HIV Apheresis Tags (HIVAT) Aided Elimination of Viremia

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Original Research

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Abstract

Introduction

HIV viremia is the essential element for progression of an initial HIV infection into AIDS and death. The currently approved management relies primarily on chemotherapy repressing the HIV replication in the infected CD4+ cells, although with severe systemic adverse effects. The problem is that it does not physically eliminate viruses, which then not only keep infecting healthy cells of these patients, but also promote infectivions of other people.

Specific Aim

An overall objective of our work is biomolecular engineering of virus apheresis tags (VAT) that eliminate viremias without adverse effects. The specific aim of this project was biomolecular engineering of Human Immunodeficiency Virus Apheresis Tags (HIVAT): $CD4-Au-Fe_3O_4$, $CD4-SiO_2-Fe_3O_4$, $anti-gp120-Au-Fe_3O_4$, and $anti-gp120-SiO_2-Fe_3O_4$.

Healthy Donors and Patients

Per the Institutional Review Board's approval and in compliance with Declaration of Helsinki, healthy donors and patients were presented with Patient Bill of Rights and provided Patient Informed Consent, while all the procedures were pursued by the licensed physicians.

Materials and Methods

CD4, gp120, gp41, gp160, anti-gp120, p24 were transgenomically expressed. Superparamagnetic core-shell particles (SPM-CSP) were synthesized. SPM-CSP were used as the nucleation centers for assembling the expressed molecules upon them to create virus apheresis tags (VAT). VAT were injected into the blood or lymph acquired from the HIV+ and HBV+ patients followed by apheresis at 0.47 - 9.4 T. VAT efficacy in eliminating viremia was determined through immunoblots, NMR and q-RT-PCR.

Results

Treatment of blood or lymph of the HIV+ patients' with VAT followed by virus apheresis resulted in rapid elimination of the HIV viremia. Efficacy of apheresis was contingent upon the gravity of viremia versus doses and regimens of VAT. Importantly, administration of VAT also effectively improved levels of non-infected CD4+ lymphocytes.

Discussion / Conclusions

Herein, we present in vitro the proof of concept for a new, effective treatment with virus apheresis tags (VAT), specifically Human Immunodeficiency Virus Apheresis Tags (HIVAT), of the HIV+ patients' blood and lymph, which is eliminating the HIV viremia.

It can be easily adapted as treatments of viremias perpetrated by other deadly viruses, which we vigorously pursue.

Introduction

According to the World Health Organization, approximately 36.7 million people suffered from AIDS in 2016 and approximately 1 million of them died that year, while almost 1.8 million became newly infected. [1-3]

Currently, there are no prophylactic HIV vaccines approved and / or recommended neither by WHO, nor by FDA in the United States, although the vigorous research

continues. [3-8]

HIV viremia is the essential element of progression of an initial HIV infection into AIDS and death. Within 6 weeks from the moment of infection, the HIV viremia rapidly increases up to 10^8 copies of RNA copies per milliliter of plasma. At the same time, the population of CD4 cell T lymphocytes, the primary target for HIV, declines down to 500 cells per microliter. Presence of HIV p24 shortly precedes other HIV proteins gp160, gp120, gp41. That is followed by raising IgM subsequently switched to IgG. Thereafter, there is a short period of time of the viremia decrease and CD4 population increase. Nevertheless, the HIV viremia persists at all stages. And so do HIV cell reservoirs. However, since 8-10 weeks until death, the HIV viremia is constantly increseasing up to 10^8 copies of RNA copies per milliliter of plasma and the healthy CD4+ populations are constantly apoptotically declining. This is associated with the complete anninhilation of the CD4 cell population, while patients suffer advancing stages of AIDS and death. [9-35]

Currently, there are no therapeutic HIV vaccines and / or immunotherapies approved and / or recommended neither by WHO, nor by FDA, despite the vigorous research. Although, attempts to develop therapeutic vaccines or therapeutics relying upon soluble CD4 and neutralizing anti-gp120 antibodies, as well as on genetic engineering of CD4, CXCR5, CXCR4 are vigorously pursued. [36-48]

At the present time, therapy of the HIV+ patients relies upon administration of drugs repressing HIV propagation mechanisms: entry inhibitors (e.g., enfuvirtide or maraviroc), reverse transcriptase inhibitors (e.g., zidovudine or tenofovir), integrase inhibitors (e.g., elvitegravir) or protease inhibitors (e.g., darunavir). Although, all currently approved chemotherapies cause very serious adverse effects. Moreover, the choice of the therapeutic cocktails has different impact upon the HIV-infected cells. [3, 49-52]

The main problems with aforementioned therapies are, that they do not directly eliminate the HIV viremia, i.e., they do not physically remove the virus from the patients' bodies, and they do not remove the HIV infected CD4+ cells. Therefore, HIV keep replicating and infecting healthy CD4+ cells, so that the HIV+ patients not only keep suffering progression of the disease due to disabling of the immune system, but also potentially continue infecting others through blood and lymph (primary sharing needles), as well as, all physiological secretions (primarily sex). [1-3]

Specific aim

An overall objective of our work is biomolecular engineering of virus apheresis tags (VAT) that facilitate elimination of viremia without adverse effects. The specific aim of this project was biomolecular engineering of Human Immunodeficiency Virus Apheresis Tags (HIVAT): CD4-Au-Fe_3O_4, CD4-SiO_2-Fe_3O_4, anti-gp120-Au-Fe_3O_4, and anti-gp120-SiO_2-Fe_3O_4.

Healthy Donors and Patients

Per the Institutional Review Board approval and in compliance with the Declaration of Helsinki, all healthy donors and patients were presented with the Patient Bill of Rights and provided Patient Informed Consent with their identities entirely concealed, while all the procedures involving them were pursued by the licensed physicians.

Six healthy volunteers (three women, three men) had no past or present diseases in their medical records, so they were not taking any medications. Results of their routine laboratory tests of blood, with emphasis on ESR and immunoglobulin profiles, and of urine, with emphasis on potential presence of cells and proteins, and basic tests for assuring absence of viral infections, were within laboratory norms.

Three oncology patients (all males) were diagnosed with Kaposi sarcomas. [53-54] Their blood routine laboratory tests revealed low counts of CD4+ lymphocytes, but increased ESR. These results prompted us to test for Human Immunodeficiency Virus (HIV), Herpes Simplex Virus (HSV), Cyto-megalo-virus (CMV), Human Papilloma Virus (HPV). The HIV tests came out positive, but the HSV, HPV, and CMV tests came out negative. These patients had not received any therapy prior to admission.

Five oncology patients (three women, two men) were diagnosed Multiple myelomas. Their blood routine laboratory tests revealed high ESR. These results prompted us to test for protein profiles, which revealed high immunoglobulin. Their urine routine laboratory tests revealed protein presence, identified as Bence-Jones protein. These patients were treated by aspiration of pleural and peritoneal effusion as the first therapeutic steps, but had not received prior chemotherapy.

Three oncology patients were diagnosed with Hepatocellular carcinoma and admitted for surgical treatment with intrahepatic artery infusion. Chronic Hepatitis B was in their medical histories. Four oncology patients were diagnosed with Acute or Chronic Hepatitis B, while admitted for other surgical therapy of non-liver cancers.

