residual HIV-1 variant longitudinally detected in plasma of a patient on suppressive antiretroviral therapy

G.K. Sahu, S. Rassler, N. Khoury, G. Skowron

Division of Infectious Diseases, Department of Medicine, Roger Williams Medical Center, Providence, RI, USA

Background: Residual viruses (RVs) persist in plasma below 20–50 vRNA copies/mL in patients on suppressive ART. The biological characteristics and the mechanism of production of these viruses still remain unclear. In this study, we have attempted to characterize these viruses *in vitro* by reconstructing their genomes from residual plasma vRNA isolated from a patient on prolonged ART.

Methods: A 57-year-old white (non-Hispanic) male HIV+ patient (identified here as patient G) with plasma viral loads of <50 vRNA copies/mL on ART was recruited in November 2012 in the RWMC clinic for an HIV-1 reservoir study by following an IRB-approved protocol. We longitudinally collected a total of six 50 mL blood samples from him and isolated residual plasma vRNAs from each sample. Residual viral genomes were reconstructed by an overlapping RT-nested PCR method, followed by sequence analyses and testing for their functionalities *in vitro*.

Results: We found that the reconstructed molecular clones of RVs lack antiretroviral drug-resistant mutations as well as APOBEC-induced G-to-A hypermutations. These vDNA clones when transfected into TZM-bl cells could release HIV-p24 into the culture media at extremely low levels, but importantly, HIV-p24+ media showed infectivity in the TZM-bl reporter cells. The low level virus production was due to a unique mutation (GU-to-GC) present in the conserved 5′-major splice donor (MSD) motif of the corresponding vRNAs. This novel viral variant was detected in the patient's plasma intermittently over a period of 28 months.

Conclusions: Our data suggest that RVs circulating in plasma during ART below 50 copies/mL in patient G have potential to spread infection in the body after therapy interruption. The lack of drug-resistant mutations and G-to-A hypermutations in RVs, as well as the longitudinal detection of viral 5′-MSD variant with reduced fitness in the patient's plasma strongly reinforce the notion that RVs are released from one or more stable "active" reservoirs in the body during therapy. The viral 5′-MSD mutation could be used as a unique tool in locating the source of residual plasma viruses in patient G.

PP 3.6

Impact of a decade of sustained antiretroviral therapy started during HIV-1 seroconversion on blood and gut reservoirs

S. Kinloch[1], E. Malatinkova[2], W. De Spiegelaere[2], P. Bonczkowsk[2], M. Kiselinova[2], K. Vervisch[2], W. Trypsteen[2], M. Johnson[1], D. de Looze[2], L. Vandekerckhove[2]

[1]Royal Free Hospital, Division of Infection and Immunity, Royal Free Campus, University College London, London, UK, [2]HIV Translational Research Unit, Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University and University Hospital Ghent, Ghent, Belgium.

Background: Early ART limits the viral reservoir size and enhances the possibility of a functional cure. We have assessed the impact of such a strategy over a decade of successful treatment on the HIV-1 DNA reservoir.

Methods: Cross-sectional study of HIV-1 reservoir size (total and integrated HIV-1 DNA) and dynamics (2-LTR circles and cell-associated HIV-1 unspliced RNA (usRNA)) performed using digital PCR in PBMCs of 84 HIV-1 patients (four cohorts: long-term treated patients with ART initiated during seroconversion, SRCV on ART, n=25; or chronic infection, Chronic ART, n=32; long-term non-progressors, LTNP, n=17; and ART-naïve recent seroconverters, Recent SRCV, n=10). Time on ART, CD4 count and CD4/CD8 ratio were collected. Rectal biopsies were taken on one occasion.

Results: Median total HIV-1 DNA copies were: 92, 48, 137 and 1901 copies/10^6 PBMCs in SRCV on ART, LTNP, Chronic ART and Recent SRCV, respectively. Lower levels of total (P=0.041) and integrated HIV-1 DNA (P=0.003) were detected in SRCV on ART versus the Chronic ART cohort, however, not as low as in LTNP. Integrated HIV-1 DNA levels were comparable in the Recent SRCV and Chronic ART cohorts. Levels of usRNA were lower in early compared to chronically treated patients (P=0.007). SRCV on ART exhibited a higher CD4/CD8 ratio as compared to the chronic ART cohort (P=0.009). Rectal biopsies showed comparable levels of HIV-1 DNA in the two ART-treated cohorts and LTNPs.

Conclusion: A beneficial effect of early treatment on the reservoir size, residual transcription and CD4/CD8 ratio is present with early treatment intervention after a decade of successful ART.

PP 3.7

Integrated HIV DNA accumulates more rapidly in non-controllers than controllers

M.R. Pinzone, E. Graf, L. Lynch, B. McLaughlin, F.M. Hecht, G. Nunnari, S. Migueles, U. O'Doherty

Division of Infectious Diseases, University of Catania, Italy,

Background: HIV persistence in latent reservoirs represents a major barrier to HIV eradication. No study has directly addressed the dynamics of HIV reservoir accumulation over time in patients off ART.

Methods: Levels of integrated HIV DNA, a surrogate for reservoir size, were longitudinally measured in eight elite controllers (EC), six

chronic progressors (CP) and four patients on ART. Genomic DNA was extracted from PBMCs and integrated HIV DNA measured by Alu-HIV PCR. Cytotoxic responses were monitored over time in EC measuring infected CD4 T-cell elimination (ICE) and granzyme B activity. Differences between time points were tested using paired t-tests or Wilcoxon Signed-Rank.

Results: Integrated HIV DNA increased over time in EC, from 5.1 (IQR 1.83–17.5) to 16.9 (IQR 5.6–40.2) copies/million PBMCs after 4.13 years (P=0.0078) and increased more dramatically in CP, from 73.5 (IQR 29.75–118) to 1570 (IQR 670–1971) copies/million PBMCs after 6.1 years (P=0.0019), but not in patients on ART, 1179 (IQR 632–1287) *vs.* 1038 (IQR 555–1074)/million PBMCs after 4.5 years. The median annual fold-change was significantly higher in CP compared to EC (10.5 *vs.* 1.28 per year, P=0.006). CTL function as assessed by ICE (72.3–75.4%, P=0.44) and granzyme B activity (43–56%, P=0.13) did not significantly change over time in EC.

Conclusions: In the absence of ART, integrated HIV integration accumulates over time both in CP and, more slowly, in EC despite robust CTL activity. Low, albeit continued reservoir accumulation in EC might be due to a ongoing, low level rounds of replication in immunologic sanctuaries where CTL are excluded. Meanwhile, the rate of accumulation is more brisk in chronic progressors who have poor CTL function. The increase in integrated HIV DNA over time further reinforces the importance of treating non-controllers early to limit reservoir size and raises the question of whether it is reasonable to treat controllers at a certain threshold.

PP 3.8

SNPs within the HIV-1 LTR associate with increased virus persistence

N.T. Sullivan[1,2], M.R. Nonnemacher[1,2], V. Pirrone[1,2], R. Feng[3], B. Moldover[4], W. Dampier[1,2], S. Passic[1,2], J. Williams[1,2], B. Aiamkitsumrit[1,2], W. Zhong[1,2], B. Blakey[1,2], S. Shah[1,2], J.M. Jacobson[1,2,5,6], B. Wigdahl[1,2,5]

[1]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [2]Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [3]Department of Biostatistics and Epidemiology, Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA, [4]B-Tech Consulting, Ltd., Philadelphia, PA, USA, [5]Center for Clinical and Translational Medicine, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [6]Division of Infectious Disease and HIV Medicine, Department of Medicine, Drexel University College of Medicine, Philadelphia, PA, USA

Background: HIV-1 is continuously mutating, even in well-controlled individuals on cART. SNPs in the LTR are of interest due to their potential impact on the regulation of viral gene transcription and replication. Studies have previously demonstrated that SNPs in particular transcription factor (TF) binding sites can significantly alter viral transcription and correlate with changes in disease severity. To understand the molecular evolution of HIV-1 in patients longitudinally prior to and after initiation of cART, the Drexel Medicine CNS AIDS Research and Eradication Study (CARES) Cohort in Philadelphia, PA was used to identify SNPs correlated with clinical disease parameters.

Methods: PBMCs were isolated from patients for PCR, sequencing and bioinformatic analysis. Linear mixed model analysis was used to identify SNPs that correlated with disease parameters. Jasper analysis predicted the TF binding surrounding LTR nucleotide position 108 and electrophoretic mobility shift assay (EMSA) determined the direct TF/DNA interactions in T cells.

Results: Numerous hotspots were identified throughout the LTR. Position 108 change from A to G, residing in a confirmed COUP/AP1 binding site, was highly correlated with increase in viral load and a decrease in CD4+ T cell counts, even in individuals on continuous cART. EMSA demonstrated differential TF/DNA binding. Oligonucleotides with an A at position 108 showed three district complexes and a G at position 108 showed four distinct complexes. Not only was a new complex formed with position 108 but the overall complex formation was increased. Supershift analysis supports the presence of numerous TFs as predicted by the multiple TF binding sites predicted in this region by JASPER analysis.

Conclusions: Even on cART, changes in the prevalence of SNPs in the LTR can be correlated with alterations in clinical disease severity involving enhanced viral persistence that may be driven by increased levels of transcription.

PP 3.9

Detection of replication competent HIV from latently infected CD4+ T cells

A. Fun, M.R. Wills, A.M.L. Lever

Department of Medicine, University of Cambridge, UK

Background: Accurate measurement of the latent HIV reservoir is critical for the evaluation of novel eradication strategies. Drawbacks of the 'standard' viral outgrowth assay are the high cost and poor sensitivity due to low expression of CCR5 on most donor PBMCs. RIVER, a UK wide collaborative on eradication is the first phase II trial designed to test a combination of a prime-boost vaccination strategy followed by treatment with an HDAC inhibitor in order to reduce the size of the latent HIV reservoir. This study aimed to develop a reliable assay to quantify the replication competent latent HIV load in CD4+ T cells with a significant cost reduction and increased sensitivity for R5-tropic virus.

Methods: Resting CD4+ T cells were obtained from whole blood using density-gradient PBMC isolation followed by negative selection of CD4+ T cells and depletion of activated cells. The resulting resting CD4+ T cells were activated with PHA and allogeneic irradiated PBMCs and co-cultured with a clonal cell line expressing CD4, CXCR4 and CCR5. Viral production was detected by HIV-1 p24 ELISA.

Results: The new assay has significantly lowered costs from £3800 to £750 per assay and is more sensitive than co-culture with donor PBMCs. Replication competent virus was detected in 19/23 patients. Re-stimulation with PHA of negative cultures often resulted in viral outgrowth in additional cultures. However, in samples from patients without a detectable reservoir all cultures remained negative upon re-stimulation. The robustness of the assay was validated using eight samples from the same patient taken over a period of 4 months.

Conclusion: The cell line based outgrowth assay reduced both labour and cost and has an improved sensitivity compared to a donor PBMC based assay. Multiple rounds of PHA stimulation result in a larger proportion of the latently infected population being activated, indicating that the standard assay is only a measure of the minimal reservoir size.

PP 3.10

Low viral reservoir treated patients (LoViReT): clinical predictors of low HIV-1 DNA

M. Salgado[1], C. Gálvez[1], J. Dalmau[1], J. Carrillo[1], V. Urrea[1], B. Clotet[1–3], J. Blanco[1,3], J. Martinez-Picado[1,4]

[1]AIDS Research Institute IrsiCaixa, Badalona, Spain, [2]Fundació Lluita contra la SIDA, Badalona, Spain, [3]Universitat de Vic-Central de Catalunya, UVIC-UCC, Vic, Spain, [4]ICREA, Barcelona, Spain

Introduction: Small sized reservoirs are predominantly found in HIV-1 controllers and individuals who received cART during primary HIV-1 infection. Herein, we established a cohort of 'Low Viral Reservoir Treated' patients (LoViReT) with a very low HIV-1 DNA levels in peripheral blood despite having initiated cART during chronic HIV-1 infection. We looked for clinical and immunological variables that could explain the low level proviral reservoir observed in these subjects.

Methods: We recruited 319 patients on cART for ~3 years. HIV-1 proviral reservoir was analyzed in PBMCs with ddPCR using two different primer sets, gag and ltr. HIV-1- env trimers antibody titers were determined using flow cytometry. Statistical analysis was carried

out with non-parametric methods and Spearman's correlation coefficient.

Results: Total HIV DNA was detected in 96.2% of the samples, with a median proviral DNA of 136 copies/10^6 cells (IQR: 63–301). Patients were stratified in two groups: above and below this median. We observed lower HIV-1 DNA in younger patients with shorter time from diagnosis and lower levels of zenith plasma viremia. Additionally, low reservoir was associated with higher nadir CD4 T cells and higher CD4/CD8 ratio. We also found that patients who initiated therapy with raltegravir harbored a significantly lower reservoir.

LoViReT patients represented 20 subjects (6.3%) with HIV-1 DNA<10 copies/10^6 cells, including 12 undetectable patients. These subjects were younger, had been diagnosed more recently, and had higher nadir CD4 T cells when compared with the rest of patients. We also observed that LoViReT patients had smaller envelope trimer-specific antibody titers than patients with high latent reservoir.

Conclusion: LoViReT patients harbor an extremely low HIV-1 proviral reservoir during suppressive cART despite not being involved in any HIV eradication-based strategy. A more conserved immune system seems to characterize this infrequent cohort.

PP 3.11

Population genetic approaches to estimating the size of the HIV reservoir

J. Hattori[1], V. Boltz[1], W. Shao[2], M. Kearney[1], F. Maldarelli[1]

[1]HIV Drug Resistance Program, National Cancer Institute, Frederick, MD, USA, [2]Leidos Biomedical Research, Frederick, MD, USA

Introduction: Understanding the genetic characteristics of HIV populations is essential to characterizing the size the replicating HIV population as combination antiretroviral therapy (cART) is initiated. The size of the replicating viral population, which represents the reservoir of infectious HIV, has been reported to be substantial prior to cART, but variation in size among patients has not been extensively investigated. We used next generation sequencing (NGS) to obtain more accurate estimates of replicating population sizes and to investigate range in sizes of replicating HIV reservoirs prior to cART.

Methods: Longitudinal plasma samples from five chronically infected antiretroviral naïve individuals were subjected to NGS using a new library construction and processing strategy to obtain sequences with the Illumina platform of c. 500-nt within the HIV RT. Replicating population sizes were estimated using temporal and coalescent methods, and linkage disequilibrium measures calculated (DNASP).

Results: Plasma samples from antiretroviral naïve patients (n=5) with chronic HIV infection (duration of infection>1 year, median CD4=546 cells/μL, viral RNA=3.9 $\log_{10}$ copies/mL) were subjected to NGS, >15,000 sequences (average of 1,277 sequences/time point) were obtained. Estimates of replicating population sizes were consistently >1E5, and a c. 100 fold range (1.7E5–2.4E7) of infectious cells per individual was detected among patients studied. Linkage analyses revealed only 3–6 pairs of alleles in linkage disequilibrium (Fisher exact P<<0.0001) per patient time point, indicating extensive recombination.

Conclusions: HIV populations are well mixed prior to cART and have a broad range of replicating population sizes among patients. With cART, the range in sizes of HIV reservoirs capable of replicating is expected to be similarly wide.

PP 3.12

Cell-associated HIV-1 DNA and viral load evaluation in HIV-1 infected children before and after combination antiretroviral therapy initiation

M. Moragas, P. Aulicino, D. Mecikovsky, R. Bolgna, L. Sen, A. Mangano

Hospital de Pediatría S.A.M.I.C. "Prof. Dr. Juan P. Garrahan", Buenos Aires, Argentina

Background: Viral load (pVL) and CD4+ T cells counts are the main and effective measurements for HIV-1 infection monitoring. However, these parameters do not manifest the behavior of the viral intracellular DNA forms (CA-HIV-1 DNA) in the HIV-1 reservoirs. The aim of this study was determine CA-HIV-1 DNA levels and study its association with pVL in HIV-1 infected children before and after combination antiretroviral therapy (cARV).

