Chimeric Trojan protein insertion in lentiviral membranes makes lentiviruses susceptible to neutralisation by anti-tetanus serum antibodies

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Abstract

This study describes the initial testing of a novel strategy for neutralisation of lentiviruses using the fundamental biology of enveloped viruses' assembly and budding. In the field of gene therapy, viral vector surface proteins have been manipulated in order to redirect host cell specificity by alteration of pseudo-types. We tested whether known viral pseudo-typing proteins or surface proteins known to be recruited to the HIV envelope could be engineered to carry neutralising epitopes from another microorganism onto the lentiviral surface. Our results identify ICAM1 as a novel vehicle for lentiviral pseudo-typing. Importantly, we show that in a model lentiviral system ICAM1 can be engineered in chimeric form to result in expression of a fragment of the Tetanus toxoid on the viral membrane and that these viruses can then be neutralised by human serum antibodies protective against Tetanus. This raises the possibility of delivering chimeric antigens as a gene therapy in HIV infected patients.

Introduction

In 2015 UNAIDS estimated that 36.9 million people were living with HIV infection and that there were 1.2 million AIDS related deaths http://www.unaids.org/sites/default/files/media_asset/20150901_FactSheet_2015_en.pdf). Much is being learned from recent clinical trials but efforts to find either a vaccine or a cure have been so far unsuccessful [1-3]. This leaves infected individuals facing a possible lifetime on anti-retroviral drug regimes, which though revolutionary are challenging and costly to implement and are not without side-effects [4, 5]. HIV biology presents many challenges to medical advances including its rapid systemic spread from site of entry, the massive antigenic diversity generated by its mode of replication and the damage it causes to the immune system. Furthermore, HIV latency, though incompletely understood, has been demonstrated to be a source of viral re-emergence upon cessation of drug therapy and of drug resistant strains of HIV [6-8].

In this paper we would like to propose a novel strategy to circumvent the ability of HIV to evade immune clearance and present data using lentiviral models that support initial proof of concept; we call this strategy Trojan Insertion. HIV infection is characterised by continual cycles of immune evasion due to the rapid selection of escape mutations [9-12]. Our strategy involves forcing HIV virions to express on their surface immunogens from other pathogens, to which there is a pre-existing memory response which can rapidly and decisively clear the emerging virus.

Lentiviruses like HIV are enveloped by the host-cell plasma membrane, which coats the virus as it buds from the cell. Some host cell plasma membrane proteins may be actively recruited to sites of HIV budding and can increase infectivity [13, 14]. This might be exploited if host cells can be made to express immunogens on their surface in a form that can be incorporated onto budding virions. To this end we have designed chimeric proteins, which we call Trojans,

which consist of a transmembrane domain from proteins known to be incorporated into lentiviral envelope membranes artificially fused to an immunogenic extracellular domain.

The extracellular antigen selected for our model experiments was the Tetanus Fragment C (TetFrC) antigen. Tetanus Toxoid has been historically used with great success for immunisation of humans against *Clostridium Tetani.* The serum of immunised humans has been shown to contain anti-toxoid neutralising antibodies that can be administered as a passive therapy against tetanus infection [15]. In addition, a modified fragment of the Tetanus Toxin has been shown to be an effective adjuvant fusion molecule to stimulate T cell responses against human cancers [16, 17].

We decided to test two potential membrane anchors for fusion to the TetFrC extracellular domain. The first is the Vesicular Stomatitis Virus glycoprotein (VSVg). This is a virus attachment and fusion protein, which confers viral tropism for a wide variety of cell types and has been shown to be successfully incorporated into the surface membrane of lentiviral vectors [18, 19]. These properties have been successfully utilised for pseudo-typing many recombinant lentiviral vectors. This protein has been genetically engineered to bear the 52 kDa protein Streptavidin in place of its native attachment and fusogenic extracellular domains. Kaikkonen *et al*. showed that virions expressing Streptavidin in this way on their membrane could be targeted to a particular cell type using bridging biotinylated antibodies against cell type-specific markers [20].

The second transmembrane anchor is derived from ICAM1. This protein has been shown to be recruited to the HIV surface via a direct interaction with the HIV protein gag and as a result may be present at relatively high levels on free virions [13, 21]. ICAM1 has been extensively studied

and the exonic sequences contributing to its transmembrane and cytoplasmic domains are well-defined.