Materials & Methods
Blood, lymph, plasma, effusion, virus

Acquisition, processing, storage, and culture of blood, erythrocyte-free blood, peritoneal or pleural effusions, lymph, and plasma were pursued according to the original, detailed protocols published earlier and only briefly outlined here. [8-27, 53-68]

Blood was drawn according to the standard clinical procedure by venipuncture into citric acid / dextran receiving buffer. It was stored at 4 deg. C until further processing. For virus binding assays, the blood was depleted of fibrinogen and calcium (later re-adjusted to physiological levels). Erythrocyte-free blood was prepared by magnetophoresis aided by antibodies conjugated with magnetic beads. Plasma was prepared by simple sedimentation and collecting supernatant or alternatively sampled during plasmapheresis. T cell fractions were

prepared by activated sheep erythrocyte resetting. B cells were removed by complement receptor activated lysis. Desired cell fractions were enriched by FACS after labeling with fluorescent antibodies followed or by MACS after labeling with superparamagnetic antibodies. Plasma and B cells were isolated from erythrocyte-free blood by MACS after labeling with anti-CD19 and anti-CD20 magnetic antibodies.

Lymph was acquired according to the standard clinical procedure during surgeries on the open abdomens. The viruses and cells from lymph were prepared according to the protocols for erythrocyte-free blood.

All the samples were processed on various ways depending on the research plans: secured for long term storing or processed immediately upon being drawn.

For storing, the samples were prepared primarily by cryo-biobanking. The samples were equilibrated with 10% DMSO in the patients' serum at 4 deg C. They were frozen according to the gradual lowering temperature down to -35 deg C, -70deg C, -196 deg C per 24 h on each step (Cryo-immobilization apparatus was constructed and protocols for it developed thanks to the NSF Grant to MM). These samples were stored indefinitely without compromised quality. When needed, these samples were thawed according to the reverse-to-freezing protocol for processing (The cryo-apparatus aa). Alternatively, HIV was collected on filter papers for genomic studies. [23-25] Alternatively, total RNA was prepared and either stored as such or converted into cDNA for storing and / or shipping. [60-66]

HIV was propagated from the aforementioned aspiration as specifically described in the original protocols. [56-60]

For immediate processing, the HIV+ patients' viremia samples in blood, lymph, effusion, or plasma were tested by reverse transcription and polymerase chain amplification of the sequences nested by the primers designed upon the published HIV-1 sequences in [GenBank] from the samples generated by isolation of total RNA. [SEQ ID NO: 6-8] [23-26, 58-62, 64-66] The healthy donors' blood, lymph, and plasma served as the controls and processed in parallel to those of the HIV+ patients.

The samples acquired from the HIV+ patients, whose HIV counts were adjusted to the concentrations needed for particular tests, were spiked with VAT, with doses and regimens indicated in the Results. The infected samples in vials were incubated at the desired time at 37 deg. C, 5 % CO_2, while on the gyroscopic tables.

Depending on the plans for further tests, lysis of the cells was pursued in two buffers: NP40 and RIPA, as outlined in the forthcoming section. In NP40, they were prepared for immunoprecipitation (IP). In RIPA, they were prepared for immunoblotting (IL).

HIV were aspirated from the peritoneal or pleural effusions of the HIV+ oncology patients, who were diagnosed with Kaposi sarcoma. Of these sterile effusions, 100 microliters containing the HIV copy number determined by RT-PCR, was injected into in the H9 culture (ATCC) and propagated strictly according to the detailed published protocols. [58-62]

Human CD4 – Human Immunodeficiency Virus Entry Receptor (HIVER)

Human CD4 was manufactured on different ways: cell lysis - immunoprecipitation – immunoblotting, magnetic activated molecular sorting – affinity chromatography, and genomic isolation - amplification - recombination. T lymphocytes from healthy volunteers were initially selected by sheep erythrocyte resetting precipitation followed by B cell complement lysis. Later, these fractions were enriched by CD4+ selection with our anti-CD4 superparamagnetic antibodies on MACS or our anti-CD4 fluorescent antibodies by FACS. [8, 18-20, 63-66, 75] [SEQ ID NO: 1-5]

For immunoprecipitation, the enriched fractions of the CD4+ cells were lysed with NP40. The soup was mixed with our anti-CD4, superparamagnetic, genomically engineered antibodies, incubated for 1 h at 4 deg. C, and inserted into magnetic field at room temperature for 15 minutes. Diamagnetic content was rinsed off, while in the field. The fraction retained by anti-CD4 was released after the magnetic field ceased. Aliquots of this fraction were electrophoresed on PAGE and transferred onto the PVDF membranes (Amersham). The immunoblots were tested by the standard OKT4 antibody produced by the ATCC cell lines, which were initially grown in the recommended cell culture conditions, but we later modified to grow in sera-free media (ATCC).

For immunoblotting, the enriched fractions of the CD4+ cells were lysed with RIPA. The lysates were electrophoresed by PAGE and validated as immune-precipitated ones.

Alternatively, primarily for future expression of the CD4 selected domains, total mRNA was isolated from these cells and stored. [68-69] After importing the CD4 mRNA main transcripts' sequences from GenBank, the primers flanking the CD4 coding sequence were designed with the aid of 5Prime software (NIH) and synthesized. [SEQ ID NO: 1-5] [23-26] These primers served to create cDNA templates, which were cloned into the plasmids comprising CMV promoter and metal binding domains, hetero-bifunctional linker domains linking chelates. [63-66, 76-77] The choice of the coding cloning vector was contingent upon the future superparamagnetic molecule to be rendered for nuclear magnetic resonance diagnosis and for magnetic separation. Propagated plasmids were electroporated into the human myelomas, which were established from the oncology patients diagnosed with Multiple myelomas, to express the monomeric, soluble CD4. The cells were conditioned to grow in the serum-free RPMI media in roller bottles at 37 deg. C, 5% CO_2. Therefore, supernatant could be easily used to test secreted recombinant receptors expressed from all the transcript variant versions by NMR and FCM. [63-64, 76-77]

Human Immunodeficiency Virus gp160, gp120, gp41, p24

HIV glycoproteins gp160, gp120, gp41, p24, were affinity absorbed from the effusions of the HIV+ oncology patients

admitted primarily for treatment of Kaposi sarcomas or from cultures, as well, as transgenically expressed. [23-26, 63-66, 75] Templates were generated by two ways. Total mRNA was isolated from HIV+ producing CD4+ lymphocytes of the HIV+ patients as described. [SEQ ID NO: 6-8] To make gp160, gp120, gp41, p24, total mRNA was converted into ds cDNA by reverse transcription and polymerase chain reaction aided by the primers for coding sequences for gp160, gp120, gp41, p24 having sequences imported from GenBank and synthesized. The yielded amplicons were inserted into plasmids comprising CMV promoters and metal binding coding sequences or bifunctional linker binding domains as in the details described elsewhere. [63, 77] These plasmids were propagated in Escherichia coli grown in Luria-Bartani media on shakers at 37 deg. C. After isolation of plasmids on Maxipreps (Qiagen), the restriction sites were tested and plasmids cut opened to accept the inserts coding for gp160, gp120, gp41. The new plasmids (pCMV-gp160, pCMV-gp120, pCMV-gp41, pCMV-p24) were electroporated into the myeloma cells in cultures established from effusions of the oncology patients diagnosed with Multiple myelomas. Either HAT or RPMI1640 based culture media were supplemented with the filtered effusion fluids rather than bovine sera. Alternatively, the HIV proteins were isolated directly from the patients' effusions or cultures by affinity columns or magnetic activated molecular sorting (MAMS). [71-72]