Methods: Twenty-one children infected with HIV by vertical transmission, followed up from 2008 to 2013, were studied. Samples were tested prior to cART (pre-cARV) and at 18 months (± 6 months) after cARV initiation (post-cARV) with lopinar/ritonavir and abacavir, didanosine or lamivudine. CA-HIV-DNA levels were quantified by a semi-nested real time PCR with Taqman probes targeting LTR-gag region in PBMCs (LLOD 8 copies/10^6 PBMC). The pVL data was collected.

Results: The median pVL at pre-cARV and at post-cARV was 5.7 (IQR 5.2–6.1) and 1.7 (IQR 1.7–2.2) $\log_{10}$copies ARN/mL, respectively. The median CA-HIV-DNA levels at pre-cARV were 1204 (IQR 346.8–2182.0) copies/10^6 PBMC, while at post-cARV they were 164.2 (IQR 63.9–359.3) copies/10^6 PBMC. This showed an important reduction in the levels of both variables: 70% in pVL and 86% in CA-HIV-1 DNA levels. Correlation between CA-HIV-1 DNA and pVL at pre-cARV was not found, while at post-cARV a positive correlation was found (r=0.49, P<0.05). Of 17 children studied at post-cARV, viral suppression, defined as consecutive pVL<400 copies HIV-1 ARN/mL, was observed in 14 (78%), with a median CA-HIV-1 DNA levels of 110 (IQR 63.3–1026) copies/10^6 PBMC. The four children who did not have viral control, median CA-HIV-1 DNA levels were 308.4 (IQR 239.6–370.4) copies/10^6 PBMC.

Conclusions: Our results suggest independent behavior between pVL and CA-HIV-1 DNA levels before treatment initiation. However, after 1 year of cARV, a significant reduction of both CA-HIV-1 DNA and pVL was observed and also a proportional counts of both.

PP 3.13

Post-mortem analysis of HIV-1 reservoir after allogeneic transplantation using stem cells

M. Nijhuis[1], A. Bruns[1], M. Salgado[2], A.J. Stam[1], P.M. Ellerbroek[1], K. Tesselaar[1], P. van Ham[1], T.C.M. de Jong[1], G. Hutter[3], S.S. Zeerleder[4], J.T. van der Meer[4], L.L.A. Brosens[1], J. Martinez-Picado[2,5], J. Kuball[1], A.M.J. Wensing[1] for the EpiStem Consortium

[1]University Medical Center Utrecht, Utrecht, the Netherlands, [2]AIDS Research Institute irsiCaixa, Badalona, Spain, [3]Cellex, Dresden, Germany, [4]Academic Medical Center, Amsterdam, the Netherlands, [5]ICREA, Barcelona, Spain

Background: In the Berlin patient, cure of HIV infection was observed following stem cell transplantation (SCT) with homozygous CCR5Δ32 donor cells. In contrast, in the Boston patients, transplanted with a regular donor, rebound of HIV was observed after treatment interruption despite loss of detectable HIV DNA in PBMCs. It is unclear from which reservoir HIV rebounded.

Methods: We investigated the impact of SCT on the size of the HIV-1 reservoir in EpiStem patient #11. SCT for acute myeloid leukemia was performed using an HLA-matched unrelated CCR5Δ32 donor. The patient was re-transplanted with cells from a CCR5Δ32 heterozygous donor after graft failure at 10 weeks. Before SCT we performed: (1) phenotypic and ultradeep genotypic (gp120-V3) co-receptor analysis; (2) quantitative analysis of the HIV reservoir in different CD4+ T-cell subsets ($T_{N,}$ T_{CM}, T_{TM}, T_{EM} and T_{SCM}) and bone marrow using ddPCR; (3) quantitative viral outgrowth assay (qVOA); (4) single copy assay (SCA) on plasma. Post-SCT viral dynamics and post mortem viral reservoir analysis on tissue were performed using ddPCR.

Results: Patient #11 was on effective cART for 18 years and harboured a subtype B CCR5-tropic virus population (false positive rate, 33–49%). Before SCT, no viral RNA was detected in routine diagnostics while 2 copies/mL plasma were observed in SCA. qVOA showed presence of replication competent virus (1.6 IUPM). Proviral DNA was detected in PBMCs (295 copies/10^6 cells), bone marrow (80 copies/10^6 cells), and CD4+ T cells with stem cell-like properties

(490 copies/10^6 cells), naive T cells (579 copies/10^6 cells) and memory T-cell subsets (T_{CM}, T_{TM} and T_{EM}, 2237, 2854, and 4687 copies/10^6 cells, respectively). Five weeks after the second SCT, at time of full chimerism, proviral DNA declined to undetectable levels (<1 copies/10^6 PBMCs). Unfortunately, the patient thereafter died with a pneumonitis. Post mortem analysis revealed that proviral DNA could be detected in lymph node tissue (40 copies/10^6 CD4 cells) but not in ileum.

Conclusion: Within EpiStem, we show that after 18 years of effective cART HIV DNA can readily be detected in various T-cell subsets. In the neutropenic phase post-SCT, HIV DNA could no longer be detected in PBMCs nor in ileum. In contrast, viral DNA was still found in lymph node tissue indicating that this tissue may serve as a long-standing viral reservoir after SCT.

PP 3.14

HIV antibodies as markers of HIV systemic reservoir and replication activity

S.M. Keating, V. Jain, S.G. Deeks, C.D. Pilcher, M.P. Busch

Blood Systems Research Institute, CA, USA, Department of Laboratory Medicine, University California San Francisco, CA, USA, Department of Medicine, UCSF, CA, USA

Background: Sensitive antibody (Ab) measurements are used to determine HIV infection. These tests can be modified to identify individuals who are within the seroconversion period and recently infected. Using these assays, we have shown significant decreases in Ab measures due to viral suppression or control. Longitudinal specimens from treated individuals can demonstrate Ab seroreversion due to reduction of circulating virus and viral reservoirs. We hypothesize that the concentration of Abs correlates with viral load and viral reservoir.

Methods: Modified HIV Ab assays were used to measure quantity and avidity of HIV Ab: less-sensitive (LS: dilution in buffer; increased cutoff) and avidity modifications (AI) VITROS Anti-HIV1+2 and the Limiting Antigen Avidity (LAg) assay. We investigated 29 early-treated (ET) and 35 late-treated (LT) infected individuals at five timepoints before and after treatment. We measured changes of Ab over time, viral load measurements, and cell-associated RNA (CA-R) and proviral DNA (PV-D) measurements at the final timepoints.

Results: Ab from pre-treatment timepoints from chronically infected individuals correlate with viral load (LS- and AI VITROS $P<0.005$; LAg $P<0.05$). The seroreversion in all Ab are faster in the LT compared to the ET group ($P<0.001$) and for the LT, Ab concentrations continue to decline with viral suppression. The PV-D and CA-R measurements correlate with LAg and LS-VITROS (both $P=0.01$). These differences were not observed in the ET or LT subgroup analysis.

Conclusions: There is a reduction in HIV Ab concentration and avidity with time on treatment and this correlates with pre-ART viral copies and post-ART viral reservoir. Measuring the quantity and quality of HIV Ab are a useful indirect marker of reservoir size/activity for monitoring viral persistence in eradication and treatment intensification studies.

PP 3.15

Ultradeep sequencing characterization of HIV-1 diversity in primary infection

C. Delaugerre, G. Gaube, M. Salmona, S. Gallien, M.L. Chaix

Hôpital Saint Louis, INSERM U941, Paris, France

Background: HIV-1 diversity during primary infection is associated with viral immune control. We assay the genetic diversity of the *envelope* gene V3 region using next-generation sequencing (NGS) in different compartments in patients at the time of primary infection.

Methods: Quantification of HIV-1 DNA in peripheral blood mononuclear cells (PBMC, $n=7$), non-spermatozoid cells (NSC, $n=3$), and lymphoid cells of the rectal tissue ($n=3$) was performed in eight patients at the time of primary infection (7/8 were MSM, 1 Fiebig II, 2 Fiebig IV and 5 Fiebig V). HIV-1 RNA in plasma (PL, $n=8$), seminal fluid (SF, $n=8$) and saliva (SAL, $n=3$) was also quantified. NGS of viral DNA and RNA was performed on amplicons (408pb) using Roche/454 GS-Junior. Generated sequences were analyzed by: (a) calculating the maximal diversity index (MDI); (b) reconstructing *in silico* variants (haplotypes); (c) building Neighbor Joining trees; (d) and searching for compartmentalization using tree and pairwise distance based tests. A threshold of 1% was chosen for considering a haplotype.

Results: Median [IQR] HIV-1 DNA was 4.1 [3.1–4.1] log/10^6 PBMC and 3.7 [3.5–3.8] log/10^6 rectal cells. Median HIV-1 RNA was 6.2 [5.5–6.95] log/mL in PL, 4.9 [4.2–5.3] log/mL in SF, and 4.88 [4.4–5.3] log/mL in SAL. For each patient, a median of 4550 sequences per compartments were analyzed. The median MDI per patient varied from 0.7% to 6.6% suggesting a very low diversity, confirmed by the clonal aspect of all the phylogenetic trees. A median of five haplotypes [range: 3–19] per compartments was created. In the earliest stages of infection, a major haplotype was present in all compartments, representing more than 70% of the sequences. Four significant viral compartmentalization events were found.

Conclusions: Viral dissemination and massive replication occur within the first month after HIV infection. A homogenous clonal viral population in each compartment and between compartments was demonstrated using deep sequencing.

PP 3.16

Correlation between HIV-2 RNA and HIV-2 total DNA levels

C. Charpentier[1–3], M. Bertine[1–3], G. Collin[1–3], F. Damond[1–3], V. Avettand-Fenoel[4], J.C. Plantier[5], A. Storto[1], M. Naudin[6], S. Matheron[1,2,7], D. Descamps[1–3], and the HIV-2 ANRS CO5 Cohort

[1]INSERM, IAME, UMR 1137, Paris, France, [2]Université Paris Diderot, IAME, UMR 1137, Sorbonne Paris Cité, Paris, France, [3]AP-HP, Hôpital Bichat-Claude Bernard, Laboratoire de Virologie, Paris, France, [4]Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, EA 7327, Paris, France; Assistance Publique-Hôpitaux de Paris, Service de Virologie, Hôpital Necker-Enfants Malades, Paris, France, [5]Laboratoire de Virologie, CHU Charles Nicolle, Rouen, France; EA 2656 GRAM, Université de Rouen, Rouen, France; Laboratoire associé au Centre National de Référence du VIH, CHU Charles Nicolle, Rouen, France, [6]INSERM, ISPED, Centre INSERM U897-Epidemiologie-Biostatistique, Bordeaux, France Université Bordeaux, ISPED, Centre INSERM U897-Epidemiologie-Biostatistique, Bordeaux, France, [7]AP-HP, Hôpital Bichat-Claude Bernard, Service de Maladies Infectieuses et Tropicales, Paris, France.

Background: Few data are available regarding HIV-2 reservoir. The aim of the study was to assess the size of reservoir in different populations of HIV-2-infected patients.

Methods: HIV-2 total DNA was assessed in ARV-naïve and ARV-treated patients in virological failure (VF) included in the HIV-2 ANRS CO5 cohort. HIV-2 total DNA and RNA quantifications were performed using "in-house" real-time PCR assays: adapted from the HIV-2 RNA Biocentric kit with a LOQ=7.5 copies/PCR for DNA and with a LOQ=100 copies/mL for RNA.

Results: Among the 57 ARV-naïve patients, median CD4-cell-count was 475/mm^3 (IQR=381–659), plasma viral load (pVL) and HIV-2 total DNA were below the LOQ in 74% and in 12% of the patients, respectively. Median pVL was 1,458 copies/mL (IQR=313–3,574); median HIV-2 total DNA was 2.7 $\log_{10}$ copies/10^6 PBMC (IQR=2.5–3.2). No difference was observed in HIV-2 total DNA or RNA values between patients infected with group A and with group B ($P=0.22$; $P=0.06$).

Among the 50 patients with VF, treated since a median of 8 years (IQR=4–13), median CD4-cell-count was 232/mm^3 (IQR=137–361), 14% and 2% had pVL and HIV-2 total DNA below the LOQ, respectively. Median pVL was 832 copies/mL (IQR=192–4,011) and median HIV-2 total DNA was 3.2 $\log_{10}$ copies/10^6 PBMC (IQR=2.6–3.5). HIV-2 total DNA and RNA values were significantly higher in patients infected with group A than with group B (3.3 *vs.* 2.7 $\log_{10}$

copies/10^6 PBMC, *P*=0.03; 1,658 *vs.* 659 copies/mL, *P*=0.04). We observed a strong positive correlation between HIV-2 RNA and DNA levels: r=0.74, CI95%=0.57–0.85.

In ARV-treated patients, a trend to a lower proportion of patients with HIV-2 total DNA below the LOQ and a significant higher median of HIV-2 total DNA were observed than in ARV-naïve patients (*P*=0.06; *P*=0.04; respectively).

Conclusions: This is the first description of correlation between HIV-2 RNA and DNA levels. The size of reservoir was significantly higher in ARV-treated patient in VF than in ARV-naïve patients, as described in HIV-1 infection.

PP 3.17

Integrated and total HIV-1 DNA can predict quantitative viral outgrowth in patients on long-term ART

W. De Spiegelaere[1], M. Kiselinova[1], M.J. Buzon[2,3], E. Malatinkova[1], M. Lichterfeld[2,3], L. Vandekerckhove[1]

[1]HIV Translational Research Unit (HTRU), Department of Internal Medicine, University and University Hospital Ghent, Ghent, Belgium, [2]Infectious Disease Division, Massachusetts General Hospital, Boston, MA, USA, [3]Ragon Institute of MGH, MIT and Harvard, Boston, MA, USA

Background: Accurate markers to assess the replication competent HIV reservoir are urgently needed to support HIV-1 cure efforts. Despite the development of diverse PCR and cell culture-based assays, few comparative studies have been performed on patient samples.

Methods: We designed a study to evaluate and compare HIV-1 reservoir markers in 25 patients. These were selected from a previous study (*n*=164) in order to select patients with a high, intermediate or low HIV-1 burden, based on total HIV-1 DNA and cell associated HIV-1 RNA. Patients were on ART for a median of 9 years (IQR 6–12). Total and integrated HIV-1 DNA, unspliced (us) HIV-1 RNA, and 2LTR circles were quantified by digital PCR in peripheral blood, and a viral outgrowth assay (VOA) was performed.

Results: The VOA correlated with Integrated HIV-1 DNA (*P*=0.05, R^2=0.44) and total HIV-1 DNA (*P*=0.019, R^2=0.54), but not with usHIV-1 RNA, nor with 2LTR circles. Integrated HIV-1 DNA correlated with usHIV-1 RNA (*P*=0.001, R^2=0.28) and total HIV-1 DNA (*P*=0.002, R^2=0.85). Bland Altman analysis to assess the agreement between the assays revealed a quantification bias of the VOA at 2.88 log (95% CI 1.91–3.85) and 2.23 log (95% CI 1.20–3.27) compared to integrated and total HIV-1 DNA, respectively. However, this bias was not constant, as the difference between both methods changes with the reservoir size. Using the Bland Altman model, we propose a prediction model to predict viral outgrowth output from HIV DNA measures in patients on ART.

Conclusion: Our study reveals important correlations between the VOA and HIV-1 DNA measures, suggesting that the total pool of HIV-1 DNA may predict the size of the replication competent virus in ART suppressed patients. However, this study was restricted to patients on long term ART who started treatment during chronic HIV infection. Future studies should investigate whether HIV DNA and the VOA also correlate in early treated patients or after reservoir purging interventions.