Having designed our chimeric Trojan proteins we set out to test whether they can be used to coat lentiviral vectors and whether viruses so coated can be neutralised by a simulated human immune response. Our data show that the Trojan proteins can transferred by human lentiviral vector packaging and T-cell lines both transiently and stably. Furthermore, we show that lentiviral vectors packaged in the presence of Trojan proteins are measurably infectious and this infectivity can be neutralised by human anti-tetanus serum antibodies.

Materials and Methods

Chimeric Constructs

Chimeric cDNA constructs TV, TI, SV and SI (Figure 1) were designed with a four domain structure consisting of a signal peptide, sequences encoding a 5' FLAG® epitope tag (Sigma Aldrich), an extracellular domain, and finally a transmembrane/cytoplasmic domain to direct plasma membrane. The signal peptides were either from the baculovirus Gp64 protein (constructs TV and SV) or from human ICAM1 (constructs TI and SI).

TetFrC sequence was provided by J. Rice (Southampton University UK) [16, 22]. Sequences for gp64 signal peptide, VSVg transmembrane and cytoplasmic domain were provided by M. Kaikkonen (University of Kuopio, Finland) [20]. ICAM1 signal and transmembrane and cytoplasmic domain sequences were identified from published sequences [23] and NCBI entry CCDS12231.1.

Chimeric cDNA constructs were produced by GeneArt® (Life Technologies). Chimeric cDNA constructs TV and TI were subcloned using *Eco*RV and *Nhe*I restriction enzymes (New England Biolabs, UK) to the pRRLsc_C_W self-inactivating integrating lentiviral vector plasmid with a central polypurine tract/central termination sequence. This vector encodes a lentiviral transfer cassette with expression driven from an hCMV promoter with a Woodchuck hepatitis virus posttranscriptional regulatory element. Resulting plasmids were designated as TV and TI respectively and were subjected to Sanger sequencing to confirm chimeric gene sequences. To create an enhanced green fluorescent protein (*eGFP*)-expressing control virus (LVGFP), the pRRLsc_CEW transfer plasmid was used and has been previously described [24].

Streptavidin cDNA was recovered by FLAG® tag primer extension proof-reading PCR amplification of Streptavidin sequences from plasmid pCMV-SA-VSV-GED (provided by M. Kaikkonen, University of Kuopio, Finland) with first round primers forward

CAAGGACGATGACGACAAGGACCCCTCCAAGGAC and reverse ATCCCGGGCTGCTGAACGGCGTCGAG and for second round amplification forward primer ATAGGATCCATGGACTACAAGGACGATGACGACAAG. PCR products were digested with *Xma*I and *BamH*I and subcloned into plasmids TV and TI using *BamH*I and *Age*I enzyme sites to create SV and SI, respectively. Sanger sequencing was used to confirm chimeric gene sequences.

Cell lines and Culture

Human Embryonic Kidney (HEK) 293T and human fibrosarcoma HT-1080 cell lines were obtained from ATCC. PM1 T-cell line was obtained from National Institute for Biological Standards and Control (UK). All cell lines were cultured in High Glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) (PAA, UK), with stable Glutamine. Unless otherwise indicated medium was supplemented with 10 % foetal bovine serum (FBS) (Gibco, UK), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco, UK)

Transient expression of chimeric constructs

$1x10^6$ HEK 293T cells were grown for 24 h. Cells were then transfected with 5 μg lentiviral transfer expression cassette plasmids (TV, TI, SV and SI) using Lipofectamine® (Life Technologies) according to manufacturer's instructions. On day 1 post-transfection cells were removed from flasks using Trypsin EDTA (Gibco UK), washed and returned to new flasks with fresh media. On day 3 post-transfection cells were harvested using 0.5 mM EDTA in PBS (Sigma Aldrich) and stained with either 5 μg/mL of mouse M2 anti-FLAG® antibody (Sigma Aldrich) or neat mouse anti-TetFrC hybridoma supernatant 31e11 (kindly provided by C. Watts, University of Dundee UK). Negative control cells, mock transfected in the absence of plasmid, were stained with 5 μg/mL isotype control antibody mouse IgG1. The secondary antibody in

each case was goat anti-mouse Alexa Fluor®647 (GaM647, Life Technologies). After staining cells were fixed with 2 % paraformaldehyde and singlet cells analysed by flow cytometry using a FACS Canto II machine (Becton Dickinson). FACS Plots and associated measurements were generated using FlowJo software version 8.8.6 (Treestar Inc.).