Anti-CD4, anti-gp120, anti-gp160, anti-gp41, anti-p24 genomically engineered antibodies (GEAs)
All HIV+ patients manifested anti-HIV antibodies, which were detected in their blood and lymph with the clinical diagnostic tests. [50] One of these patients manifested also anti-CD4 antibodies. Taking advantage of these laboratory results, we adopted coding sequences from the plasma and B cells of these patients, while using them for biomolecular engineering of genomically engineered anti-virus antibodies (AVAs), which in the process were also rendered magnetic and / or fluorescent. All the procedures of manufacturing of antibodies was pursued as previously described and briefly outlined here. [62-65, 71-72]

The plasma and B cells were selected from blood of the HIV+ patients by MACS and FACS using our anti-CD19 and anti-CD20 magnetic antibodies. [61] Total mRNA was isolated and stored. After importing the human HC and LC lead conserved sequences [Kabat], the primers were designed with the aid of the 5Prime software [NIH] for heavy and light variable chains and synthesized. [61, 68-69] After reverse transcription, these primers primers served to create cDNA templates, which were cloned into the plasmid containing CMV promoter and metal binding domains. Plasmids were propagated in Escherichia coli grown in the Luria-Bartani media in cultures maintained on the shakers at 37 deg. C. After purification on MaxiPreps [Qiagen], the plasmids were electroporated into the cells in cultures of myelomas, which were established from the effusions of the oncology patients, who were diagnosed with Multiple myelomas. The cells were cultured in RPMI1640 media, but modified on such a way that they were supplemented with the filtered effusions of the patients, but not bovine sera. Later, the cells were conditioned to grow in serum-free media in roller bottles at 37 deg C and 5% CO2, and later conditioned to the CO2-free environment. Therefore, supernatant could be easily used to test specificity of secreted antibodies. Only Fv, assembled as scFv or dcFv with metal binding domains MBS, were used to attach to superparamagnetic nanoparticles (SPM-CSP). [71-72, 75-76] The binding domains were chosen to provide strong direct binding to Au, Ni, Co, Fe and / or Fe3O4 or SiO2 activated shells of core-shell superparamagnetic molecules as outlined herein. [82-83] Metal binding domains (MBS) facilitated rendering them not only superparamagnetic, but also fluorescent, if MBS were saturated with Eu or Tb.

Superparamagnetic domains and molecules
Superparamagnetic molecules (SPM) are prepared to comprise solid homogenous or core-shell architecture. The choice of these superparamagnetics is driven by their future applications. The solid superparamagnetic particles are used for in vitro diagnosis and research. The core-shell particles are manufactured for in vivo, in-patients diagnosis and therapy. Their inner core provides superparamagnetic properties. Their outer shells comprise biologically inert elements to protect the patients from potentially leaking, toxic magnetic material and to offer interfacing layer to link them with guiding molecules in VAT. [62, 71-72, 68-69, 73-74, 80-81]

The solid, homogenous, magnetic nickel and iron metal binding domains / particles are synthesized according to classical protocols modified for this project. [60, 73-77] Therefore, the magnetic properties of genomically engineered molecules (GEM) are gained either by incorporation of superparamagnetic entities into the structure of genomically engineered molecules or by attaching of superparamagnetic particles (SPM) to the genomically engineered molecules. Chemical reactions involved in linking biomolecules and metalic entities are described in the details. [60, 80-81]

The core-shell particles comprise Fe_3O_4 or Ni, Co cores and Au or SiO_2 shells engineered according to classical protocols modified for this project. [60] Briefly, the cores are synthesized by mixing aquous solutions of $FeCl_3$ $x6H_2O$ / $FeCl_2x4H_2O$ in ½ molar ratio, followed by adding 1m NaOH and stirring initially at room temprerature, that was gradually increased to 90 deg. C for 1h. The process is completed by multiple cycles of rinsing with water. The superparamagnetic particles are then retained by magnets and dispersed in water as ferrofluid. The gold shells are prepared according to the modification of the classical Turkevich procedure. [80] The aliquots of the ferrofluid are mixed with 01.% $HAuCl_4$, that is followed by adding 100 mM NH_2OH. The thickness of the shells is determined empirically by monitoring time and changes in absorption at 400 nm being indicative of depletion of Au. The sillica shells are prepared according to the modification of the classical Stober procedure. [81] The solution is added

to the aliquots of the ferrofluid and multiple cycles of incubation for 1h at 90 deg. C, that are followed by multiple rinses with water. Covalent attachments of CD4 and anti-gp120 to shells follow. [82-83]

Nuclear magnetic resonance spectroscopy (NMR)
Magnetic, activated cell sorting (MACS)
Magnetic apheresis
The cells were labeled with the superparamagnetic antibodies as described in details elsewhere. [62-63, 71-72, 76-77] Briefly, the antibodies were dissolved and all washing steps carried in phenol-free, Ca+ / Mg+- free, PIPES buffered saline solution, supplemented with 20 mM glucose, 10% human serum. The aliquots were dispensed into the magnetism-free NMR tubes (ShiVATi). The relaxation times T1 and T2 were measured in resonance to the applied pulse sequences on the NMR spectrometers: DMX 400 WB, MQ20, MQ60, AVANCE II NMR (Bruker) or the Signa clinical scanners (General Electric).

The VAT aka GEM-SPM or AVA-SPM were used to isolate the labeled molecules and/or cells from the solution. The labeled cells rendered superparamagnetic properties, which facilitated their isolation on the magnetic, activated cell sorter (MACS) operated at 0.47 T – 9.4 T and / or clinical MRI instruments operating at 0.5 T – 3 T and / or NMR scanners operating at 0.47 T- 4.7 T – 9.4 T (Bruker) (Thanks to the NIH and NSF grant support to MM). Measurements were also performed at T1 and T2 using Bruker mq60 (60 MHz, sample tube 7.5mm diameter) and mq20 (20 MHz, sample tube 10mm diameter). Measurements were also validated from performed in the clinical scanners at 1.5 T and 3 T (Bruker or GE) with the samples loaded into multiwall plates, imaged, and quantified from the image brightness with ImageJ (NIH). Apheresis of the VAT aka GEM-SPM tagged Human Immunodeficiency Virus (HIV) was conducted on various ways in preparation for research, diagnostic, or clinical applications: external field, magnetic filters, magnetic needles, and magnetic traps.