PP 3.18

A theoretical framework to guide clinical trial design to estimate efficacies of latency-reversing agents

R. Ke[1], J.M. Conway[2], A.S. Perelson[3]

[1]Department of Mathematics, North Carolina State University, USA, [2]Department of Mathematics, Pennsylvania State University, USA, [3]Theoretical Biology and Biophysics (T-6), Los Alamos National Laboratory, USA

Background: Recently, HIV eradication studies have focused on developing latency-reversing agents (LRAs) to activate HIV expression in latently infected cells and ultimately purge the HIV latent reservoir. However, measuring the size of the latent reservoir reduction experimentally has been challenging, and it is not clear which steps in the latency-reversing processes determine the rate of latent reservoir reduction. This makes evaluating the efficacy of candidate latent reversing agents and predicting long-term treatment outcomes difficult.

Methods and Results: We constructed a mathematical model to describe the dynamics in latently infected cells under LRA treatment. Using the model, we show that the rate of latent reservoir reduction is strongly dependent on three key parameters: the rate at which latently infected cells become activated, the rate at which LRA activated cells return to latency and the death rate of LRA activated cells. We further extended the model to describe clinical sampling procedure and examined the accuracy of estimating the three key parameters using data collected from three commonly used assays: the viral outgrowth assay, q-PCR to measure cell-associated unspliced HIV RNA and q-PCR to measure plasma virus. We show that parameter estimation based on the viral outgrowth assay is unreliable due to stochastic variations in the low number of latently infected cells in a clinical sample. The q-PCR measurement of plasma viremia provides the best estimates, if the rate of viral production in LRA activated cells is at least 5% of the rate in productively infected cells. We further explore how combining data from multiple assays and increasing assay accuracy would improve estimation of the key parameters.

Conclusions: The theoretical framework we have developed could be used in designing future clinical trials and experiments to evaluate the efficacy of candidate latency-reversing agents and predict long-term treatment outcomes.

PP 3.19

Evidence of ongoing HIV replication during suppressive antiretroviral therapy

L. Bertoni Giron[1,2], S. Tenore[1], M.C. Araripe Sucupira[1], L.M. Janini[1], S. Pillai[2], R. Sobhie Diaz[1]

[1]Federal University of São Paulo, São Paulo, Brazil, [2]Blood Systems Research Institute, San Francisco, CA, USA

Background: HIV-1 genetic diversity and divergence result from replication in the setting of immune pressure. Antiretroviral therapy (ART) is proposed to abrogate viral genetic evolution when it achieves undetectable viral load. We hypothesized that current ART regimens are not fully suppressive and allow HIV genetic evolution to proceed.

Methods: Cell-associated HIV gp41 DNA was ultra-deep sequenced from 12 HIV-infected individuals pre-ART, and 4 years post continuous suppressive-ART. Trimmed filtered reads were analyzed using QuRe software to reconstruct viral variants, which were subject to phylogenetic analysis. HIV population genetics at each time point was analyzed using measures of divergence and diversity based on pairwise distance, and selection pressure was inferred using dN/dS tests.

Results: There was no correlation between pre-ART viral load and number of sequence reads (*P*=0.54). There was a positive correlation between number of reconstructed viral lineages and pre-ART viral load (*P*=0.01) but no correlation with the number of reads (*P*=0.44). Phylogenetic analysis revealed that HIV diversity was similar between time points despite several years of ART (pre-ART range 0.006–0.04, post-ART range 0.003–0.03; mean pre-ART *vs.* post-ART: 0.014 *vs.* 0.015 repeated measures *t*-test *P*=0.2). Viral divergence during ART with respect to baseline approached statistical significance (pre-ART range 0.007–0.02, post-ART range 0.007–0.16; mean pre-ART *vs.* post-ART: 0.014 *vs.* 0.038 repeated measures *t*-test *P*=0.07). dN/dS analysis showed lack of selection pressure (mean ratio pre-ART 1.17, post-ART 1.1 repeated measures *t*-test, *P*=0.7) despite high range observed (pre-ART 0.27–2.37; post-ART 0.14–3.61).

Conclusions: High HIV replicative rates as inferred by pre-ART viral loads relates to a higher number of reconstructed viral lineages

and increased viral divergence after ART suppression, suggesting that at least in some cases, ART treatment is not completely suppressive.

PP 3.20

Peripheral blood CD4+ T cells and intestinal lamina propria mononuclear cells contribute to viremia following an analytical treatment interruption: a follow-up analysis of the panobinostat trial

K. Barton[1], B. Hiener[1], A. Winckelmann[1,2], T. Aagaard Rasmussen[2], M. Tolstrup[2], W. Shao[3,4], R. Olesen[2], P.W. Denton[2], A. Solomon[5], L. Østergaard[2], S.R. Lewin[5,6], O. Schmeltz Søgaard[2], S. Palmer[1]

[1]Centre for Virus Research, Westmead Millennium Institute, Westmead, Australia, [2]Department of Infectious Diseases, Aarhus University Hospital, Denmark, [3]Advanced Biomedical Computing Center, Leidos Biomedical Research, USA, [4]Frederick National Laboratory for Cancer Research (FNLCR), Frederick, MD, USA, [5]Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, [6]Department of Infectious Diseases, Alfred Hospital, Monash University, Melbourne, Australia

Background: Administration of panobinostat to HIV-1 infected individuals on long-term antiretroviral therapy (ART) activates HIV-1 transcription. The extent to which the induced HIV-1 RNA transcripts and related HIV-1 DNA sequences contribute to viremia upon treatment discontinuation is unknown. In this study, we compared HIV-1 sequences in CD4+ T cells from the blood and intestinal lamina propria mononuclear cells (LPMCs) during panobinostat therapy to sequences collected from the plasma during a post-panobinostat analytical treatment interruption (ATI).

Methods: CD4+ T cells were obtained before, during, and after panobinostat administration (n=15). Five participants participated in an ATI and donated LPMCs. We used single-proviral/genome sequencing to determine the genetic composition HIV-1 DNA and RNA. Phylogenetic analyses were conducted using MEGA 6.0.

Results: We identified an expansion of clonal cell-associated HIV-1 DNA in the peripheral blood, which is indicative of previous cellular proliferation that matched viral RNA sequences from the ATI. Cell-associated HIV-1 DNA sequences from CD4+ T cells from both blood and LPMCs were closely related to sequences detected in plasma during the ATI (>99% similarity; blood 38 sequences, LPMC 12 sequences). Furthermore, we identified cell-associated HIV-1 RNA sequences in CD4+ T cells from blood and LPMCs collected during panobinostat treatment that were closely related to sequences from the ATI (>99% similarity; blood 6 sequences LPMC 1 sequence).

Conclusions: Clonally expanded HIV-1 is capable of contributing to viremia. Importantly, we found that cell-associated HIV-1 DNA in the blood and LPMCs contribute to plasma viremia following discontinuation of ART. Furthermore, HIV-1 RNA from virus that later emerged during ATI was expressed in both tissues during panobinostat treatment. Thus, both of these important reservoirs of latent virus should be prioritised in remission and curative strategies.

PP 3.21

Duplex droplet digital PCR to study the composition of HIV-DNA in blood cells

F.R. Simonetti, P. Cattaneo, A. Lai, S. Gioffrè, C. Balotta

Department of Biomedical and Clinical Sciences Luigi Sacco, University of Milan, Italy

Background: As most of proviruses are defective, PCR-based methods lead to an overestimate of the true size of the reservoir. These defective species accumulate with replication and increase through clonal expansion, but their composition is only partially known. We developed a duplex ddPCR to further characterize HIV-DNA in PBMC.

Methods: We studied the following groups of participants: chronic infection (CHI, n=14), chronic infection on cART with HIV-RNA <37 copies/mL for more than 4 years (CHI-ART, n=25), acute infection in Fiebig stages I–V (AHI, n=8) and treated acute infection (AHI-ART, n=7). Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) and digested by MscI. Duplexed ddPCR assays were designed to measure single and double positive (DP) copies of RU5 and *gag*, both *tat/rev* exons, and *tat/rev* exon-1 and RU5. CCR5 gene copies were measured to determine cell equivalents. An unpaired *t*-test was used to compare differences between groups; correlations were assessed by Pearson's coefficient.

Results: Within CHI-ART subjects, LTR, *gag* and *tat/rev* DNA copies showed strong positive correlations (r range 0.67–0.88, P values all <0.0001). As expected, AHI-ART group reached lower DNA levels than CHI-ART (P<0.02 for all assays). A greater fold reduction (FR) was observed in DP-*tat/rev* copies compared to LTR, *gag* and *tat/rev* levels in both acute and chronic treated subjects (10.8 *vs.* 3.0 FR). In chronic patients, LTR counts decreased by only 1.9 FR, likely due to accumulation of defective variants. In the CHI-ART group, the fraction of DP-*tat/rev* copies to total LTR count was significantly smaller than fractions of *gag* and *tat/rev* (mean 16.6% *vs.* 30%, P<0.0001).

Conclusions: Duplex ddPCR assays can identify a fraction of HIV-DNA that more likely contributes to the size of the reservoir, closing the gap between previous methods based on PCR and viral outgrowth assays.

PP 3.22

Diversity changes in blood HIV-1 DNA reservoir after combination of chemotherapy and autologous hematopoietic stem cell transplantation for lymphoma

H. Delagreverie[1,2], M. Salmona[1,2], L. Gerard[3], E. Oksenhendler[3], C. Delaugerre[1,2]

[1]Laboratoire de Virologie, Hôpital Saint Louis, APHP, Université Paris Diderot, Paris, France, [2]INSERM UMR941, Université Paris Diderot, Paris, France, [3]Immunopathologie Clinique, Hôpital Saint Louis, APHP, Université Paris Diderot, Paris, France

Background: After high-dose chemotherapy and autologous stem cell transplantation (HDC/ASCT) for lymphoma in HIV-1 infected patients, viral DNA persists in PBMCs, but reservoir diversity has not been assayed so far.

Methods: Longitudinal blood samples from 13 retrospective HIV-1-infected lymphoma patients treated with HDC/ASCT in Hôpital Saint Louis, Paris were assayed for HIV-1 DNA (HIV DNA Cell, Biocentric). Samples from two patients suppressed on 3TC-ABC-DRV/r with relapsed DLBCL were selected for ultra-deep sequencing (UDS) of a 373bp region in gp120 (454GS Junior, Roche) at two time points before and two after HDC/ASCT. Viral diversity was investigated by neighbour-joining (NJ) analysis (MEGA, 1000 bootstraps), time-scaled maximum clade credibility (MCC; Beast) and compartmentalization tests.

Results: 85 samples (median 7/patient) were assayed for HIV-1 DNA and did not display overall nor patient-specific quantitative changes in blood reservoir levels before and after HDC/ASCT (median 2.88 [2.44–3.20] $\log_{10}$ copies *vs.* 2.56 [2.09–2.73] $\log_{10}$ copies/10^6 PBMC, respectively). UDS of gp120 in two patients yielded ≈10,000 sequences/sample, clustered into haplotypes for further analysis. In one patient infected with CRF02_AG who started cART 7 months before HDC/ASCT, overall diversity decreased but there was no evidence of a shift in reservoir HIV-1 populations in PBMCs over time. Alternatively, in a patient infected with subtype B and on cART for 7 years before ASCT, haplotypes from each time point were distinct in NJ and MCC, and post-ASCT haplotypes did not descend from pre-ASCT time point 1.

Conclusion: Although blood HIV-1 DNA quantification rises quickly back to its former level after HDC/ASCT while on cART, pointing at homeostatic proliferation from autologous infected grafts and tissue reservoirs, UDS of proviral populations in blood unveiled qualitative changes. This shift in diversity supports the ability of high-dose chemotherapy to notably alter HIV-1 DNA reservoir.

PP 3.23

Abstract withdrawn

PP 3.24

HIV-1 mediated insertional activation of STAT5B promotes the formation of a viral reservoir in T regulatory cells

D. Cesana[1], F.R. Santoni de Sio[1], L. Rudilosso[1], P. Gallina[1], A. Calabria[1], L. Passerini[1], S. Nozza[2], E.Vicenzi[3], G. Poli[3], G. Tambussi[2], E. Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy, [2]Department of Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy, [3]AIDS Immunopathogenesis Unit, San Raffaele Scientific Institute, Milan, Italy

It has been suggested that HIV-1 by integrating near cancer-associated genes could promote the expansion and persistence of infected cells in patients under antiretroviral therapy (ART). However, the molecular mechanism/s of insertional mutagenesis used and the physiological impact on the cells harboring these integrations are completely unknown.

Here, we found that in peripheral blood mononuclear cells (PBMC) from 54 HIV-1 infected patients under ART, *BACH2* and *STAT5B* were targeted by a significantly higher number of integrations ($P<0.0001$) and with the same orientation of gene transcription compared to other lentiviral integration datasets. Furthermore, aberrant chimeric transcripts containing viral sequences fused to the first protein coding exon of *BACH2* or *STAT5B* and predicted to encode for unaltered full-length proteins were found in PBMC of 34% of HIV-1 patients under ART (30/87). Tracking the expression HIV-1/*STAT5B* transcripts in purified T cell subpopulations and monocytes ($n=6$) we found a specific enrichment of chimeric HIV/STAT5B mRNAs in T-regulatory (reg) and T-central memory (cm) cells in all patients tested ($n=6$). *In vitro* experiment on CD4+ T-cells isolated from healthy donor showed that forced expression of these transcription factors significantly increased their proliferation rate and do not alter the immune-suppressive function of T-reg cells.

Hence, our findings provide novel evidence that HIV-1 takes advantage of insertional mutagenesis to favor its persistence in the host by activating *STAT5B* and *BACH2*. Indeed, the selective advantage conferred by these integrations should favor the survival and proliferation of T-reg and T-cm cells which are long lived, potentially able to diminish the immune surveillance against infected cells thus favoring long-term viral persistence.

PP 3.25

Diverse proviral structure of HIV integrants in clonally expanded cells

F.R. Simonetti[1,2], J. Spindler[1], X. Wu[3], F. Maldarelli[1], J.M. Coffin[4]

[1]HIV Drug Resistance Program, National Cancer Institute, National Institutes of Health, Frederick, MD, USA, [2]Department of Biomedical and Clinical Sciences Luigi Sacco, University of Milan, Italy, [3]Leidos Biomedical Research, Frederick, MD, USA, [4]Tufts University, Department of Molecular Biology and Microbiology, Boston, MA, USA

Background: Clonally expanded populations of HIV infected cells persist during prolonged cART. Recently, we identified several highly expanded lineages, including a clone with an integrant in the HORMAD2 gene that accounted for c. 50% of all of the infected cells. Integration into specific introns of BACH2 or MKL2 increased the expansion and/or survival of infected cells. In one instance a clonally expanded provirus was infectious. The proviruses in these clonally expanded cells have not been adequately characterized. We describe characterization of other proviruses in highly expanded clones.

Methods: Longitudinal PBMC samples were obtained from volunteers with chronic HIV infection. Integration sites were determined as previously described. Proviruses integrated in HORMAD2, MKL2, and an intergenic region of the X chromosome were selectively amplified using specific primers for HIV and the flanking host DNA.

Results: The HORMAD2 integrant was present both pre- and on-cART and accounted for 50–80% of all of the infected cells after 7–8 years on cART, implying that expansion of the clone started early in infection. Proviral sequence analysis revealed a 675 nt single LTR with intact promoter elements. Analysis of a highly expanded provirus in an intergenic region of the X chromosome also revealed evidence for a solo LTR. Analysis of seven integrants in MKL2 were also characterized. One of the proviruses had a large pol-U3 internal deletion; of the remaining, four proviruses showed evidence GtoA mutation leading to multiple stop codons and three were found to lack intact tat or rev. All seven, however, had intact LTR promoter elements and retained the major splice donor sequence.

Conclusions: We found proviruses in expanded clones that were intact, hypermutated, partially deleted or consisting of a solo LTR. MKL2 proviruses, have intact LTR regulatory elements but some lack tat, which is normally required for HIV transcription.