For further quantitation of chimeric protein expression, $0.2x10^6$ 293T cells were transfected 24h post-plating with 1.6 μg of transfer cassette plasmid using Calcium Phosphate which was to be used in lentiviral packaging. Gene expression was detected at 48h by antibody staining as described above. Statistical analysis was performed using GraphPad Prism 6 statistical software (GraphPadSoftware, San Diego, CA). The levels of gene expression were compared by Ordinary One-way ANOVA with Tukey's post-hoc test for multiple comparisons.

Lentiviral Vector Production

Lentiviral vectors were produced by calcium phosphate mediated transfection into HEK-293T cells, using VSVg pseudotype for all vectors. Plasmids used for lentiviral production are as previously described [25]. Cells were transiently transfected with 12.5 μg packaging plasmid (pMDLg/pRRE), 6.25 μg pRSV-REV, 7 μg pMD2.VSV-G and 25 μg of transfer plasmid.

Viruses were titrated for Transducing Units/mL by transduction with limiting dilutions and FACS as previously described [25], using the HT1080 cell line. Cytoplasmic *eGFP* gene expression in singlet cell populations was measured directly in the FITC channel. Surface chimeric Trojan protein expression was detected by binding of the M2 anti-FLAG® antibody (Sigma) as described above. Percentage cells expressing surface FLAG® epitope above background detected in mock transduced cells was measured in the APC channel. Mean titres for each lentiviral vector were compared by Ordinary One-way ANOVA with Tukey's post-hoc

test for multiple comparisons using GraphPad Prism 6 statistical software (GraphPadSoftware, San Diego, CA).

Lentiviral transduction

For testing of production of chimeric proteins in cell lines, 5 x 10^5 cells were transduced at multiplicity of infection (MOI) of 1 in DMEM 10 % FCS in the presence of 8 µg/mL polybrene (Sigma). On day 3 post-infection half the cells were analysed for surface FLAG® expression by antibody staining and FACS as described above. Remaining cells were subjected to clonal dilution (3 cells per mL) and distributed at 200 µL per well to 96-well round bottom plates. Wells containing growing colonies were expanded until enough cells were available to be sampled for surface FLAG® epitope expression by M2 antibody binding and FACS as described above.

Immunoprecipitation and Western Blotting

Lentiviral suspensions were prepared as described above. As a positive control for immunoprecipitation cell lysates were prepared from 293T cells transiently transfected with TI and 24 (as described above). Cells were lysed at 50 x10^6 cells/mL in ONYX buffer (20 mM Tris (pH 7.4), 140 mM NaCl, 1 mM EGTA, 1 % Triton, 10 % glycerol, 50 mM iodoacetamide and protease inhibitor cocktail (Roche) according to standard methods.

Protein concentration of viral preparations and cell lysates was analysed using the Micro BCA kit (Perbio) according to manufacturer's instructions. 10 µg of protein from each sample was subjected to immunoprecipitation as previously described [26] using human anti-tetanus polyclonal serum IgGs (NIBSC reference antibody TE-3) or Isotype human polyclonal IgGs (Sigma). Recovered beads were washed and treated with PNGase F (New England Biolabs). Immunoprecipitated proteins were released from beads during denaturation as described by manufacturer into LDS sample buffer (Life Technologies) with addition of 50 µM DTT (Sigma).

After SDS-PAGE and blotting, PVDF membranes were probed with HRP-conjugated M2 anti-FLAG® antibody (Sigma), followed by chemiluminescent detection using ECL™ reagent (GE Healthcare). As an additional control 1 µg of each lentiviral preparation was left unprecipitated, denatured, treated with PNGase F and then subjected to SDS-PAGE and western blotting as for the immunoprecipitated proteins.

Neutralisation assay

$6.5x10^5$ transducing units of vector was pretreated in 100 µL PBS for 30 min with 100 µg of human anti-Tetanus polyclonal IgG antibody (NIBSC, described above), or with 100 µg of human IgG (hIgG) isotype control antibody (Sigma). 10^5 HT1080 cells were then transduced in standard growth medium for 18 h before vector was removed and cells washed with PBS to remove any unbound antibody or viral vector 48 h post-infection cells were fixed with 2 % paraformaldehyde and analysed by FACS for expression of the relevant transfer cassette as described above.

Mean percentage gene expression-positive cells above background (from mock-transduced cells) was calculated. Mean gene expression resulting from successful transductions was calculated from a minimum of 3 transduced wells for each combination of virus and antibodies. Error bars were calculated as ± Standard Deviation (SD) of the mean. Statistical analysis was performed using GraphPad Prism 6 statistical software (GraphPadSoftware, San Diego, CA). The effects of the different treatments on transgene gene expression as a proxy for virus infectivity were compared using a One-way ANOVA with Sidak's multiple comparisons post-hoc test comparing the percentage transduction by each treated vector with the corresponding PBS treated vector control.