Flow cytometry (FCM)
Fluorescent, activated cell sorting (FACS)
Multiphoton spectroscopy
The cells were labeled with the fluorescent antibodies as described in details elsewhere. [63, 71-72, 79] They were sorted on the Calibur, Vantage SE, or Aria (Becton-Dickinson). The antibodies were dissolved and all washing steps carried in phenol-free, Ca+ / Mg+- free, PIPES buffered saline solution, supplemented with 20 mM glucose, 10% human serum. Sorting was performed on Aria, Calibur, Vantage SE (Becton-Dickinson) with the sheath pressure set at 20 pounds per square inch pressure and low count rate. The sorted batches were analyzed on Calibur or Aria using FACS Diva software or on the FC500 (Beckman-Coulter). For the measurement of the fluorescently labeled cells, these settings were tuned at the maximum emission for the Eu chelated antibody at 500V with references to isotype antibodies and non-labeled cells. This assured the comparisons between populations of cells labeled with multiple antibodies without changing the settings on PMTs.

The fluorescently labeled cells or tissues were imaged with the Axiovert (Zeiss) equipped with the Enterprise argon ion (457 nm, 488 nm, 529 nm lines) and ultraviolet (UV) (364 nm line) lasers; Odyssey XL digital high-sensitivity with instant deconvolution confocal laser scanning imaging system operated up to 240 frames/s (Noran), and the Diaphot (Nikon) equipped with the diode-pumped Nd:YLF solid state laser (1048 nm line) (Microlase).

Energy dispersive X-ray spectroscopy (EDXS)
Electron energy loss spectroscopy (EELS)
X-ray reflection fluorescence spectroscopy (XRFS)
Elemental analyses were pursued by EDXS, EELS, and XRFS as described earlier. [62-63, 71-72, 79] The field emission, scanning transmission, electron microscope FESTEM HB501 (Vacuum Generators) was equipped with the energy dispersive x-ray spectrometer (EDXS) (Noran) and post-column electron energy loss spectrometer (EELS) (Gatan). The cryo-energy filtering transmission electron microscope 912 Omega was equipped with the in-column, electron energy loss spectrometer (EELS) and the energy dispersive x-ray spectrometer (EDXS) (Zeiss). The cryo-energy filtering transmission electron microscopes 410 and 430 Phillips were equipped with the post-column, electron energy loss spectrometers (EELS) and the energy dispersive x-ray spectrometer (EDXS) (Noran). The field emission, scanning electron microscope SEM1530 (Zeiss) was equipped with the energy dispersive x-ray spectrometer (EDXS) (Noran). The field emission, scanning electron microscope H3400 (Hitachi) was equipped with the energy dispersive x-ray spectrometer (EDXS) (Hitachi). The S2 Picofox XRFS spectrometer was equipped with a molybdenum (Mo) X-ray target and the Peltier cooled Xflash Silicon Drift Detector (Bruker AXS). Scan times ranged up to 1000 seconds. The ICP standard of 1000 mg/l of mono-element Gallium or Gadolinium (CPI International) was added to 500 microL of each sample to the final concentration of 10 mg/l. Instrument control, data collection, and analysis were under the SPECTRA 7 software (Bruker).

Virus apheresis tags (VAT)
The aforementioned CD4 molecules and anti-gp120 antibodies are directly absorbed onto surfaces of the aforementioned superparamagnetic molecules or reacted through bifunctional linkers to the –SH, -COOH, or –NH2 groups present on surfaces of the aforementioned superparamagnetic molecules to form genomically engineered molecules with superparamagnetic properties (GEM-SPM) in this project specifically named HIVAT. [62-63, 76]

Rapid elimination of HIV from blood and lymph of HIV+ patients
Removal of HIV was pursued on different ways. First, the VAT aka GEM-SPM treated samples in metal-free

vials were exposed to the uniform, steady magnetic field from 0.47 T – 9.4 T. The superparamagnetic content of the samples was retained by magnetic field through VAT. The diamagnetic content of these samples was aspirated. Second, the samples were filtered through the polystyrene-coated magnetic gauze inserted into the steady magnetic field from 0.47 T – 9.4 T. The VAT labeled material was retained by the magnetic field, but non-magnetic flow-through was collected. Thereafter, the VAT-tagged material was immediately released, to separate from flow-through vials, upon removal from the influence of magnetic field. Third, the VAT-treated blood or lymph was flown over large magnetic surface that was retaining VAT-tagged virus on the flow. Fourth, magnetism-induced needle was inserted into the vials and retracted after a period of time, while pulling out absorbed HIV tagged with VAT.

Assessment of efficacy was pursued by q-RT-PCR, immunoblotting, immunoprecipitation, and magnetic resonance. [15-16, 21-25, 33, 49, 68-71]

The outcomes were studied on the following ways. First, to determine the HIV count, both, superparamagnetic and diamagnetic materials, but separately, were processed through reverse transcription and polymerase chain reaction as outlined in details in this report. Second, to determine CD4 expression and display, the samples were lysed for processing with RT and PCR aided by the primers, which sequences of the five known transcripts were imported from the GenBank, and synthesized. Alternatively, the samples were labeled with superparamagnetic CD4 or anti-gp120 to be quantified by measurements of relaxivities in NMR and after magnetic activated molecular sorting (MAMS) aided pull-out followed by non-denaturing electrophoresis, immunoprecipitation, or immunoblotting.

Statistical analysis

All the measurements were run in triplicates for each sample from six patients. The numbers were analyzed and displayed using statistical analysis and image quantification software (GraphPad, ImageJ). Data were presented as mean of standard error of the mean (SEM). Statistical significance was calculated by t-test for two groups.

Result

Elemental composition of virus apheresis tags (VAT)

The key elements for success of the HIV apheresis by virus apheresis tags (VAT) were their superparamagnetic properties. These properties were determined by the core-shell Fe_3O_4-SiO_2 or Fe_3O_4-Au nanoparticles; thus contingent upon their elemental composition of the magnetic cores. Moreover, the essential element of their safety was presence of bio-neutral shells. They were meant to protect cores against leaking Fe to the environment; thus elemental composition of their shells. The elemental composition of superparamagnetic VAT was determined by Energy Dispersive X-ray Spectroscopy as displayed in FIG. 1. The sharp, multiple energy peaks for Fe, O, of the Fe_3O_4 cores and Si, O of the SiO_2 shells clearly confirm the desired elemental composition. The designed elemental composition of Fe_3O_4-Au nanoparticles was confirmed the same way.