PP 3.26

No selection of CXCR4-using variants in cell reservoirs of dual-mixed HIV infected patients receiving suppressive maraviroc therapy

J. Izopet[1–3], S. Raymond[1–3], F. Nicot[1,3], N. Jeanne[3], P. Delobel[1,2,4] and the ANRS 145 MARIMUNO Study group

[1]INSERM, U1043, Toulouse, France, [2]Université Toulouse III Paul-Sabatier, Faculté de Médecine Toulouse-Purpan, Toulouse, France, [3]CHU de Toulouse, Hôpital Purpan, Laboratoire de Virologie, Toulouse, France, [4]CHU de Toulouse, Hôpital Purpan, Service des Maladies Infectieuses et Tropicales, Toulouse, France

Background: The CCR5 antagonist maraviroc is only active against CCR5-using viruses. However, some patients were given maraviroc although they were infected by R5X4 dual-mixed viruses. In the MARIMUNO study, the patients received a 24-week maraviroc supplement to an efficient antiretroviral therapy. Thus, we investigated how the frequency of CXCR4-using variants in R5X4 dual-mixed virus populations responded to maraviroc selection pressure using ultra-deep sequencing (UDS).

Methods: We explored 22 patients from the MARIMUNO study infected with R5X4 dual-mixed viruses according to the recombinant virus assay Toulouse Tropism Test. The frequency of CXCR4-using variants was determined in peripheral blood mononuclear cells (PBMCs) before maraviroc intensification (week 0) and after 24 weeks of maraviroc (week 24). UDS was performed on a 454 GS Junior system. The sequence reads of the V3 env regions were analysed with the PyroVir software developed to provide an automated position-specific process for inferring HIV-1 tropism from V3 env UDS data.

Results: The mean total HIV-1 DNA was stable between week 0 and 24 (2.4 log copies/10^6 cells and 2.5 log copies/10^6 cells, respectively). UDS with the PyroVir genotypic algorithm detected CXCR4-using viruses in the 22 R5X4 infected patients at week 0 with a mean frequency of 59% (range: 3–100%). CXCR4-using viruses were detected in 21/22 patients at week 24 with a mean frequency of 52% (range: 10–92%). We found no correlation between the HIV DNA concentration in PBMCs and the number of CXCR4-using variants or their frequency. The frequency of CXCR4-using variants did not increase between week 0 and 24 except in one patient.

Conclusions: A 24-week course of a CCR5 antagonist does not select CXCR4-using viruses in the PBMCs of patients on suppressive therapy infected with R5X4 dual-mixed viruses. These results indicate little or no residual HIV replication that could be subjected to selection pressure.

Session 4: Anatomic and non-CD4 cell reservoirs

PP 4.0

Kinetics of HIV production by monocyte derived macrophages (MDM) in the presence of erythrocytes

L. Fazzi, C.G. Cevallos, R.D. Rabinovich, M.M. Ávila

Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS), Buenos Aires, Argentina

Background: Erythrocytes (E) of HIV+ individuals carry HIV RNA and p24Ag even if the viral load is undetectable. Macrophages are in close contact with E and therefore studying the interaction between these cell types is highly relevant. Our previous studies demonstrated that the presence of E enhances viral production in HIV infected MDM cultures. This work focuses on the kinetics of MDM HIV production in presence of E obtained from HIV- and HIV+ individuals.

Methods: MDM were obtained from healthy donors and were infected with the HIV-1 BaL strain. E from HIV(-) and HIV+ individuals were purified with Dextran. Fourteen days p.i. MDM were incubated with E or RPMI (as control) for 5, 15, 30 and 60 minutes. Viral production in MDM cultured with E was evaluated by measuring p24Ag in the culture supernatant plus p24Ag associated to E.

Results: Highest viral production in control culture was observed at the 30′ timepoint, while a significant decay was detected at the 60′ timepoint. Viral production in the presence of HIV-E reached its maximum increase at the 15′ timepoint with no later decay. When viral production of MDM in the presence of E *vs.* control was compared, a maximum difference (6-fold increase) was observed at the 15′ timepoint (P= 0.035). The amount of virus produced when MDM were incubated with HIV+E was significantly higher than that produced in the control culture (P=0.005).

Conclusions: Our results highlight the impact of erythrocytes in viral replication kinetics of HIV-1 infected MDM. As shown, viral load was greater at early timepoints when E were present in the MDM culture, suggesting that this cell type may facilitate viral budding/release. This effect was evident regardless the erythrocyte origin (HIV+E or HIV-E), indicating that erythrocytes have an important role in the pathogenesis of HIV infection in physiological conditions.

PP 4.1

Levels of SAMHD1 and natural ribonucleotides may alter anti-HIV potency of antiretroviral agents in primary CD4+ CCR5+ placental macrophages

C. Gavegnano[1,2], E.L. Johnson[1], S. Tao[1], A. Santos[1], R.F. Schinazi[1,2]*, R. Chakraborty[1]*

[1]*Center for AIDS Research, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA,USA,* [2]*Veterans Affairs Medical Center, Atlanta, GA, USA*

Background: Perinatal transmission of HIV remains a significant global health concern with ~200,000 new infections worldwide in exposed infants. Cellular protective mechanisms of antiretroviral agents (ARV) administered during pregnancy remain unclear. Understanding the pharmacology and potency of ARV in placental macrophages or Hofbauer cells (HC), a potential HIV reservoir, provides an opportunity to elucidate the dynamics between ARV and reservoir sites. Herein, antiviral potency of clinically relevant ARV and cellular factors impacting viral replication/potency, including dNTP/rNTP and SAMHD1 levels, were assessed in resting and activated HC and compared to adult macrophages.

Methods: HC or monocytes were isolated from healthy donors (placenta or buffy coat). Macrophages were differentiated with GM-CSF for 7 days. Activated or resting cells were maintained with or without GM-CSF, respectively. Cells were treated with various concentrations of ARV for 2 hours prior to infection with HIV-1_{baL} and maintained for 6 days before viral quantification (p24-ELISA). LC-MS/MS was used to quantify dNTP/rNTP levels. SAMHD1 levels were quantified by FACS.

Results: Antiviral potency of NRTI significantly (P<0.01) diminished in activated *versus* resting HC (EC_{50} 0.01–0.9 μM *versus* 0.001–0.01 μM), while antiviral potency of non-nucleoside reverse transcriptase, integrase, or protease inhibitors, remained constant. Intracellular levels of dNTP were similar in HC and macrophages, however rNTPs ATP, GTP, and CTP levels were significantly higher in resting HC *versus* resting macrophages (7–10 fold; P<0.01). CTP and UTP were significantly higher in activated HC *versus* activated macrophages (3–7 fold; P<0.05). HC expressed high levels of SAMHD1 independent of activation state, whereas activation increased SAMHD1 levels in macrophages.

Conclusions: rNTPs were significantly higher in HC *versus* macrophages potentially reflecting increased incorporation in these cells. SAMHD1, which degrades dNTPs, is high in HC independent of activation state, which may account for increased ratios of rNTP:dNTP in HC. Cellular activation significantly decreased potency of ARV in HC, underscoring necessity for antiviral agents targeting virus intracellularly. High rNTP in HC coupled with high SAMHD1 levels (which confer dNTP degradation), suggest that ribonucleoside inhibitors should be explored as novel antiviral agents to exploit this unique cell type.

PP 4.2

Immune activation in successfully treated patients with HIV-associated neurocognitive disorders: difference according to the severity of the impairment

M. Vassallo, J. Durant, R. Fabre, F. DeSalvador, C. Lebrun-Frenay, M. Laffon, M. Ticchioni, J. Cottalorda, P. Dellamonica, C. Pradier

Cannes General Hospital, Cannes, France

Background: The central nervous system (CNS) represents one important anatomic reservoir of HIV infection. Immune activation probably persists in successfully treated patients and could increase risks for non-AIDS events, including HIV-associated neurocognitive disorders (HAND).

Our aim was to investigate if immune activation differs according to the degree of HAND in successfully treated patients.

Methods: HIV-infected patients were randomly included in two prospective cohorts and underwent a neuropsychological (NP) evaluation. Test scores were adjusted for age, gender and education.

Inclusion criteria were undetectable viral load and stable treatment for at least 6 months.

Patients were divided into: unimpaired, asymptomatic neurocognitive disorders (ANI) and symptomatic HAND (sHAND), represented by mild neurocognitive disorders (MND) and HIV-associated dementia (HAD). Demographic and background parameters, immune activation markers and CD4/CD8 ratio values were recorded. A cross-sectional analysis of parameters associated with NP test results was performed.

Results: 204 patients were included (mean age 52 years, 78% male, mean CD4 620, nadir CD4 240, 28% HCV-co-infected, 16 years of infection, 2.9 years on current treatment). HAND counted for 30% of subjects, including 20% ANI and 10% sHAND. Among patients with sHAND, 80% had MND and 20% HAD. In multivariate analysis the CD4/CD8 ratio <1 was associated with CD4 nadir <200 (OR 3.14) and higher CD4+CD38+HLA+ cells (OR 1.24). Logistic regression showed that patients with ANI or sHAND were older (OR 1.05 and 1.08, respectively) than unimpaired subjects, while those with sHAND had higher risks to be HCV co-infected (OR 3.97) and having a CD4/CD8 ratio below 1 (OR 11.2). Patients with ANI diverged from unimpaired subjects only for a lower education level (OR 2.71).

Conclusions: Aviremic patients with sHAND, but not those with ANI, have higher immune activation than unimpaired subjects, suggesting different patho-physiological mechanisms.

PP 4.3

Asymptomatic cerebrospinal fluid viral escape during ART is associated with increased intrathecal immune activation

A. Eden[1], S. Nilsson[2], L. Hagberg[1], D. Fuchs[3], H. Zetterberg[1,4], B. Svennerholm[1], M. Gisslen[1]

[1]Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden, [2]Mathematical Sciences, Chalmers University of Technology, Gothenburg, Sweden, [3]Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Austria, [4]Institute of Neurology, University College London, UK

Background: Asymptomatic cerebrospinal fluid (CSF) viral escape, where HIV-1 RNA is increased in CSF while suppressed in plasma, occurs infrequently in patients on effective antiretroviral therapy (ART). It is unclear if CSF escape is benign or represents an active CNS infection. We examined the relationship between asymptomatic CSF viral escape and CSF biomarkers of axonal injury (the light subunit of neurofilament protein, NFL) and intrathecal immune activation (neopterin) in subjects on ART.

Methods: Patients on ART ≥6 months (plasma HIV-1 RNA <50 c/mL) followed with ≥2 lumbar punctures without other CNS disease were included from a longitudinal study. Plasma viral blips were allowed. HIV-1 RNA was analyzed with real-time PCR (TaqMan v2, Roche) with a lower level of quantification (LLQ) of 20 c/mL. CSF NFL and neopterin were measured by ELISA.

Results: Seventy-five (52 male) neuro-asymptomatic patients on ART with in median (IQR) 5 (3–8) available CSF samples were included. Median (IQR) treatment time was 93 (60–129) months. Twenty-seven (36%) patients had ≥1 CSF HIV-1 RNA >LLQ. Median (IQR) CSF HIV-1 RNA in samples >LLQ was 50 (32–77) copies/mL. Forty-two (56%) patients also had ≥1 plasma blip >20 copies/mL (median 44, IQR 29–71 copies/mL). CSF neopterin was 26% higher in samples with quantifiable CSF RNA (median 7.2, IQR 4.9–10.5 nmol/L) than with CSF RNA <LLQ (median 6.5, IQR 5.2–7.7 nmol/L) (P=0.0002). CSF NFL was 12% higher in samples >LLQ (median 508, IQR 350–786 ng/L) than <LLQ (median 588, IQR 339–797 ng/L), with a trend seen toward significance (P=0.06).

Conclusion: In longitudinal analysis, occasional increase in CSF HIV-1 RNA was found in a substantial minority of patients on ART. Asymptomatic CSF viral escape was associated with higher neopterin reflecting increased intrathecal immune activation. A trend towards higher NFL was also seen, suggesting a possible link between CSF virus, immune activation and neuronal damage despite ART that needs to be further characterized.

PP 4.4

HIV isolated from CSF cells of a virologically controlled patient infects astrocytes

G.H. Li, R. Traslavina, B. Smith, L. Henderson, S. Steinbach, A. Nath

Section of Infections of the Nervous System, NINDS, NIH, Bethesda, MD, USA

Background: *In vivo* studies show that astrocytes may be a critical reservoir for HIV in the brain. However, HIV infection of astrocytes *in vitro* is inefficient. We investigated if HIV strains from the central nervous system were able to infect astrocytes.

Methods: 100–150 mL of CSF was collected via lumbar drain and the cells were spun down for HIV isolation. HIV isolate was characterized in T lymphocytic cell lines, THP-1-derived macrophages and primary fetal astrocytes.

Results: We collected CSF cells via lumbar drain from six patients with plasma viral suppression; five had normal cognitive performance and one had HIV-associated neurocognitive disorder (HAND). HIV was not detected in CSF of all patients by clinical lab. We isolated an HIV virus from the HAND patient after 10 days-coculture with PBMCs from normal donors. This virus was able to infect MT2 cells and Jurkat-Tat (JKT) cells and significantly induced syncytia, but could not infect THP-1-derived macrophages. These observations indicate that the isolate was an X4-tropic virus. When the virus stock was initially incubated with astrocytes, no infection was detected. However, the virus could infect astrocytes via the transwell culture system where HIV-infected JKT cells were loaded on the top chamber and only HIV particles could reach the astrocytes. This is consistent with prior observations that immature HIV particles released from the infected lymphocytes were able to directly bind to CXCR4 on astrocytes in the absence of CD4 triggering virus-cell fusion and leading to infection. We have confirmed this mechanism in the cell-to-cell infection of HIV in astrocytes by cocultivating with the infected lymphocytes.

Conclusions: Despite adequate antiretroviral therapy low level HIV may be present in the CSF that can infect lymphocytes and astrocytes.

PP 4.5

CCR5- and CXCR4-tropic HIVs infect CD4+ hematopoietic stem and progenitor cells *in vitro* and in optimally treated people

N. Sebastian[1,2], V. Terry[3], F. Taschuk[3], L.A. McNamara[4], A. Onafuwa-Nuga[3], J. Riddell IV,[3] D. Bixby[3], K.L. Collins[1,3,4]

[1]Cellular and Molecular Biology Graduate Program, University of Michigan Medical School, USA, [2]Medical Scientist Training Program, University of Michigan Medical School, USA, [3]Department of Internal Medicine, University of Michigan Medical School, USA, [4]Department of Microbiology and Immunology, University of Michigan Medical School, USA

Hematopoietic stem and progenitor cells (HSPC) express HIV receptors and support both active and latent infection. To determine the co-receptors used by HIV to infect HSPCs *in vivo*, we sampled bone marrow from HIV+ donors with undetectable viral loads for at least 6 months. We purified two populations of HSPCs based on CD133 expression ($CD133^{high}$ and $CD133^{low}$) as CD133 marks a subset of CD34+ HSPC enriched for stem cells. In total, we isolated 41 *env* amplicons from HSPCs from 23 donors; 36 of these were unlikely from T cells based on CD3+ T cell contamination in each HSPC sample (0.0–1.6% CD3+) and HIV-1 amplicon frequency in corresponding bone marrow CD3+ T cells (Fisher's exact test, $P<0.05$). Geno2pheno co-receptor analysis revealed that CCR5-tropic subtype B env amplicons could be isolated from both $CD133^{high}$ and $CD133^{low}CD34+$ HSPCs; however, we isolated CXCR4-tropic subtype B *env* amplicons only from the $CD133^{high}$ sub-population. *In vitro,* CXCR4- and CCR5-tropic Env-pseudotyped viruses preferentially transduced $CD4^{high}$ over $CD4^{low}$ HSPCs. In addition, while viruses of both tropisms infected $CD133^{high}$ cells, we confirmed that CXCR4-tropic viruses infected cells with the highest amount of CD133 more efficiently than CCR5-tropic viruses. To assess the significance of differential CD4 and CD133 expression, we used cell surface markers that distinguish subsets with different developmental capacity (CD38, CC45RA and CD10). $CD133^{high}CD4^{high}$

cells contained the highest fraction of stem cells and multipotent progenitors, CD133^{high}CD4^{low} and CD133^{low}CD4^{high} cells contained intermediate fractions, and CD133^{low}CD4^{low} HSPCs contained the smallest fraction. We also found that CD133^{high}CD4^{low} cells were enriched in differentiated lymphoid and myeloid progenitors.