Results

Transient expression of chimeric constructs results in surface-membrane protein expression

In order to be incorporated into the lentiviral membrane chimeric proteins must first be incorporated into the host cell membrane. Since we planned to use 293T cells as packaging cells for lentiviral vector production, we wanted to first demonstrate that our novel chimeric proteins could be expressed from our lentiviral transfer expression cassette plasmids onto the surface plasma membrane of these cells.

Initially, we wanted to test whether we could use commercially produced and well-characterised M2 anti-FLAG® antibody staining as a marker for TetFrC-chimeric protein expression. To this end, 293T cells were transiently transfected with plasmids TV, TI, SV or SI, harbouring FLAG®-tagged TetFrC or streptavidin chimeric protein genes, using Lipofectamine®. On day 3 after transfection cells were harvested and analysed for chimeric protein expression (Figure 2a) by FACS analysis of surface binding of the anti-FLAG® epitope antibody M2 (black line plot), or of anti-Tetanus Fragment C hybridoma supernatant 14e11 (dashed line plot). As a negative control a sample of each transfectant was stained with an isotype control mouse IgG primary antibody (grey-filled plot).

In TV and TI transfections, surface expression of chimeric proteins was detectable using anti-FLAG® epitope antibody M2 in parallel to 14e11 anti-tetanus hybridoma supernatant. We therefore used M2 anti-FLAG® antibody staining as a marker for TetFrC expression in subsequent experiments.

In SV and SI transfections surface expression of chimeric proteins was also detectable using anti-FLAG® epitope antibody M2. As expected, the anti-tetanus hybridoma supernatant did not bind to the Streptavidin extracellular domain-bearing chimeric proteins.

In addition, samples of transfected cells were harvested with trypsin/EDTA instead of EDTA alone, and also with and without fixation to make sure that no epitopes to be detected in later experiments were trypsin or paraformaldehyde sensitive. There was no evidence for a decrease in either anti-TetFrC or anti-FLAG® epitope antibody binding with either of these treatments (data not shown).

To further quantify surface expression levels of each chimeric protein, 293T cells were transiently transfected with transfer plasmids using Calcium Phosphate, which is used in lentiviral packaging, and analysed for surface expression of chimeric proteins. Plots from representative wells are shown in Figure 2b. The percentage positive cells above background and median fluorescence intensities (MFIs) were measured for all wells and Figure 2c shows mean+/-SD for each measurement in independent transfections.

The TetFrC-VSVg chimeric plasmid (TV) produced a population of cells with mean 48±3 % surface FLAG® expression, which were detected with a MFI of 714±20 (Figure 2c). For the TetFrC-ICAM1 chimera (TI) a mean of 77±3 % of cells demonstrating anti-FLAG® staining above background was observed, and with a MFI of 1306±79 for this population. In the case of the matched Streptavidin control chimeras (Streptavidin-VSVg, SV and Streptavidin-ICAM1, SI) cells transfected with APGSV were 60±3 % FLAG® positive with a MFI of 854±50 and 293T cells transfected with SI showed 71±9 % surface FLAG®-positive cells and a MFI of 1317±468.

Statistical analysis showed that transient transfection with TV (TetFrC-VSVg) resulted in significantly lower mean percentage of FLAG®-positive cells than TI and SI but not SV; the

greatest significance was seen when comparing transient transfectants of TetFrC-VSVg (TV) with TetFrC-ICAM1 (TI). In addition, SV (Streptavidin-VSVg) transfectants had significantly lower percentage FLAG®-positive cells compared to TI (TetFrC-ICAM1) transfected cells. MFI comparison did not result in any significant difference between the 4 different chimeric proteins on the surface of positive cells. These results showed that, with some variation, each chimera could be expected to be expressed on the surface of the cell line to be used for lentiviral vector packaging.