VAT specificity and sensitivity to tag HIV

The ultimate test for this endeavor was to determine, if indeed VAT would be specific and sensitive enough for pulling specifically HIV out of blood or lymph acquired
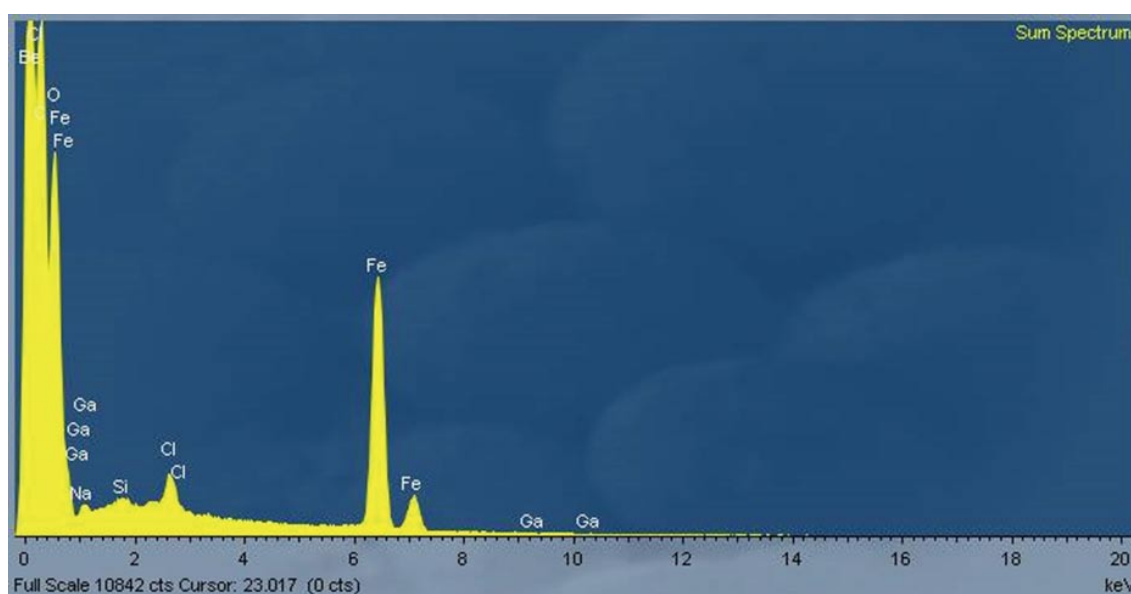

Figure 1. Elemental composition of VAT: HIVAT: CD4-SiO_2-Fe_3O_4 was determined with EDXS. Energy elemental peaks are marked (Fe – iron; C – carbon; Si – silica; O- oxygen; Ga – gallium from NIST spiked in as the internal control). This is a raw read-out, no post-acquisition manipulation, from the EDXS machine.

from the HIV+ patients. The outcomes would have to be compared versus naïve, healthy donors, or the patients carrying other viral diseases (e.h., HBV, HPV, HSV, CMV). Efficacy of VAT is obviously contingent upon specificity and sensitivity of their guiding domains: CD4 and anti-gp120 to tag gp120 - the lead targeting molecule of HIV. To determine that, blood or lymph of healthy donors, the HIV+ patients, and the HBV+ patients were having administered CD4 and anti-gp120 guided HIVAT, spun down at 1000g to remove cells, debris, and aggregates, followed by affinity columns saturated with non-competing, non-neutralizing anti-gp120, and elution. Lysates and eluates were transferred all onto PVDF membranes, and labeled with the CD4 and anti-gp120 as shown in FIG. 2. Therefore, the only source of gp120 could come from apheretic acquisition of HIV. Both were derivatized with nickel, cobalt, or iron nanoparticles. Only gp120 was labelled very specifically, so that it appeared as one, single band on the PVDF membrane with the remaining portions of the lanes entirely and completely clear. The lanes carrying lysates from healthy donors and HBV+ patients, as well as mutated CD4 and gp120 remained bands' free. Intensity of the bands was contingent upon the concentration of gp120; thus indicative of gravity of the HIV viremia.

Efficacy VAT aided HIV apheresis from bloodand lymph of HIV+ patients

The VAT aided efficacy test was meant to determine, if indeed VAT would be capable for pulling most, if not all, of HIV out of blood or lymph acquired from the HIV+ patients. The easiest and the fastest way to monitor viremia and efficacy of apheresis are accomplished by measuring relaxation time rates in NMR or MRI. [72, 79] To determine that, CD4 and anti-gp120 guided VAT aka HIVAT was administered into blood or lymph of the HIV+ patients, spun down at 1000 x g to remove cells, debris, and clusters, followed by affinity columns saturated with non-neutralizing, non-competing anti-gp120, and elution. Relaxation time rate changes induced by VAT bound to

HIV were recorded in NMR after separating each into 2 separate vials: one containing HIV apheresed by VAT and the other non-bound eluates. This approach allowed us to quantify efficacy of HIVAT aided apheresis (apheresed to non-apheresed ratio) as reported in FIG. 3. Relaxivities for water, blood, and serum of healthy volunteers served as the additional controls. In assessment of the results, it is very important to realize that relaxation time rates are contingent upon many factors including, but not limiting to field strength, frequency, relaxivity, molecular weight, binding constants, etc. Therefore, setting the base line measurements for water, serum, lymph and blood are essential, as well multiple controls performed by measuring relaxation times for healthy donors, as well as after administratin of VAT engineered with mutated CD4 or isotype anti-gp120, and also being tested on the other viruses having the completely different targeting molecule e.g., HBV. The readings are then relative to these base line values. Further, they can be then correlated to q-RT-PCR data, to make NMR data additionally validated quantitatively. As shown, VAT are sensitive and specific as an effective disease gravity measure in all heights of the HIV viremia. As the data show, the HIVAT was primarily contingent upon the molar ratios of HIVAT. With the sufficiently high dose of HIVAT, it could eliminate the HIV from blood and lymph of HIV+ patients, so that HIV was not detected by immunoblotting, q-RT-PCR, and NMR. The data demonstrate rapid reduction of the HIV viremia in blood of the HIV+ patients thanks to administration of VAT, specifically HIV targeting genomically engineered molecules engineered with superparamagnetic properties (GEM-SPM) aka Human Immunodeficiency Virus Apheresis Tags (HIVAT).

Dose dependence of elimination of HIV viremia from blood and lymph of HIV+ patients

Rapid reduction of the HIV viremia in blood of the HIV+ patients thanks to administration of VAT, specifically Human Immunodeficiency Virus Apheresis Tags (HIVAT)
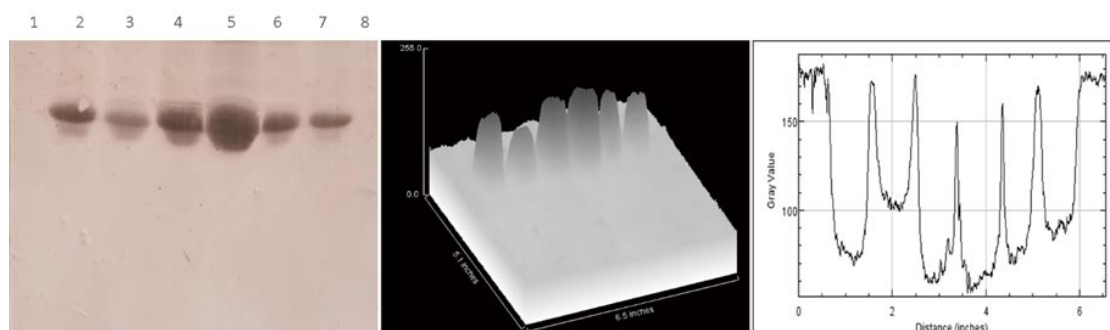