In sum, our data indicate that CD4 marks an HSPC subset that is enriched for multipotent progenitors and stem cells. Moreover, both CXCR4- and CCR5-tropic HIVs target HSPCs capable of persisting in HIV-infected people.

PP 4.6

Most tissues of a plasma-negative HIV autopsy cohort contain HIV DNA and many exhibit tissue pathology

R. Rose[1], S. Lamers[1], D. Garcia[2], E. Singer[3], M.S. McGrath[2]

[1]Bioinfoexperts, LLC, Thibodaux, LA, USA, [2]AIDS and Cancer Specimen Resource and University of California, San Francisco, CA, USA, [3]National Neurological AIDS Bank and Department of Neurology, University of California Los Angeles, David Geffen School of Medicine, Los Angeles, CA, USA

Background: The high rate of comorbidities in ART+HIV+ patients suggests that viral persistence in anatomical sites may contribute to tissue pathology. We identified subjects with undetectable viral load at death, assayed a diverse panel of their autopsy tissues for HIV, and assessed tissue histopathology to discover if residual anatomical HIV levels during cART were potentially related to tissue injury.

Methods: 20 HIV+/ART-treated participants were identified from the National Neurological AIDS Bank (NNAB) and AIDS and Cancer Specimen Resource (ACSR) autopsy cohort who had undetectable plasma and CSF viral loads at autopsy. Extensive medical histories, including ART adherence, was compiled for each participant. Detailed histopathological findings were noted in autopsy specimens (n=212, including up to six brain and six lymphoid tissues per subject). All tissues were assayed for the presence of HIV DNA using digital droplet PCR. A subset of tissues was assessed for HIV RNA using an *in situ* hybridization assay.

Results: The mean patient age and span of HIV infection was 46.5 years and 12 years, respectively. Fifteen of the 20 patients developed cancer. Abnormal pathology was identified in the spleen, lung, lymph node and liver in 90% of the participants. Aorta and kidney were abnormal in 50 and 60% of the participants, respectively. 75% of participants exhibited atherosclerosis and all brain tissues exhibited slight to severe pathology. 66% of the tissues studied contained HIV DNA copies >200/million cell equivalents. Hybridization studies localized HIV RNA to macrophages within cancer tissues. CD68/CD163+ macrophages surrounded HIV+ cells in the brain.

Conclusions: Residual HIV within diverse anatomical tissues could promote inflammatory diseases, including cancer, atherosclerosis and other organ-associated diseases. These ACSR/NNAB cohorts, along with others of their kind, are highly valuable resources for future studies of HIV reservoirs and persistence.

PP 4.7

Hematopoietic stem and progenitor cells harbor provirus with identical Gag and V3 sequences as residual plasma virus in optimally treated patients

V.H. Terry[1], N.T. Sebastian[2,3], R. Yucha[1], F. Taschuk[1], L.A. McNamara[4], A. Onafuwa-Nuga[1], J. Riddell IV[1], D. Bixby[1], K.L. Collins[1,2,4]

[1]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA, [2]Cellular and Molecular Biology Graduate Program, University of Michigan Medical School, Ann Arbor, MI, USA, [3]Medical Scientist Training Program, University of Michigan Medical School, Ann Arbor, MI, USA, [4]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, USA

Curing HIV infection requires the eradication of all infected cells; recent studies suggest that hematopoietic stem and progenitor cells (HSPCs) may serve as a reservoir *in vivo*. We collected bone marrow and peripheral blood from 46 cART treated HIV-1 infected people with undetectable viremia for at least 6 months, including two donors treated in acute phase. We PCR amplified gag and env from highly purified HSPC, PBMC and plasma virus and recovered HIV-1 amplicons from HSPC samples from 31 donors. For 23 of 27 donors tested, including a donor treated since acute infection, the frequency of provirus detected in HSPCs was higher than would be expected from T cell contamination (P<3×10–18–0.05). We examined the reproducibility of provirus detection with four participants who donated multiple times at 3–6 month intervals. We found provirus in HSPC from all three donations from one donor and from both donations from two donors. We detected provirus in HSPC from one of two donations from the fourth donor, but could not rule out T cells as the source of HIV in one donation. Thus far, we have generated cDNA from the plasma of 23 donors and recovered HIV sequences from 16. Twelve donors yielded amplifiable HIV sequences from both HSPC and plasma virus. Three of these donors had plasma viral sequences that were identical to provirus from highly purified HSPCs. One donor had a cluster of clonal plasma viral sequences that matched an HSPC-associated provirus. In ongoing studies, we have infected T cell lines with reporter viruses pseudotyped with full-length Env amplicons isolated from HSPC. In sum, provirus can be detected in HSPC isolated from a large subset of optimally treated, HIV-infected people, including one person treated since acute infection. Moreover, some HSPC proviruses show sequence identity to circulating plasma virus and have functional Env. These data support HSPCs as a reservoir of HIV-1 in optimally treated HIV-infected people.

Session 5: Immunology of HIV persistence

PP 5.0

Modulations in key defense responses of epithelial cells during co-stimulation with BCG and HIV-Nef

S. Mehto, D. Sharma, N. Gautam, S. Jameel, K. Natarajan

University of Dehli, New Delhi, India

Background: Pulmonary tuberculosis (being the most common form) is an infectious disease that carves its way to the lungs through the respiratory route but it can very well spread to other organs. Considering the residence of *M.tb* as lungs, epithelial cells are one of the foremost host cells interacting with the invading pathogen. In this study we used epithelial cells to understand the mechanism that modulates innate immunity during co-infection of HIV with BCG.

Method: A549 epithelial cells were used in this study. Cells were stimulated with HIV-1 Nef or infected with (2 MOI) BCG or both for 24 h. ROS analysis was performed using DCDHA for 30 min, 60 min and 120 min time points through Flow cytometry. Annexin V assay was performed to monitor the apoptosis. Expression of NF-kB and autophagy molecules such as BECN 1and LC-3 was monitored using western blotting. Expression level of various co-stimulatory molecules was monitored using flow cytometry.

Results: Our results show that HIV-1 early protein Nef downregulates BCG mediated ROS and co-stimulatory molecule CD80. Further decrease in Annexin V staining was observed upon stimulation with BCG, Nef or BCG and together. Interestingly, we found that the expression of key mediators of autophagy, Beclin1 and LC3, significantly decrease when epithelial cells are challenged with BCG and Nef together.

Conclusion: Collectively, our results indicate that HIV-1 Nef and BCG synergistically inhibit epithelial cell apoptosis and autophagy. Our study points out the mechanisms employed by these pathogens to evade epithelial cell defense mechanisms during co-infection.

PP 5.1

HIV alters the profile of cytokines responding to seasonal influenza vaccination

L. González, M. Roach, C. Sánchez-Mora, V. George, L. de Armas, R. Pahwa, G. Dickinson, M. Fischl, S. Pallikkuth, S. Pahwa

University of Miami Miller School of Medicine, Miami, FL, USA

Background: The aim of this study was to assess how influenza vaccine responses in HIV+ and age matched HIV- individuals correlate with age and a curated panel of inflammatory biomarkers.

Methods: 281 participants [HIV+ *n*=131; HIV- *n*=150] aged 18–83 years were given TIV during the 2013–14 or 2014–15 flu season. Blood was collected pre-vaccination (T0) and at day 7 (T1), 21 (T2) and 180 (T3) post-vaccination. Antibody responses to flu vaccine ags were assessed by hemagluttination inhibition assay (HIA). Vaccine Responders (R) were defined as those achieving a 4-fold increase in HA Ab titer at T2 relative to T0; those who failed were Non-Responders (NR). Plasma was assessed for 15 cytokines, three markers of microbial translocation, and three markers of cardiovascular inflammation by Luminex Multiplex bead assay or ELISA.

Results: HIA titer to H1N1 Ag at T0 showed seroprotection (>1:40) in 69% HIV- and 74% HIV+ (range 80–2560). Because of low frequency of R when T0 titer was high (>1:360) we selected participants with T0 titer of ≤360 (HIV- *n*=126, R 53%; HIV+ *n*=100, R 38%). In HIV-, age was inversely correlated with Ab titer at T0 (P=0.004) and T2 (P<0.001) whereas in HIV+, there was no correlation of age with T0 Ab titer but an inverse relationship was evident for T2 titer (P=0.043). Among HIV-, age was positively correlated with T0 IL-17a, MCP-1, TNF-α, CRP, Neopterin, and sCD163 (P<0.05 for all). However, the age-related inflammatory profile in HIV+ showed T0 IL-8, sVCAM, CRP, Neopterin, sCD163, and sTNFRII (P<0.05 for all) were significant. In HIV+ NR, the cytokines IFNα2 and IL-8 were negatively associated with Ab titers at T0 (P=0.010 and P=0.050, respectively) and T2 (P=0.004 and P=0.022, respectively) whereas in HIV+ or HIV- R this association was not observed.

Conclusions: HIV+ and HIV- show different age-related baseline cytokine profiles and a cytokine signature of responsiveness or unresponsiveness was evident suggesting viral persistence plays a role in flu vaccine responses.

PP 5.2

Virologic and immunologic correlates of viral control after ART-interruption in SIV-infected rhesus macaques

M. Paiardini, L. Micci, E. Ryan, R. Fromentin, N. Chomont, R. Sekaly, J. Lifson

Emory University, Atlanta, GA, USA

Background: ART does not eradicate HIV and the virus rebounds upon ART interruption. A sustained control of HIV replication off-ART has been described in a subset of subjects starting ART early after infection (post-treatment controllers; PTC). The determinants of post-ART HIV control are still unclear, particularly in tissues. Here, we used SIV-infected rhesus macaques (RM) to investigate the features associated with post-ART SIV control.

Methods: We identified five SIV-infected RM that, after 7 months of ART started at 2-months p.i., controlled viral rebound (<200 copies/mL) after structured treatment interruption (STI). Blood (PB), rectum (RB), and lymph node (LN) samples were collected from these animals and from RMs that, under the same conditions, experienced a robust SIV rebound after STI (non controllers; NC). Total SIV-DNA and RNA was measured on PB CD4 T cells and gut tissues by qPCR; immunological parameters were determined by flow cytometry. Predictors associated with PTC status were evaluated by odds ratio analyses.

Results: At pre-ART, PTC had reduced SIV-RNA levels in plasma and RB, lower SIV-DNA content in PB CD4 T cells and RB, reduced T-cell activation, and higher CD4 counts than NC (P<0.01 for all). Gut CD4 T cells were similar, but PTC had higher frequencies of Th17 cells. On-ART, PTC had significantly lower residual viremia (<3 copies/mL) and SIV-DNA content in PB CD4 T cells. SIV-specific CD8 T cell compartment was comparable between the two groups. Remarkably, PB and RB SIV-DNA contents rapidly increased in NC after ART interruption, while remain stable or progressively decreased in PTC. Finally, partial control of SIV rebound in PTC resulted in higher CD4 levels and reduced inflammation during the entire off-ART period.

Conclusions: Lower pre-ART viremia, cell-associated SIV-DNA, and T cell activation with concomitant preservation of Th17 cells are highly associated with prolonged viral control post-ART interruption in SIV-infected RMs.

PP 5.3

Administration of panobinostat is associated with increased IL-17A mRNA in the intestinal epithelium of HIV-1 patients

A. Bjerg Christensen[1], A. Dige[2], J. Randel Nyengaard[3,4], J. Agnholt[2,3], P.W. Denton[1,3,5]

[1]Department of Infectious Diseases, Aarhus University Hospital, Skejby, Denmark, [2]Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark, [3]Department of Clinical Medicine, Aarhus University, Denmark, [4]Stereology and Electron Microscopy Laboratory, Centre for Stochastic Geometry and Advanced Bioimaging, Aarhus University Hospital, Aarhus, Denmark, [5]Aarhus Institute for Advanced Studies, Aarhus University, Denmark

Background: Intestinal CD4+ T cell depletion is rapid and profound during early HIV-1 infection. This leads to a compromised mucosal barrier that prompts chronic systemic inflammation. The preferential loss of intestinal Th17 cells in HIV-1 disease is a driver of the damage within the mucosal barrier and disease progression that is not reversed during ART. Given the pathogenesis of HIV-1 in the intestines, understanding the effects of new therapeutic strategies within this organ is a priority.

Methods: We conducted a single-arm, phase I/II clinical trial designed to evaluate the therapeutic effect of the histone deacetylase (HDAC) inhibitor panobinostat on HIV-1 persistence despite successful ART (NCT01680094). We examined colonic biopsies collected before and during oral panobinostat treatment from nine individuals for the effects of panobinostat on viral DNA (ddPCR) and viral RNA (RNAScope ISH). Given that HDAC inhibitors have broad effects beyond virus reactivation (e.g. modulation of immune pathways), we also examined intestinal T cell activation (flow cytometry) and inflammatory cytokine mRNA production (RNAScope ISH).

Results: As reported for peripheral blood cells from these patients, there were no panobinostat-associated cohort-wide changes in viral DNA in the intestinal biopsies. Productively infected cells were detected in biopsy sections but were too rare for quantification. We observed a decrease in the frequency of intestinal lamina propria T cells expressing the activation marker CD69. Notably, we observed an increase in IL-17A mRNA expression specifically within the intestinal epithelium that was associated with panobinostat treatment (P=0.04).

Conclusions: Panobinostat had a clear biological impact in the intestines of HIV-1 patients as shown by the decreased activation and increased IL-17A expression in the intestinal epithelium. These results suggest that panobinostat therapy may influence the restoration of mucosal barrier function in these patients.

PP 5.4

Molecular profiling of antigen-specific peripheral T follicular helper cells from HIV-infected donors using influenza vaccination model

L. de Armas, N. Cotugno, L. Pan, S. Pallikkuth, S. Pahwa

University of Miami, Miami, FL, USA

Background: T follicular helper (TFH) cells have been shown to be a preferred cellular subset for HIV reservoirs, however the mechanisms governing HIV persistence in this cell type are unknown. A peripheral

TFH population characterized by expression of the chemokine receptor, CXCR5 has been shown to share some functional properties with GC TFH. The objective of the current study was to evaluate gene expression in H1N1-specific pTFH from HIV-infected and uninfected donors following influenza vaccination.

Methods: Cryopreserved PBMC from HIV-infected (*n*=6) and uninfected (*n*=6) individuals 21 days post-influenza vaccination from the 2014–15 flu season were thawed and stimulated with H1N1 antigen (5 ug/mL) overnight (16–18 h) in the presence of fluorochrome-conjugated anti-CD40L antibodies. PBMC were stained with antibodies to CD4, CD45RO, CCR7, CXCR5, and a live/dead marker and were FACS-sorted using BD FACS Aria. 50 H1N1-specific pTFH and non-pTFH cells characterized by CD4+CD45RO+CCR7+CD40L+ and CXCR5+ or negative, respectively, were sorted directly into CellsDirect one-step PCR buffer containing primers for specific target amplification. The resulting cDNA was run on Fluidigm BioMark platform to evaluate gene expression of 96 genes using Taqman gene expression assays by RT-PCR. SingulaR, R-based statistical analysis package, was used to compare gene expression from different populations by ANOVA.

Results: H1N1-specific pTFH from HIV-infected donors exhibited significantly ($P<0.05$) higher expression of genes involved in activation (*CD69, IL2RA, STAT1, STAT5A, TRIM5*) and immune regulation (*FOXP3, TIGIT, ITCH*) compared to HIV-uninfected individuals. H1N1-specific (CD40L+) pTFH from HIV-infected individuals also exhibited significantly higher expression of genes, including multiple transcription factors and activation markers compared to CD40L+ non-pTFH. On the other hand, CD40L (neg) pTFH from HIV-infected individuals did not exhibit an activated gene signature and had few differentially expressed genes compared to HIV-uninfected. No differences in the frequency of CD40L+ pTFH were observed between HIV-infected and uninfected vaccinees ($P=0.59$).