Figure 2. Left pane. Immunoblot of electrophoresed and transferred lysates captured by VAT from the healthy donor 1 loaded into the lane 1, the HIV+ patients (2- 7), lanes 4-5 (HIVAT: CD4-Au-Fe$_3$O$_4$), the lanes 6-7 (HIVAT: CD4-SiO$_2$;-Fe$_3$O$_4$), the lane 2 (anti-gp120-Au-Fe$_3$O$_4$), the lane 3 (anti-gp120-SiO$_2$-Fe$_3$O$_4$), and from the HBV+ patient 1 loaded into the lane 8, while all were labeled with anti-gp120 derivatized with SPM. This is a raw image acquired in white light illumination, with no post-acquisition manipulation. VAT guiding domains incorporating CD4 mutations and isotype anti-gp120 resulted in empty lanes. Middle pane. Three-dimensional quantification of the immunoblot in the left pane. Right pane. Top values quantification of the immunoblot in the left pane.

aka genomically engineered molecules engineered with superparamagnetic properties (GEM-SPM) is presented in FIG. 4. The GEM portions of VAT comprise recombinant CD4 or anti-gp120. Therefore, they are uniquely and reliably specialized in docking the HIV through its gp120 infection lead. The SPM portions of VAT comprise superparamagnetic domains. Therefore, they efficiently aid retention of HIV, anchored through its gp120 docked into CD4 of VAT, by magnetic field. Efficacy of this apheresis is contingent upon the ratio between the number of HIV in blood or lymph and the number of VAT administered to the specific sample. The higher the viremia the higher the number of VAT has to be administered to reduce the viremia. The higher the viremia the more rounds of VAT administration are necessary to bring down the viral count

to undetectable levels. Equivalent results were acquired for treatment of lymph with VAT. Although, the antibodies linked to form VAT were nearly as effective as the CD4 guided ones. However ultimately, the efficacy of the HIV rapid removal is primarily contingent upon the gravity of HIV viremia (HIV particle counts) versus dose (VAT particle count) and regimen (maintaining or frequency of VAT guided aphereses).

Long term repression of HIV viremia
Rapid reduction of the viral count has long term consequence for the height of the HIV viremia in time (thus corresponding to the time frame of progression of the disease towards AIDS) as shown in FIG. 5. The presented results compare how viremia, in blood acquired from the
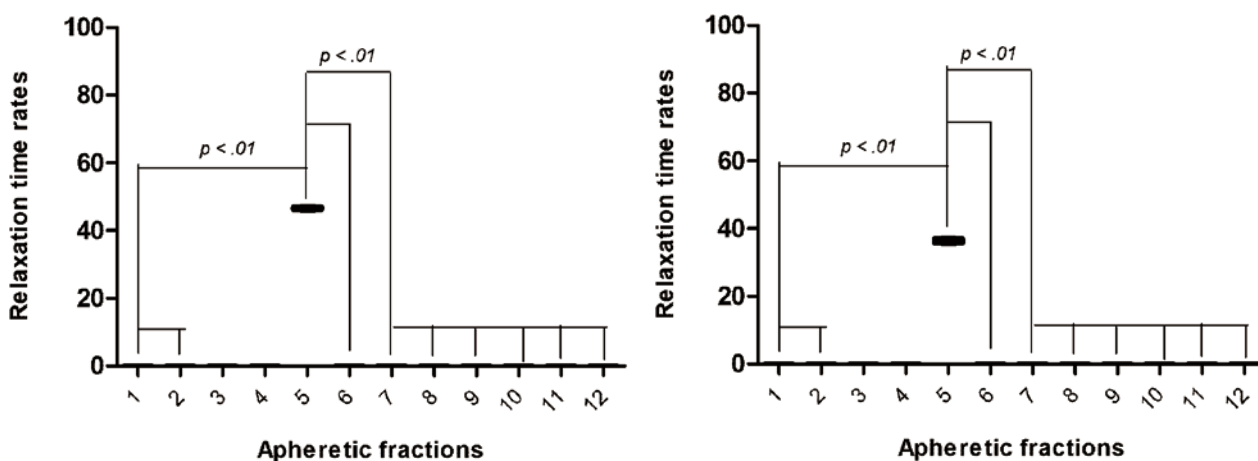

Figure 3. Left pane. Quantified changes in relaxation time ratesy (y axis: relative units) measured in blood from the healthy donor 1 (x axis: Apheretic fractions) (1-4), HIV+ patient 1 (5-8), and HBV+ patient 1 (9-12) after administration of VAT followed by apheresis. All relaxation time readouts of the samples containing the VAT apheresed material (1, 3, 5, 7, 9, 11) are compared with the residual after apheresis (following even numbers). VAT: CD4-Au-Fe$_3$O$_4$ (1-2, 5-6, 9-10), mut CD4-Au-Fe$_3$O$_4$ (3-4, 7-8, 11-12) adjusted to equmolarity. All samples were measured in triplicates for each VAT and for each patient. The data are representative for all measurements performed. Right pane. Quantified changes in relaxivity measured in lymph from the healthy donor 3 (1-4), HIV+ patient 3 (5-8), and HBV+ patient 3 (9-12) after administration of VAT followed by apheresis. All labels the same as in left pane. All samples were measured in triplicates for each VAT and for each patient. The data are representative for all measurements performed.
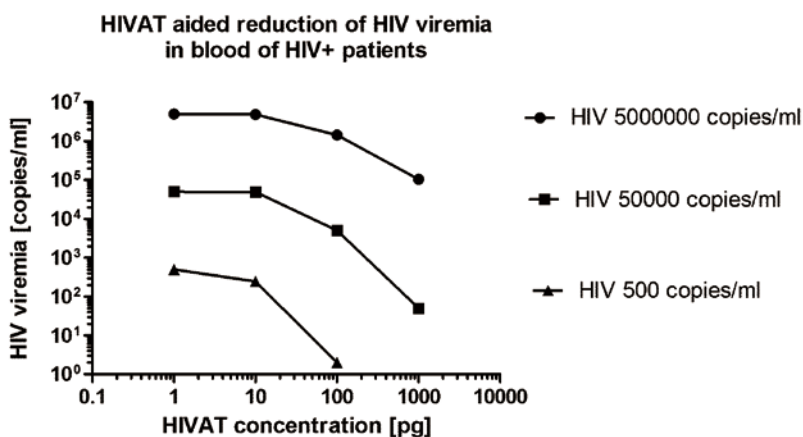

Figure 4. Rapid reduction of the various heights of HIV viremia in blood of the HIV+ patients was attained by means of VAT aka genomically engineered molecules with superparamagnetic domains 9GEM-SPM0 aided apheresis. The HIV copy numbers were determined in blood by polymerase chain reaction with the HIV specific primers.

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HIV+ patients and maintained in cultures, progresses in absence of administration of VATs and how it is repressed thanks to the daily administration VATs and apheresis. Without administration of VAT aka GEM-SPM, viremia may reach 10^8 copies in a milliliter of blood or lymph in a few weeks. With administration of VAT and VAT, the HIV viremia is repressed down to only a few hundred copies of HIV or undetectable by PCR levels contingent upon the concentration and number of cycles of the administered VAT aka GEM-SPM.

Prophylactic administration of VAT reducing raise of HIV viremia

Human Immunodeficiency Virus Apheresis Tags (HIVAT) have also preventive effects onto the progress of the HIV viremia as shown in FIG. 6. These effects were revealed, when the human CD4+ lymphocytes in cultures with or without VATs were spiked with 100 copies of HIV adjusted in 100 microliter of blood or lymph from the HIV+ patients. In a week, the HIV copy number in culture without VAT increased to a few thousand copies. During the same time,

the HIV copy number in the culture containing VAT prior to infection and treated by cycles of magnetic apheresis was retained at very low, down to PCR undetectable levels.

HIVAT repression of new infections of CD4+ cells

Reducing the HIV viremia thanks to administration of HIVATs resulted in the dramatic reduction of the newly infected CD4+ lymphocytes as shown in FIG. 7. The graph shows, that the number of CD4+ of newly infected was significantly lower in the samples in which HIVAT were applied, than in the samples in which its administration was absent. Therefore, VAT were very effective decoys protecting CD4+ cells against being infected by HIV.

VAT aided, long term protection of the CD4+ cells' population

Progression of the HIV infection into AIDS is primarily driven by the cytopathic effects upon CD4+ cells. Administration of VAT caused the dramatic improvement of the CD4+ cells' population as demonstrated in the FIG. 8. The CD4+ cell counts in blood and lymph cultures

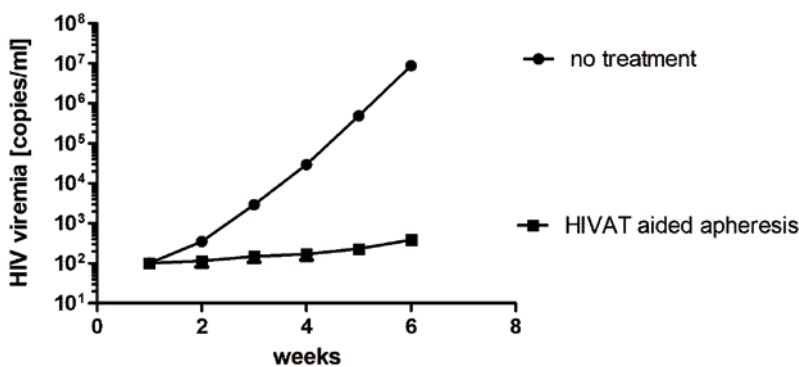**HIVAT aided represion of HIV viremia in blood of HIV+ patients over time**

Figure 5. HIV viremia in blood of the HIV+ patients was repressed over time by administering HIVAT aka HIV targeting genomically engineered molecules featuring superparamagnetic properties (GEM-SPM). The HIV copy number was determined in blood by polymerase chain reaction with the HIV specific primers.

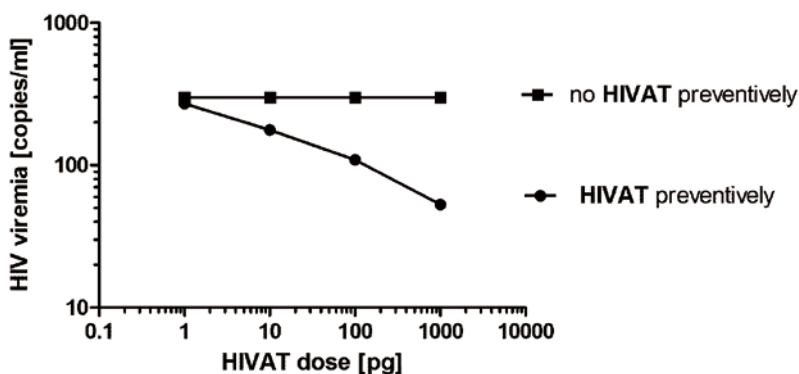**HIVAT aided prevention against HIV viremia in blood of HIV+ patients**

Figure 6. Prophylactic administration of VAT aka HIVAT prior to the HIV infection resulted in significant reduction of the HIV viremia upon infection. The HIV copy number was determined in blood by q-RT-PCR with the HIV specific primers.

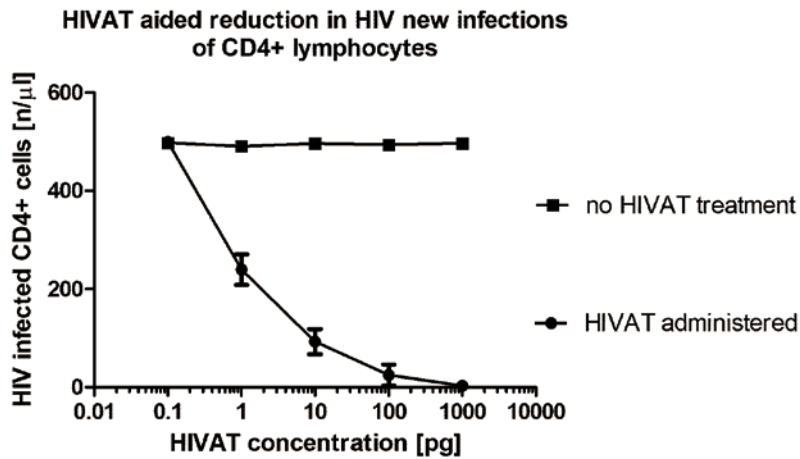**HIVAT aided reduction in HIV new infections of CD4+ lymphocytes**

Figure 7. Significantly reduced number of CD4+ lymphocytes, which became newly infected with HIV, if infection occurred after administration of HIVAT aka genomically engineered molecules featuring superparamagnetic properties (GEM-SPM). The CD4+ lymphocyte count was conducted by flow cytometry after labeling with anti-CD4 antibodies rendered fluorescent with phycoerythrin.

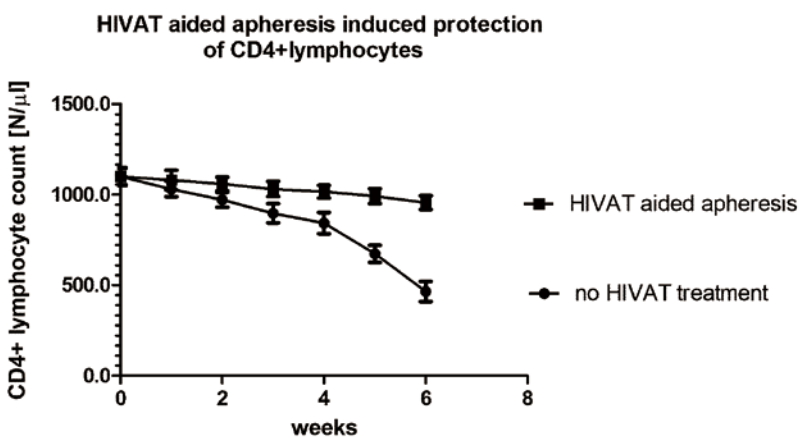**HIVAT aided apheresis induced protection of CD4+lymphocytes**

Figure 8. CD4+ lymphocyte population is protected by administration of HIVAT aka VAT, which is repressing the viral count, therefore is reducing CD4+ lymphocyte cytopathic depletion. The CD4+ lymphocyte count was conducted by flow cytometry after labeling with anti-CD4 antibodies rendered fluorescent with phycoerythrin.

acquired from the healthy donors were quickly depleted upon the HIV infection with 100 microliters of the blood or lymph from the HIV+ patients. However, when the parallel samples were treated with HIVAT followed by daily session of apheresis and replenishing of fresh batches of VAT, populations of the CD4+ cells were retained near borders of the low norm levels. Relevance of this procedure to the clinical practice would be giving the time for the infected patients' immune system to assemble immune response; thus surely reduce progression of the disease.