Discussion: H1N1-specific pTFH from HIV-infected donors exhibit a gene signature of enhanced activation as well as immune regulation compared to HIV-uninfected. Given the role TFH have been shown to play in maintenance of the HIV reservoir, these data suggest the transcriptional state of activation in antigen-stimulated pTFH may favor HIV replication and persistence.

PP 5.5

Pathogenicity of CD16+ monocyte-derived dendritic cells during HIV-1 infection

V.S. Wacleche[1,2], P. Ancuta[1,2], J.P. Goulet[3], A. Gosselin[2], M.C. Gaudreau[1,2], J.P. Routy[4,5]

[1]Université de Montréal, Faculty of Medicine, Department of Microbiology, Infectiology, and Immunology, Canada, [2]CHUM-Research Center, Montréal, Quebec, Canada, [3]Caprion, Montréal, Quebec, Canada, [4]Division of Hematology, McGill University Health Centre, Canada, [5]Immunodeficiency Service, Montreal Chest Institute, McGill University Health Centre, Montreal, Quebec, Canada

During HIV-1 infection, circulating CD16+ monocytes represent a more advanced stage of monocyte differentiation with a pro-inflammatory profile and an exacerbated frequency that is not normalized under ART. Monocytes are precursors for dendritic cells (DC), but functional differences between CD16+ versus CD16- monocyte-derived DC (MDDC) during HIV infection remain unknown. Here, we investigated CD16+/CD16- MDDC genome-wide transcriptional profiles in relationship with their ability to present antigens and disseminate HIV. Monocyte subsets were isolated by negative selection using magnetic beads (Miltenyi) and subsequently by flow cytometry (BD-AriaII). MDDCs were obtained by culture in presence of GM-CSF/IL-4. Genome-wide transcriptional profiling were performed using the Affymetrix Microarray technology in matched CD16+/CD16- MDDC samples from *n*=5 subjects exposed or not to HIV and LPS. The trans-infection ability (FACS, ELISA, PCR) and immunogenic potential (CFSE dilution assay) were evaluated by co-culturing MDDC with autologous CD4+ T-cells in the presence or absence of HIV (NL4.3BaL) and antigens (SEB, CMV, *C. albicans, S. aureus*). Gene Ontology and Gene Set Variation Analysis revealed unique transcripts and biological pathways expressed by CD16+ versus CD16- MDDC under constitutive conditions and upon exposure to HIV or LPS. A meta-analysis using the NCBI Interaction database identified differentially expressed HIV dependency factors. Consistent with transcriptional differences, CD16+ versus CD16- MDDC exhibited a superior ability to trans infect autologous CD4+ T-cells and a reduced ability to induce antigen-specific T-cell proliferation and Th17 polarization. These results emphasize the critical role played by CD16+ MDDC in HIV dissemination, with relevance for viral reservoir establishment in long-lived CD4+ T-cells and persistence under ART.

PP 5.6

Co-expression of multiple inhibitory receptors on CD8+ T cells in viremic and ART-suppressed HIV-1(+) individuals

B. Macatangay, C. Klamar Blain, F. Hong, J. Bui, A. Cillo, J. Mellors

University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Background: Inhibitory receptor (IR) expression on CD8+ T cells is associated with regulation of HIV-1-specific function. We tested whether co-expression of multiple IRs on CD8+ T cells in individuals on ART is associated with HIV persistence.

Methods: We evaluated 57 combinations of PD1, 2B4, TIM3, LAG3, CD160, and CTLA4 in total, naïve (T_N; CD45RA+CCR7+), central (T_{CM}; $CD45RA^-CCR7+$) and effector memory (T_{EM}; $CD45RA^-CCR7^-$) CD8+ T cells in HIV(+) viremic (n=14) and suppressed (n=30) individuals and age-matched HIV(-) controls (n=8) using flow cytometry and Boolean gating. PBMC and plasma were tested for cell-associated HIV RNA (CAR) and DNA (CAD) and residual viremia using qPCR targeting the 3′ integrase region of *pol*.

Results: Suppressed donors had a median CD4+ T count of 642 cells/mm^3 and had been suppressed for a median of 7.4 years. Viremic donors had a median CD4+ T cell count of 240 cells/mm^3 and median HIV-1 RNA of 10500 copies/mL. In HIV(+) and controls, frequencies of total CD8+ T cells co-expressing combinations of 2B4, CD160, TIM3, and PD1 was higher compared to the %CD8+ T cells that co-expressed combinations including LAG3 and CTLA4 ($P<0.001$; Mann–Whitney). The %CD8+ T cells (total and T_{EM}) expressing 2B4/CD160/PD1 was higher in HIV(+) donors (viremics>suppressed) compared to controls (14.25% *vs.* 6.5 *vs.* 3.2). %2B4/CD160+ CD8+ cells (total, T_{CM}, and T_{EM}) were also higher in HIV(+) donors than controls (15.95% *vs.* 5.4 *vs.* 1.4). Despite the higher frequencies in HIV, %2B4/CD160/PD1+ and %2B4/CD160+ CD8+ T cells did not correlate with the levels of residual viremia, CAR, and CAD. %CD8+ T cells expressing other IR combinations were similar in HIV(+) and controls.

Conclusions: 2B4/CD160+ and 2B4/CD160/PD1+ CD8+ T cells are higher in HIV infection despite virologic suppression on ART, but the higher frequencies do not correlate with markers of HIV persistence. Studies blocking multiple IRs are needed to better understand the role of IRs in HIV persistence.

PP 5.7

Immune activation profile associated with comorbidities in successfully treated HIV infected patients

C. Psomas[1,2], M. Younas[1], C. Reynes[3], C. Fernandez[2], V. Le Moing[2,4], C. Barbuat[5], N. Nagot[6], P. Portales[7], J.F. Eliaou[7], J. Reynes[2,4], P. Corbeau[1,8]

[1]Institute of Human Genetics, CNRS UPR 1142, Montpellier, France, [2]Infectious Diseases Department, Montpellier University Hospital, France, [3]Institute of Functional Genomics, UMR 5203, Montpellier University, France, [4]UMI 233, IRD-Montpellier University, France, [5]Infectious Diseases Department, Nîmes University Hospital, France, [6]Department of Medical Informatics, Montpellier University Hospital, France, [7]Immunology Department, Montpellier University Hospital, France, [8]Immunology Department, Nîmes University Hospital, France

Background: Immune activation in HIV-1-infected patients persists under suppressive antiretroviral treatment and may fuel comorbidities such as atherothrombosis, osteoporosis, metabolic syndrome, neurocognitive disorders and liver steatosis. Our hypothesis was that treated patients present with distinct profiles of immune activation and that each profile is linked to a specific comorbidity. We first explored the profile of immune activation that was associated with the presence of a metabolic syndrome.

Methods: We measured by flow cytometry and ELISA the level of activation of CD4+ and CD8+ T cells, B cells, monocytes, NK cells and endothelial cells as well as of inflammation with a total of 68 soluble and cell surface markers in 120 virologically suppressed individuals and 20 healthy donors (aged ≥45 years). We used a hierarchical clustering analysis to classify the patients according to different markers of immune activation, and logistic regression with odds ratios (OR) and 95% confidence intervals (CI) to measure the association between immune activation profiles and metabolic syndrome.

Results: We observed evidence of inflammation and immune activation in all the cell subpopulations analysed. Patients were clustered in five distinct immune activation profiles. Each one of these five profiles could be characterized by a marker of CD8+ T cell, NK cell, monocyte, endothelial cell activation or of inflammation, respectively, and could be distinguished between the other profiles by a signature of eight biomarkers. Only one of these immune profiles was significantly associated with marks of metabolic syndrome: hypertriglyceridemia (OR 4.18, 95% CI 1.08–16.19, *P*=0.038), hyperinsulinemia (OR 12.17, 95% CI 1.79–82.86, *P*=0.011) and lipodystrophy (OR 4.87, 95% CI 1.36–17.39, *P*=0.015).

Conclusion: We have uncovered an immune signature that might be useful for the prevention and early diagnosis of metabolic syndrome in HIV-infected patients. A better knowledge of the links between immune activation profiles and their consequences might highlight biomarkers predictive of comorbidities, as well as new therapeutic targets in HIV-induced immune activation or other situations of chronic hyperactivity of the immune system including aging.

Session 6: Pharmacology of HIV persistence

PP 6.0

Differential efficacy of antiretroviral drugs in HIV-1 infected human microglia

E.L. Asahchop[1], G.B. Baker[2], G. Rauw[2], C. Power[1,2]

[1]*Department of Medicine (Neurology), University of Alberta, Edmonton, Alberta, Canada,* [2]*Department of Psychiatry, University of Alberta, Edmonton, Alberta, Canada*

Background: HIV-1 infects the human brain within days of primary infection and establishes a viral reservoir. Microglia and perivascular macrophages are productively infected by HIV-1 forming the principal viral reservoir in the brain. The relative susceptibility of this viral reservoir to ART is unknown. We investigated antiretroviral therapy (ART) efficacy in HIV-infected human microglia, bone marrow-derived macrophages (BMDMs) and peripheral blood mononuclear cells (PBMCs). In addition, the concurrent extracellular and intracellular ART concentrations were assessed.

Methods: Human fetal microglia (HFM), and BMDMs were prepared from 16–20 week old fetuses. Peripheral blood mononuclear cells (PBMCs) were also isolated from adult healthy donors. Cultured HFMs, BMDMs and PBMCs were infected with HIV-1_{YU-2} at a multiplicity of infection (MOI) ranging from (0.1–1.0). HIV-infected cells were treated with zidovudine (AZT), etravirine (ETR), raltegravir (RAL), darunavir (DRV) or maraviroc (MRV). The EC_{50} levels were determined at day 4 or 5 post-infection by measurement of p24 in the supernatant. We ascertained the extracellular and intracellular concentrations of ART in differentiated human THP-1 cells using HPLC MS.

Results: HFM and BMDM cultures consistently expressed MHC Class II. Treatment of HIV-1 infected HFMs and BMDMs revealed the following EC_{50} levels at day 5 post-infection of HFMs: AZT (93.2 nM), RAL (7.4 nM), MVC (1.9 nM), DRV (105.5 nM), and ETR (12.1 nM). AZT and RAL EC_{50} levels in BMDMs were 63.9 nM and 50.4 nM, respectively. The EC_{50} levels for AZT, RAL, ETR and MVC in PBMCs at day 4 post-infection were: 7.4 nM, 2.7 nM, 2.7 nM and 6.3 nM respectively. Cytotoxicity was not observed for the above ARTs. Exposure of RAL and DRV (100 nM) to differentiated THP1 cells showed that the extracellular concentrations were 63.6 nM and 33.6 nM respectively, while the intracellular concentrations were 4.7 nM and 4.4 nM, respectively.

Conclusions: EC_{50} values for AZT, ETR and DRV in HIV-infected HFMs were exhibited substantially higher than those observed in PBMCs. Intracellular:extracellular concentration ratios were low for RAL and DRV. These results underscore consideration of assessing ART concentrations in different viral reservoirs in efforts to eradicate HIV-1.

PP 6.1

Donor-to-donor variation in the host gene expression response to SAHA

B. Moesker[1], B. Reardon[2], N. Beliakova-Bethell[2], A. Singhania[1], M.S. Breen[1], C.H. Woelk[1]

[1]*Clinical and Experimental Sciences, University of Southampton, Southampton, UK,* [2]*Department of Medicine, University of California San Diego, La Jolla, CA, USA*

Background: The "shock and kill" approach to eliminating the latent HIV reservoir depends on efficient reactivation of HIV provirus. Multiple studies have observed variability in the levels of HIV reactivation after treatment of latently infected CD4+ T cells with the histone deacetylase inhibitor SAHA. We hypothesize that the heterogeneity in HIV reactivation is partly related to donor-to-donor differences that are reflected in host gene expression. The objective of this study was to identify genes displaying a high level of donor-to-donor variability upon treatment with SAHA.

Methods: Primary CD4+ T cells from six healthy seronegative volunteers were incubated with 0.34, 1.0, 3.0 or 10.0 µM SAHA for 24 hours, or left untreated, after which RNA was extracted and gene expression was measured using Illumina HT-12 v4 BeadChips. To identify donor-to-donor variation specific to the SAHA treatment, we initially selected only those genes that were expressed at the same level in the untreated condition for each donor, and then ranked genes according to highest variation between donors upon SAHA treatment.

Results: At each SAHA dose, over 400 variable genes were identified with differences in gene expression greater than 2-fold between donors. Genes with relevance to HIV latency included *CCNT2/Cyclin T2*, a regulatory subunit of p-TEFb; *JMJD1A/KDM3A*, a lysine demethylase specific for H3K9me1/2; *SIN3B,* a transcriptional repressor of c-MYC; and *HMGA1*, a subunit of the inactive 7SK RNA/p-TEFb complex. Gene ontology analysis revealed an enrichment of genes encoding for proteins involved in ubiquitination and proteasomal degradation, suggesting that protein turnover may play a role in donor-to-donor variation.

Conclusions: It appears that SAHA has different effects in different donors at the gene expression level. Future work is required to determine if such differences lead to variation in the ability of SAHA to activate HIV in different donors.

Session 7: Drug discovery

PP 7.0

The utility of the Connectivity Map (CMAP) for HIV cure strategies

Y.P. Gunawardana[1], N. Beliakova-Bethell[2], C. Spina[2], D. Margolis[3], A. Bosque[4], V. Planelles[4], C.H. Woelk[1]

[1]*Faculty of Medicine, University of Southampton, Southampton, UK,* [2]*Department of Pathology, University of California San Diego, La Jolla, CA, USA,* [3]*Department of Medicine, University of North Carolina Chapel Hill, Chapel Hill, NC, USA,* [4]*School of Medicine, University of Utah, Salt Lake City, UT, USA*

Background: Major goals of HIV cure research are to identify latency-reversing agents (LRAs) and to characterize LRAs with unknown mechanism of action (uMOA). The Connectivity Map (CMAP) can address these goals and represents a collection of microarray gene expression profiles generated by treating cell lines with 680 different drug compounds. It is essential to evaluate compatibility between the CMAP (cell lines, microarray and fixed dose) and HIV cure studies (primary CD4 T cells, RNA-Seq, variable doses).

Methods: The following studies were used to assess the utility of the CMAP for HIV cure research: (1) microarray dose response study of primary CD4 T cells from six donors treated with 0, 0.34, 1, 3 and 10 μM of vorinostat; (2) RNA-Seq study of primary CD4+ T cells from four donors treated with 0.34 μM of vorinostat; and (3) differentially expressed genes (DEGs) between latently infected and uninfected cells in the following models: Iglesias-Ussel, Mohammadi, Planelles, Spina, and latently infected cell lines. For all data sets, the top 100 up and down DEGs were queried against the CMAP using gene set enrichment analysis.

Results: The CMAP was able to positively correlate the query profile with vorinostat for studies 1 and 2 when the gene expression data had been generated in primary CD4 T cells (instead of cell lines), by RNA-Seq (instead microarray) and at any dose. The CMAP was then used to identify novel LRAs by searching for negative correlations to the DEGs identified from latency model data. Although the top CMAP hit was different for each model, there were two compounds in common to all five latency models in the top 100 negatively correlated compounds.

Conclusions: CMAP is robust to RNA measuring technique, cell type and dose differences, and therefore represents a useful utility for characterizing LRAs with uMOA. The CMAP may also be used to identify novel LRAs and the compounds identified in this study will be tested for their ability to activate HIV.

PP 7.1

In vitro analysis of different PKC agonists: latency reversal, T-cell activation, cytokine production and isoform selectivity

R. Barnard, D. Tellers, B. Howell, E. Cook, M. Swanson, S. Vemula, J. Li, S. Carroll, D. Hazuda

Merck, West Point, PA, USA

Background: In this study, we performed an *in vitro* analysis of a number of different PKC agonists to determine the PKC isoform selectivity required for HIV latency reversal, T-cell activation potential and cytokine induction. Furthermore, we sought to identify PKC target engagement biomarkers to facilitate *in vivo* safety studies.

Methods: Nine different PKC agonists were tested in enzymatic assays. EC_{50} values for latency reversal were calculated using a Jurkat T-cell model of HIV latency. EC_{50} values were also calculated for prostratin in 2C4 cells lacking specific PKC isoforms. CD69 and CD25 expression in primary T-cells was determined by flow cytometry and a number of inflammatory cytokines measured by multiplex mesoscale analysis.

Results: Nine PKC agonists tested were active in a Jurkat T-cell HIV latency model (EC_{50} 9 nM to 5000 nM). In enzymatic experiments, the majority of PKC agonists tested had activity on PKC isoforms alpha, beta I/II and gamma. However, selective siRNA knockdown of these isoforms had no effect on prostratin-stimulated HIV expression in Jurkat 2C4 cells.

A positive correlation was identified between the EC_{50} required to activate HIV expression in 2C4 cells and the EC_{50} value for CD69 expression in resting primary T-cells. At these concentrations, we noted significant expression of a number of inflammatory cytokines.

To identify biomarkers of PKC agonism, RNAseq analysis was performed on Jurkat T-cells stimualted with prostratin.

Conclusions: Agonism of multiple PKC isoforms is required for efficient HIV latency reversal in a Jurkat model system. However, among the PKC agonist tested, all elicited CD69 expression and cytokine release at the concentration of latency reversal and cytokine release. In order to facilitate *in vivo* safety studies, we developed a CD69 mRNA biomarker assay to monitor target engagement and facilitate understanding of desired viral flush versus undesired T-cell activation.

PP 7.2

Suppression of the HIV-1 reservoir with a potent Tat inhibitor

S. Valente[1], C.F. Kessing[1], G. Mousseau[1], S. Mediouni[1], R. Fromentin[2], N. Chomont[2], L. Trautmann[3]

[1]Department of Immunology and Microbial Sciences, Scripps Research institute, Jupiter, FL, USA, [2]Université de Montréal, Centre de Recherche du CHUM, Montréal, QC, Canada, [3]Military HIV Research Program, Silver Springs, MD, USA

Despite the success of HIV antiretroviral therapy (ART), HIV persists still in latently and productively infected $CD4^+$T cells and macrophages in treated individuals. Novel therapeutic agents are needed to limit latent HIV disease.

HIV Tat protein activates transcription and its inhibition blocks viral exponential production. We showed that didehydro-cortistatin A (dCA) binds Tat and potently reduces viral RNA production (EC_{50} as low as 0.7 pM). dCA was also shown to induce a state of deep latency from which viral reactivation was impaired in cell line models of latency and in latently infected $CD4^+$T primary cells explanted from ART suppressed individuals, suggesting that the HIV promoter is epigenetically repressed. Using these latter primary cells we developed a latency model and confirmed that viral rebound occurs when antiretrovirals (ARVs) are removed, recapitulating what is observed in patients. In this model prior treatment with ARVs+dCA can almost completely prevent viral rebound up to 21 days when all drugs are removed. Moreover, reactivation with prostratin in the presence of ARVs+dCA was 100% inhibited. Additionally, the number of integrated proviruses in the infected $CD4^+$T cells decreased in cells treated with ARVs+dCA compared to ARVs alone, suggesting a reduction of cell-to-cell transmission over time.

Macrophages play critical roles in HIV trafficking to the brain contributing to persistence. Access of ARVs to the brain is restricted and HIV immune surveillance inefficient, thus eradication strategies based on viral reactivation might have limitations in this reservoir. We will present results of dCA activity in human primary macrophages infected *in vitro*.

Our results suggest that dCA combined with ART may abrogate residual HIV production from cellular reservoirs, block viral reactivation, reduce reservoir replenishment, and ultimately decrease the size of the latent reservoir.

Session 9: New therapeutic approaches 1

PP 9.0

Multi-dose romidepsin in SIV-infected rhesus macaques (RMs) reactivates latent, replication-competent virus in the absence of antiretroviral therapy (ART)

B. Policicchio

University of Pittsburgh, Pittsburgh, PA, USA

Background: Persistent viral reservoirs represent a major obstacle in eliminating HIV-1 from infected individuals. A reservoir reactivation strategy is the "shock and kill" approach, in which latent virus is reactivated with histone deacetylase inhibitors (HDACi) and eliminated through effective CTL responses. Therefore, our goals were to: (1) develop a nonhuman primate model of SIV control with conventional ART; (2) assess the HDACi romidepsin's (RMD) ability to reactivate SIV in controller RMs.

Methods: Four RMs were IV-infected with the SIVsmmFTq infectious molecular clone. All RMs received ART for 9 months from 65 days post-infection. ART was halted and RMD was administered over 4 hours in three rounds to three RMs. Plasma viral load was monitored with a single copy assay. PBMC histone acetylation and changes in the levels of T cells and their immune activation and proliferation status were assessed by flow cytometry.

Results: Conventional ART resulted in robust control of virus replication (<10 copies/mL), without viral blips in all RMs. At ART cessation (9 months post-treatment), the virus transiently rebounded (up to 10^6 copies/mL) and was then controlled to undetectable levels (<10 copies/mL). RMD administration resulted in significant virus rebounds (up to 10^4 copies/mL) peaking at 5–12 days posttreatment followed by gradual viral decline. RMD was well-tolerated and resulted in both a massive surge in T cell activation during the first week posttreatment, along with a noticeable, short-term decrease in overall quantities of circulating T cells.

Conclusions: We developed a new RM model of virus control with conventional ART and demonstrated that RMD can reactivate SIV *in vivo*, in contrast with previous *in vitro* studies. The levels of virus replication and timing of the virus rebound after RMD administration suggest the reactivated virus is replication-competent. Altogether, our results suggest HDACis may play a role in virus reactivation from the reservoir.

PP 9.1

Limitations of employing antibody drug conjugates (ADCs) for targeting HIV infected cells as a strategy for hiv cure

J. Strizki[1], D. Graham[1], M. Lu[1], G. Wu[1], M. Breslin[1], N. Davis[2], E. Escandon[2], L. Fayadat-Dilman[2], Y. Zheng[2], R. Barnard[1], R. Garbaccio[1], A. Manibusan[3], S. Bhowmik[3], D. Gately[3], Y. Sun[3], D. Gorman[2]

[1]Merck Research Labs, West Point, PA, USA, [2]Palo Alto, CA, USA, [3]Ambrx, San Diego, CA, USA

Background: The exquisite selectivity and potency of antibody drug conjugates (ADCs) make them attractive candidates for targeting latently or persistently infected cells. In this study, the potential of anti-ENV ADCs to specifically target and kill HIV infected cells was explored.

Methods: Anti-HIV ENV ADCs were generated using a proprietary site specific payload conjugation to cathespin or phosphatase cleavable linkers. Anti-CD71 and anti-human IgG-saporin (anti-ZAP) antibodies were used as controls. Binding affinity to gp120, cellular internalization, and cytotoxicity were evaluated in T-cell lines, and primary infected T-cells.

Results: Among payload candidates screened, the DNA alkylator, duocaromycin (Duo) demonstrated the greatest toxicity in T-cells and was selected for direct conjugation mAbs. In binding studies, the anti-gp120 antibodies rapidly bound ENV-expressing Jurkat cells, were efficiently internalized and catabolized. In these cells, treatment with ADC-DUO, or anti-gp120/anti-Hu Zap, demonstrated moderate to significant toxicity; respectively. However, in studies performed with HIV infected primary T-cells, ADCs demonstrated little or no HIV-specific cytotoxicity. Upon further investigation, internalization analysis revealed that, unlike Jurkats, gp120-mAb complexes were not efficiently internalized in primary T-cells. Additionally, quantification of cell associated gp120 was about two fold lower in primary cells compared with the Jurkat line, suggesting a lower potential for internal payload delivery.

Conclusions: Despite activity in Jurkat cells, anti-HIV ADCs failed to demonstrate significant HIV-specific cytotoxicity in primary infected T-cells. Lack of cytotoxic effect may be related to both the low numbers of gp120 molecules expressed on primary T cells, as well as limited internalization of gp120-ADC complexes. These findings do not support the further exploration of ADCs as potential therapeutic agents for HIV targeted cell killing.

PP 9.2

Sequential treatment with 5-aza-2'deoxycitidine and deacetylase inhibitors reactivates HIV

C. Van Lint[1], S. Bouchat[1], N. Delacourt[1], A. Kula[1], G. Darcis[1,2], F. Corazza[3], J.S. Gatot[1], A. Melard[4], C. Vanhulle[1], B. Van Driessche[1], K. Kabeya[5], M. Pardons[1], V. Avettand-Fenoel[4], N. Clumeck[5], S. De Wit[5], O. Rohr[6,7], C. Rouzioux[4]

[1]Service of Molecular Virology, Department of Molecular Biology (DBM), Université Libre de Bruxelles (ULB), Gosselies, Belgium, [2]Service des Maladies Infectieuses, Université de Liège, Centre Hospitalier Universitaire (CHU) de Liège, Domaine Universitaire du Sart-Tilman, B35, 4000 Liège, Belgium, [3]Laboratory of Immunology, IRISLab, CHU-Brugmann, Université Libre de Bruxelles (ULB), Brussels, Belgium, [4]Service de Virologie, EA7327, Université Paris-Descartes, AP-HP, Hôpital Necker-Enfants-Malades, Paris, France, [5]Service des Maladies Infectieuses, CHU St-Pierre, Université Libre de Bruxelles (ULB), Brussels, Belgium, [6]IUT Louis Pasteur de Schiltigheim, University of Strasbourg, Schiltigheim, France, [7]Institut Universitaire de France (IUF), Paris, France

Background: Reactivation of HIV gene expression in latently infected cells is proposed as an adjuvant therapy to decrease the reservoir size. Here, we evaluated the therapeutic potential of demethylating agents in combination with deacetylase inhibitors (HDACIs) in reactivating HIV from latency *in vitro* and *ex vivo*.

Methods: HIV-1 reactivation potential of latency-reversing agents (LRAs) was assessed first *in vitro* in latently-infected T-cell lines (by quantification of HIV-1 transcription, expression and production) and next *ex vivo* in CD8+-depleted PBMCs and resting CD4+ T-cell cultures from 58 cART-treated aviremic HIV-1+ patients (by quantification of HIV-1 production).

Results: We showed that the DNA methylation inhibitor 5-aza-2'deoxycytidine (5-AzadC), but not 5-azacytidine, induced HIV expression *in vitro*. We next demonstrated *in vitro* that a sequential treatment of 5-AzadC+HDACIs used at clinically tolerable doses synergistically induced HIV expression, highlighting for the first time the importance of treatment schedule for LRAs combined treatments. Importantly, we showed the physiological relevance of this synergy and sequential aspect in *ex vivo* cultures of primary cells from HIV+ cART-treated patients. The combined treatments did not induce global T-cell activation. Finally, we demonstrated a positive correlation between the total HIV DNA and the reactivation capacity by LARs *ex vivo*. However, we identified patient cell cultures characterized by a very low or extremely high reactivation capacity relative to their reservoir size.

Conclusions: In conclusion, we reported for the first time that, in addition to the combinatory aspect, the sequential aspect of LRA administration might be critical for anti-latency clinical trials aimed at decreasing the reservoir size. We also observed patient-specific reactivation variations that probably reflect the heterogeneity of the reservoirs and the multiplicity of the mechanisms that underlie HIV latency.

PP 9.3

Computational detection of off-target effects of CRISPR/Cas9-associated gRNAs

W. Dampier[1,2,3], Ch.H. Chung[1,2], M.R. Nonnemacher[1,2], B. Wigdahl[1,2]

[1]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [2]Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [3]School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PAa, USA

Background: Despite antiretroviral therapy, HIV-1 infection remains a life-long clinical problem due to reservoirs harboring proviral DNA in a latent/persistent form. Recently, gene-editing strategies utilizing

the CRISPR/Cas9 system have been developed to eradicate the HIV-1 genome from infected cells. These results offer a new avenue toward the elimination of HIV-1 to cure HIV/AIDS. CRISPR/Cas9 directed cleavage is mediated through the design of a 20 nucleotide guide RNA (gRNA) which is complementary to the target cleavage site. However, due to the promiscuous nature of the gRNA targeting, it is important to screen for off-target binding sites that may cause unwanted DNA damage within the human genome.

Methods: Current tools such as BLAST are insufficient for this task and will produce numerous false negatives; furthermore, they cannot account for known polymorphisms in the human genome. This is further complicated by the non-linear nature of the binding penalties associated with gRNA recognition. In order to resolve these issues, we have developed a new database containing all potential cleavage sites within the entire human genome along with all known single nucleotide polymorphisms in dbSNP.

Results: Using a suffix tree, we have indexed the more than 300 million potential cleavage sites across the human genome, which allows for a nearly instantaneous search for potential off target effects across the genome. Tree construction occurs on the order of O(N) ~ Nlog(N) time where N is the size of the database and tree search occurs in O(m) ~ m where m is the length of the query. In practice, the entire human genome and dbSNP were indexed within 72 hours and a query takes less then 10 ms per gRNA.

Conclusions: This database will greatly increase the ability of researchers to design gRNA that can cleave HIV-1 while avoiding off-target effects. Future studies will be performed to validate the off-target predictions using biomedical research laboratory techniques.

PP 9.4

Strategies to overcome active and latent HIV: attack is the best way for defense

H. Shadad

Faculty of Medicine, Tanta University, Tanta, Gharbeya, Egypt

Background: There are two phases of HIV infection in the body, the active phase (the apparent form in plasma viral load) and the latent phase (the hidden form in HIV reservoirs) that contains the genetic code of HIV. They are located mainly in CD4+ T cells (cellular reservoirs) and throughout the body, including the brain, lymphoid tissue, bone marrow, and the genital tract (anatomic reservoirs). They remain invisible to the body immune defenses, and are not sensitive to anti-HIV drugs. Hence, HIV is able to remain a chronic, life-long infection.

Methods: We could overcome HIV via two strategies. The first one is to control the plasma viral load (the apparent form) by three methods. (1) highly active antiretroviral therapy (HAART) that decreases the viral load to undetectable level by preventing HIV from multiplying. (2) Autologous monocyte-derived dendritic cells (MD-DCs) pulsed with heat inactivated autologous HIV-1 that leads to a decrease in viral load after HAART interruption in vaccinated patients with a concomitant increase in HIV-1–specific T cell responses. The vaccine is safe and well tolerated. (3) Heat therapy of human serum. It is possible to inactivate HIV by heat therapy of blood, plasma and other serum samples at 54–56°C for 5 hours. The experiment showed that heat therapy does not alter protein binding. The second strategy is to eradicate the HIV reservoirs (the hidden form). This could be done by two routes. (1) Using the first strategy (HAART) is very slow and not effective because the half-life of the reservoirs is extremely long (44 months). At this rate, eradication of those reservoirs would require over 60 years of treatment with the first strategy. (2) Reactivation of latent HIV reservoirs.

Results: The first strategy turned HIV infection from a death sentence to a manageable disease. As it allows the immune system to stay healthy but it is supposed to be used lifelong; however, this is not an ideal situation for HIV-infected individuals because of drug cost and worries about resistance. We are still in need of new strategies for overcoming HIV such as the second strategy concerned with eradication of reservoirs.

Conclusions: I think that attack is the best way for defense. If we could reactivate the latent reservoirs i.e. convert the HIV from the latent phase (hidden form) to the active phase (the apparent form) that will be recognized by the immune system in continuation of the first strategy to prevent new or spreading infection, we could say that we become very close to eradicate HIV. That reactivation process could be done by stimulating latently infected cells to replicate and express the virus, such cells will die more rapidly (HIV-induced cell death) instead of waiting for decades until they are reactivated.