Discussion

Since the patients' infection with HIV, the viral count rapidly increases up to 10^8 copies per milliliter or 10^5 per microliter of plasma in 4 – 6 weeks. [1-3] This is associated with rapid decrease of the CD4+ cell population down to 500 or $5x10^2$ CD4+ T lymphocytes per microliter of blood during the same period of time. This makes more than 200 copies of HIV per 1 CD4+ cell, which have to be annihilated to prevent continued infections. Cytopathic effects, which result from HIV hijacking of the CD4+ molecular mechanisms for replication is the underlying pathomechanism. Briefly, the following chain of events takes place, the higher HIV viral count, the more CD4 cells become infected, the more CD4 cells die, the more

immunocompromised patients become, the more rapid progression of AIDS these patients suffer from, the more likely opportunistic infection strike, and the earlier the patients die. Therefore, in our opinion, the primary, the most essential element of any therapy of the patients infected with HIV should be, to repress the number of HIV in blood and lymph, from the earliest stages of this infectious disease through all stages of advancing disease to death. In clinical practice, the goal should be to maintain viremia at the lowest levels, not only by chemotherapeutic inhibition of molecular mechanisms of the CD4+ cells, but primarily by physical reduction of the viral particles from the earliest stages of the HIV infection. Yet, no such procedure existed to date. This is exactly, what we have accomplished by designing and manufacturing VAT: HIVAT in this project. Administration of HIVAT into blood and lymph acquired from the HIV+ patients resulted in eliminating viremia or at least reducing it down to the levels undetectable by the most sensitive tests to date: q-RT-PCR, immunoblotting, and NMR. As our data show, we can efficiently reduce the HIV viremia in blood and lymph of the HIV+ patients with high specificity and high sensitivity, but also repress it low levels, while monitoring the therapeutic efficacy of HIVAT.

In addition to viremia, HIV are hiding within cells, replicate in them, and propagate through cytopathosis,

budding, and syncytia. Repressing these processes is the purpose of administering various cocktails of chemotherapies. However, these strategies do not eliminate physically the HIV infected cells. Therefore, complementary to HIVAT, we have developed the procedure eliminating the HIV infected cells. [8] Nevertheless, both novel approaches can well support chemotherapies currently apprived; thus complement each other.

Magnetic forces involved in the interactions of the VAT superparamagnetic domains with fields of the magnets are decisive factors for efficacy of the virus apheresis. Herein, we presented only the part pertinent to apheresis of HIV. These forces will differ, if applied for VAT having different relaxivities, other viruses, and magnetic fields having different strengths. In those realms, the aforementioned factors have to be calibrated accordingly, the same way as we have them calibrated herein. [60, 73-74, 77]

We propose streamlining of VAT administration for elimination of HIV viremia within different clinical trials' scenarios. First, VAT may be used in addition to HAART, while most likely allowing us to reduce the doses of administered chemotherapeutics. Second, if administered pre-emptively, they may potentially work as prophylactic vaccine. Third, for patients, who are contraindicated to receive the chemotherapy, VAT may become their main rescue route. Successful completion of the clinical trials in this regard would pave the road for streamlining this approach as therapy into medical practice.

In preparation to streamlining of VAT into the clinical trials, we consider safety of the participants as the most important factor. The main immunogenic domain of this HIVAT is HBsAg - HBV VLP vaccine. HBsAg is the first vaccine administered in the USA to newborns within hours from delivery per the CDC guidelines and with the FDA approval (Recombivax, Engerix). This is followed by the scheduled two booster shots and potential further booster shots administered to the patients, whose antibody titer fell down. Therefore, HBsAg as the immunogenic domain of VAT is not going to induce any further adverse effects than the HBV vaccine would have done already by itself, when administered during the vaccination schedule. Adverse effect may differ contingent upon the VAT guiding domain: human CD4 versus anti-gp120. Human CD4 is the molecule omnipresent on the T helper class of lymphocytes. It is expressed through 5 splicing mRNA variants. The CD4 docking domain is fairly conserved. Any mutants of this domain, the same way as of CXCR5, makes the person carrying them resistant to the HIV infection. Therefore, these people can be withdrawn from the HIV vaccination and vaccine based therapy based upon simple pharmacogenomic tests. Moreover, VAT can be personalized, while taking the CD4 sequence from the patient qualified for VAT therapy. Anti-gp120 antibodies may become immunogenic, if incorporated as VAT guiding domains. This is the same way as therapeutic human and / or humanized antibodies may raise HAHA. Mouse antibodies, even after being humanized, may still often lead to HAMA. So can antibodies from other species. All

of them may annihilate therapeutic efficacy and cause adverse effects. For those reasons, immunotherapy is often preceded by immunosuppressants. Moreover, classes of anti-gp120 will also affect potential adverse effect due to immunogenicity depending, if IgG, Fab, scFv, diFv, or dcFv. Therafter, promoting HIV infections by anti-HIV antibodies was already reported. Furthermore, the polyvalent antibodies may also result in formation of aggregates with severe adverse consequencecs depending if HIV apheresis is initiated extra- or intra-corporeally.

Finally, the data which we have presented here are so encouraging and the procedures so simple to adjust, that can be easily adapted by a person skilled in pharmaceutical biotechnology, cell molecular biology, and genomic medicine, as treatments of viremias perpetrated by other deadly viruses.

Conclusions

Herein, we present the new, effective treatment with virus apheresis tags (VAT), specifically Human Immunodeficiency Virus Apheresis Tags (HIVAT), of the HIV+ patients' blood and lymph, which is eliminating the HIV viremia.

It can be easily adapted as treatments of viremias perpetrated by other deadly viruses, which we vigorously pursue.

Abbreviations

HIV - Human Immunodeficiency Virus; AIDS - Acquired Immunodeficiency Syndrome; SPM – superparamagnetic; CD4 - Cluster of Differentiation 4; gp120 - glycoprotein 120; NKC – natural killer cell; CTL – cytotoxic lymphocyte; EDXS – energy dispersive x-ray spectroscopy; EELS – electron energy loss spectroscopy; XRFS – x-ray reflection fluorescence spectroscopy; FCM – flow cytometry; NMR – nuclear magnetic resonance; FACS – fluorescent activated cell sorting; MACS – magnetic activated cell sorting; MAMS – magnetic activated molecular sorting; SPM-CSP - superparamagnetic core-shell particles; GEM – genomically engineered molecules; SPM – superparamagnetic nanoparticles; VAT - virus apheresis tags; HIVAT - Human Immunodeficiency Virus Apheresis Tags.

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Intellectual Property
All the designs, manufacturing processes, and utility methods are protected at USPTO and WIPO on behalf of Marek Malecki MD PhD as Primary Inventor.

Conflict of interest
None.

Federal, State, Industrial Sponsorship
None.

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Sequence Listing
See attachment.