PP 9.5

Novel CD4-based bi-specific chimeric antigen receptors: toward a functional cure of HIV infection

S. Bolivar-Wagers, B. Dey, M.H. Ghanem, L. Liu, B. Patel, V. Bundoc, E.A. Berger

Laboratory of Viral Diseases, NIAID, National Institutes of Health, Bethesda, MD, USA

Background: Durable control of HIV after cessation of antiretroviral therapy is a much sought-after goal toward a 'functional cure' of infection. We are developing a strategy based on targeted killing of HIV-infected cells by genetically modified T cells; when adoptively transferred back to the infected person, these cells will potentially provide the long-term control of infection needed for a functional cure.

Methods: We designed chimeric antigen receptors (CARs) with extremely high potency and breadth, and devoid of potential undesired activities. The CARs contain novel bi-specific extracellular targeting domains composed of sequences from invariant human proteins, and directed against distinct highly conserved determinants on the HIV Env glycoprotein. The targeting domains consist of human CD4 (extracellular domains 1 and 2) linked to the carbohydrate recognition domain (CRD) of a human C-type lectin, which specifically recognizes the high-mannose glycans on gp120. The targeting domains were followed by a transmembrane and intracellular signaling domains of CD28 and CD3 zeta to exert activation and cytolytic functions. To test activity, T cells expressing experimental and control CARs were mixed with HIV-infected autologous PMBC; HIV suppression was assessed by measuring p24.

Results: Compared to a monospecific CD4 CAR, the bi-specific CD4-CRD CARs exhibited extraordinary potency; very similar patterns were observed with genetically diverse HIV-1 isolates. Importantly, the CRD moiety prevented the CD4 component from acting as an entry receptor and rendering transduced CD8+ T cells susceptible to HIV-1 infection.

Conclusion: The minimal immunogenicity predicted for invariant all-human sequences, coupled with likely limits on virus escape imposed by targeting two highly conserved Env determinants, highlight the potential of these CARs toward an HIV functional cure.

PP 9.6

Impairment of the long-term ability of dolutegravir-resistant viruses to integrate

M.A. Wainberg, N. Osman, H. Thi Pham, B. Spira, T. Mesplede

McGill AIDS Centre, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada

Background: Resistance against the integrase strand-transfer inhibitor dolutegravir can be associated with the R263K resistance substitution in HIV integrase. Previous studies have shown that R263K decreases viral replication capacity and integration in short-term infectivity assays. Given the importance of integration in the establishment of latency, we investigated the effects of R263K on HIV integration in long-term infections.

Methods: HIV integration was measured by Alu-mediated QPCR over 5 weeks infection of PM1 cells with wild-type or R263K-mutant viruses. Levels of integration were normalized against beta-actin gene and expressed relative to integration of the wild-type virus after 1 week. Means +/- standard deviations were calculated and Student?s t-test was used to evaluate difference significance.

Results: The R263K substitution was associated with a progressive decline in the levels of integrated HIV DNA.

Conclusions: The R263K resistance substitution impairs the long-term ability of HIV to integrate, possibly decreasing the size of the viral reservoir in individuals who develop resistance against dolutegravir. Therapeutic strategies using dolutegravir to decrease the HIV reservoir should be explored.

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PP 10.0

Induction of HIV from latency by a novel molecule

N.M. Archin[1], D. Boden[2], J. Kirchherr[1], B. Allard[1], K. Sholtis[1], J.D. Kuruc[1], G. Kraus[2], D.M. Margolis[1]

[1]University of North Carolina, Chapel Hill, NC, USA, [2]Janssen Pharmaceuticals, Beerse, Belgium

Background: Even with the earliest institution of therapy, a pool of long-lived HIV infected cells is established within days of infection and is refractory to ART and the immune response. Compounds that can successfully disrupt this latent pool of virus are critical in cure strategies seeking to reactivate virus while augmenting the immune response as a mean to clear persistent HIV infection. We evaluated the ability of JNJ611, a small peptide shown to lack HDAC inhibitor or mitogen activity, to act as a latency-reversing agent (LRA) in resting CD4+T cells isolated from aviremic participants.

Methods: The ability of JNJ611 to activate the HIV LTR was first evaluated in the J89, 2D10 and CI50 Jurkat cell line models of latency encoding the enhanced green fluorescence protein (EGFP) as a marker for LTR activity. EGFP was measured by flow cytometry and toxicity assessed by side/forward scatter gating or Alamar Blue assays. HIV Gag RNA was measured in pools of total or highly purified resting CD4+ T cells isolated from aviremic, durably suppressed participants following overnight exposure to JNJ611. Finally, the ability of the compound to induce replication competent virus from purified resting cells was evaluated in limiting-dilution outgrowth assays using standardized, published methods.

Results: JNJ611 induced a 4–8 fold induction of GFP production in CI50 and J89 cells, but only marginally induced GFP in the 2D10 cell line. The compound robustly induced HIV gag RNA expression in the resting CD4+ T cells from three out of four participants and in the total CD4 T-cells from 11 out of 11 participants. However, significant recovery of replication competent virus was only achievable in the resting CD4 T cells from three of six aviremic donors evaluated.

Conclusions: While JNJ611 almost uniformly induced HIV RNA expression, the compound displayed donor to donor variability in its ability to induce outgrowth of replication competent virus. This could reflect interpatient variation in the frequencies of defective genomes (i.e., LRA response and LTR expression without virion production), or the existence of additional obstacles to the production of replication competent HIV following LRA action in the cells of some patients.

PP 10.1

Predicting determinants of long-term HIV control with gene therapy strategies

A.L. Hill[1], L. Hu[1], L.E. Hogan[2], M.A. Nowak[1], T.J. Henrich[2]

[1]Program for Evolutionary Dynamics, Harvard University, Cambridge, MA, USA, [2]Division of Experimental Medicine, Department of Medicine, University of California, San Francisco, CA, USA

Background: Gene therapy to render lymphocytes resistant to HIV infection is a proposed strategy to achieve long-term ART-free remission (e.g. CCR5 modification or delivery of antiviral gene products). Preliminary *in vivo* studies, however, have had limited success, and there is a need to elucidate the conditions under which modified cells have sufficient selective advantages to reduce target cell density below a critical level required to maintain infection.

Methods: We designed a mathematical model of the competition between wildtype and genetically modified CD4 T cells and the accompanying dynamics of HIV infection. The model was parameterized using data on lymphocyte kinetics, HIV viral dynamics, and the effects of modification.

Results: We find that under a range of scenarios, the most likely outcome is that modified cells co-exist with wild-type cells below the level required to prevent HIV persistence. To control virus off ART, edited CD4+ T cells must have a higher proliferation rate or a longer lifespan even in the absence of virus, or, edited hematopoietic stem cells must be included. The enrichment level of edited cells is highly dependent on the strength of competition between cells for homeostatic proliferation signals. Interestingly, lower thymic contribution to wild-type T cell levels makes invasion easier and engraftment higher. Higher viral fitness can make it easier for edited cells to expand initially but harder to control infection. The potential benefit can be boosted if edited cells are also resistant to certain causes of bystander cell death, or if they provide enhanced immune function.

Conclusions: Modeling suggests that control of HIV using gene therapy is possible only under a narrow range of conditions, and that further measurement and manipulation of immune dynamics during engraftment may be necessary to improve outcomes.

PP 10.2

Neutraplex nanoparticles to target HIV reservoirs

C. Lavigne[1], E. Berger[1], S. Stals[1], T. Read[2], M.A. Langlois[2], D. Gonçalves[3], D. Girard[3], D. Breznan[4]

[1]Public Health Agency of Canada, National Microbiology Laboratory, Winnipeg, Canada, [2]University of Ottawa, Ottawa, Canada, [3]INRS-Institut Armand-Frappier, Laval, Canada, [4]Health Canada, Inhalation Toxicology Laboratory, Ottawa, Canada

Background: Monocyte/macrophages are the most important HIV reservoirs outside the bloodstream. Macrophages transport HIV into sequestered anatomically sites such as the lymphoid organs and the brain. Current antiretroviral drugs hardly penetrate into macrophages and anatomic reservoir sites therefore it is highly critical to improve drug delivery in these compartments in order to achieve viral eradication. Nanomedicine is one of the promising approaches to target HIV and enhance drug delivery in viral reservoirs. Here we evaluated the potential of the lipid-based nanocarrier Neutraplex (Nx) for the transport of HIV therapeutics into macrophages and in the brain.

Methods: Cytotoxicity of the Nx nanoparticles (NPs) was assessed using different cell models and various cytotoxicity assays. Cellular uptake of fluorescent Nx NPs was evaluated in THP-1 monocyte-derived macrophages (MDMs) by confocal microscopy. In addition, Nx NPs capability to cross the blood–brain barrier (BBB) was investigated using the immortalized adult rat brain microvascular endothelial cell model (SV-ARBEC) and effect on inflammatory response was evaluated using an *in vitro* neutrophil apoptosis assay.

Results: Nx NPs showed low cytotoxicity in THP-1 MDMs and were not found to be cytotoxic for HeLa-derived cells (TZM-bl) nor for neuronal cells (Be(2)-M17) up to the highest concentration tested. Confocal studies showed that Nx NPs are rapidly and efficiently taken up by THP-1 MDMs. In addition, Nx NPs were able to transmigrate the endothelial cell monolayer suggesting that they have the capability to deliver drugs through the BBB without affecting neuronal viability. Finally, Nx NPs did not modulate apoptosis of polymorphonuclear neutrophils indicating their low interference with inflammatory response.

Conclusions: Altogether these results indicate that the lipid-based Neutraplex nanocarrier shows potential as a delivery strategy aiming to target HIV in cellular and anatomical viral reservoirs.

PP 10.3

Utilizing the binding capacity of CRISPR/Cas9 to target the HIV-1 quasispecies

M. Nonnemacher[1,2], W. Dampier[1–3], M. Desimone[3], V. Pirrone[1,2], K. Kercher[1,2], S. Passic[1,2], J. Williams[1,2], B. Wigdahl[1,2]

[1]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [2]Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [3]School of Biomedical Engineering and Health Systems, Drexel University, Philadelphia, PA, USA

Background: HIV-1 viral persistence in light of long-term HAART is a major hurdle to a cure. Genomic editing techniques, such as the CRISPR/Cas9 system, hold the promise to permanently excise integrated virus from a host cell. Targets are defined by a 20–25 nucleotide guide RNA (gRNA) molecule complementary to the desired genomic region. However, due to the rapid mutation rate intrinsic to HIV replication, the virus in patients exists as a collection of distinct genomic variants, termed quasispecies. Presented here is a methodology for designing gRNA sequences which will cleave a patient's HIV quasispecies.

Methods: PBMC genomic DNA was isolated from patients in the Drexel Medicine CNS AIDS Research and Eradication Study (CARES) Cohort and the long terminal repeat (LTR) of the HIV-1 quasispecies was sampled using Illumina Next Generation Sequencing (NGS). gRNAs were designed using a known positional binding profile to optimally target the quasispecies present in the sample. Using the entire collection of sequences from the CARES cohort, a package of three gRNAs (CARES-3) were designed.

Results: The CARES-3 package alone could cleave 70% of the observed quasispecies in the cohort which accounts for >90% of the observable integrated virus of from any patient examined to date. Supplementing this with a package of personalized gRNAs of less than three additional sequences allowed us to target all patients to below our level of detection. Examining longitudinal samples collected over a five year period suggests that the personalized gRNAs will lose their effectiveness if treatment is delayed more than 6 months.

Conclusions: Utilizing NGS sampling techniques and computational gRNA design, a package of less than six gRNAs can be developed which will cleave all observable quasispecies within the PBMC compartment. This work presents a step towards understanding the complex task of using excision therapy to target HIV-1 quasispecies in the infected human population.

PP 10.4

Effects of heme degradation products on reactivation of latent HIV-1

Z. Melkova[1], P. Shankaran[1], V. Hajkova[1], D. Jilich[2], Y. Fujikura[1]

[1]Department of Immunology and Microbiology, 1st Medical Faculty of Charles University, Prague, Czech Republic, [2]AIDS Center Bulovka, Prague, Czech Republic

We have previously observed that heme arginate, a drug approved for human use in treatment of acute hepatic porphyria, reveals a strong synergism with PKC inducers like TNF-alpha, phorbol myristate acetate (PMA), prostratin or bryostatin-1 in reactivation of the latent provirus in ACH-2 cells.

Heme is physiologically decomposed by action of heme oxygenases into three degradation products: iron (Fe^{2+}), carbon monoxide (CO) and biliverdin that is further converted to bilirubin by biliverdin reductase. Therefore, we have studied the effects of heme degradation products on latent HIV-1 reactivation when added individually to ACH-2 cells harboring integrated HIV-1 provirus or to the A2 clone of Jurkat cells harboring HIV-minivirus expressing EGFP. Addition of iron strongly increased expression of both HIV-1 p24 Ag and EGFP in PMA-stimulated ACH-2 and A2 cells, respectively, as characterized at RNA and protein levels. On the other hand, addition of a CO-donor or bilirubin decreased the PMA-stimulated p24 expression. The reactivation of latent HIV-1 by iron or heme arginate was inhibited by antioxidants N-acetyl cysteine and vitamin E or by an iron chelator desferioxamine, suggesting that the effects were mediated by an iron-induced redox stress. Finally, we were able to demonstrate the synergistic effects of heme arginate and PMA on HIV reactivation also in peripheral blood mononuclear cells of HIV+ patients cultured *ex vivo*.

Redox stress was shown to affect epigenetic mechanisms regulating gene expression as well as to activate redox-sensitive transcription factors. Therefore, we propose a model in which heme arginate-mediated redox stress induces chromatin remodeling, affects binding of specific transcription factors to HIV-LTR and allows HIV-1 expression. These results may point towards a new direction in the latent HIV-1 reactivation and therapy.

PP 10.5

Genetic variation continues to occur in well-controlled HIV-1-infected patients

W. Dampier[1–3], M.R. Nonnemacher[1,2], J. Chang Mell[1,4], J. Earl[1,4], V. Pirrone[1,2], B. Aiamkitsumrit[1,2], W. Zhong[1,2], K. Kercher[1,2], S. Passic[1,2], J.W. Williams[1,2], J.M. Jacobson[1,6], B. Wigdahl[1,2]

[1]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA, [2]Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [3]School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PA, USA, [4]Center for Genomic Sciences, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA, [5]Department of Medicine, Division of Infectious Diseases and HIV Medicine, Drexel University College of Medicine, Philadelphia, PA, USA, [6]Center for Clinical and Translational Medicine, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Background: The widespread use of antiretroviral therapy (ART) has resulted in effective long-term maintenance with undetectable viral loads. However despite this, we have now clearly demonstrated the continued generation of new mutations during long-term ART.

Methods: A set of patients from the Drexel Medicine CNS AIDS Research and Eradication Study (CARES) Cohort, who have been sampled longitudinally for more than 7 years were studied. Samples for these patients included both pre- and post-ART control of viral replication. Genomic DNA was isolated from samples and the long terminal repeat (LTR) and Tat exon 1 were amplified from proviral sequence. Bayesian Evolutionary Analysis by Sampling Trees (BEAST) phylogenetic trees were built using this sequence information. We modeled shifts in the predominant proviral quasispecies and *de novo* variation due to mutations combined with selection pressures such as therapeutic interventions, AIDS-defining illnesses, and other factors.

Results: This estimated that HIV-1 has an average mutation rate of 5.71/Kb/year, which was reduced by 1.02/Kb/year upon introduction of ART. These results suggest the presence of a low level viral replication in some patients, even in the presence of ART. In a subset of patients, the rate of mutation was not affected by control of clinical parameters due to ART.

Conclusions: These studies represent the initial steps in quantifying rates of genetic variation across longitudinally sampled sequences from patients at multiple stages of disease progression. Notably, while long-term therapy reduced estimated mutation rates, they were still non-zero, even in the absence of detectable viral loads. The sequence variation observed may be due in part to differential activation of latent proviral DNA quasispecies and/or low-level viral replication in various reservoirs that occur even in well-controlled patient populations over prolonged time and ultimately detected in the peripheral blood compartment.

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