Lentiviral transfer cassettes bearing chimeric constructs can be incorporated into infectious lentiviral vector particles but with variable titres

In order to be able to detect whether lentiviral vectors could incorporate TetFrC chimeras and thus be susceptible to neutralisation with anti-TetFrC antibodies, we first needed to test whether transfer cassette expression in transduced cells could be used to measure infectivity. Transfer plasmids TV, TI, SV and SI were therefore co-transfected to 293T cells with lentiviral packaging plasmids to create VSVg-pseudotyped lentiviral vectors LVTV, LVTI, LVSV and LVSI respectively. On day 2 post-transfection supernatants were harvested and ultra-centrifuged to recover lentiviral particles. Lentiviral preparations were then titrated by transduction of HT1080 fibrosarcoma cells and measurement of transfer cassette expression through binding of anti-FLAG® antibody. At least 3 separate lentiviral preparations were produced for each virus and putative chimera-bearing viruses were prepared alongside a well characterised, lentiviral vector expressing cytoplasmic eGFP protein, as a positive control for virus manufacture.

The titres (Transducing Units/mL) produced for each virus are shown in Table 1. Transfer plasmids bearing chimeric constructs TV, TI, SV and SI were shown to be packaged into lentiviral particles and detection of chimeric protein expression on target cells through detection of the

FLAG® epitope could then be used to detect infectivity of lentiviral preparations. Variation in mean titres for viruses LVGFP, LVTV, LVTI and LVSI did not reach statistical significance. However, for LVSV (Streptavidin-ICAM1) the trend was for lower titres with one batch producing no detectable titre. Therefore, we proceeded by focusing on the LVTI (TetFrC-ICAM1) and negative control LVSI (Streptavidin-ICAM1) pair.

Chimeric proteins can be stably expressed on HEK293T cells and PM1 T-cells

The lentiviral packaging system used in each virus was integration competent, which means that the transfer expression cassette could spontaneously integrate into the host genome of transduced cells [27]. Therefore we decided to test whether the chimeric constructs could be transferred by infection and stably expressed on human cell lines and, in particular, on a human T-cell line.

We had already shown that the chimeric proteins were transiently expressed on HEK 293T cells, so we transduced 293T cells with LVTI and LVSI as a control. In addition, we transduced the human T cell line PM1 which is a CD4+CXCR5+ T cell line. The two cell lines were transduced with an MOI of 1 of viruses LVTI (encoding TetFrC-ICAM1) and LVSI (encoding Streptavidin-ICAM1). On day 3 post-infection a sample of cells transduced with each virus was analysed for surface chimera expression (Figure 3a and b). For 293T cells transduced with LVTI (Figure 3a left) 98 % cells were positive for surface FLAG® expression while 59 % were positive in cells transduced with LVSI (Figure 3b right). For PM1 cells transduced with the same viruses LVTI infection resulted in 67 % FLAG®-positive cells and LVSI infection produced 14 % positive cells. Attempts to infect PM1 cells with higher MOIs produced cell toxicity (data not shown) and did not increase expression levels.

In order to analyse stable chimeric protein expression on populations derived from single parent cells, cells from the infections described above were diluted to give on average less than one cell seeded per well in 96 well plates. Growing colonies were allowed to expand until enough cells were available for staining with anti-FLAG® antibody and FACS analysis; 12-15 colonies for each cell line and infection were analysed from d23 post transduction. For 293T cells there were 6 positive colonies for LVTI infection and 3 for LVSI infection. For PM1 there were 4 positive colonies with LVTI infection and one with LVSI infection.

For each cell line the highest expressing colony (by percentage FLAG®-positive cells above background) for each infection was passaged until 6 weeks post-infection and analysed by FACS for transgene expression (figure 3c and d). The LVTI TetFrC-ICAM1 chimeric transgene was expressed on 98 % of cells above background on colony 293T TI.9 but with a broad range of Fluorescence Intensity (Figure 3c left). The LVSI Streptavidin-ICAM1 control chimeric transgene on colony 293T SI.9 was also expressed with a broad range of fluorescence intensity and on 90 % of cells above background (Figure 3c right).

For the selected PM1 colonies the percentage of cells expressing the transgenes were 56 % (PM1 colony TI.20, Figure 3d left) and 63 % (PM1 colony SI.6). Each colony had a discrete peak of higher expressing cells with the MFI of the peak for TetFrC-ICAM1 expression measured at 700 and for Streptavidin-ICAM1 at 414. There were also dim and negative cells within each colony, which may represent cells that have downregulated or lost the transgenes. Overall the results show that our Trojan construct can be transferred to human T cell lines by infection and expression of the transgenes can be maintained over a 6 week period.

Chimeric proteins in lentiviral preparations can be recognised by immune human anti-Tetanus sera

We have shown that lentiviral vector can be used to confer surface membrane expression of chimeric proteins to target cells. In order to initially investigate whether FLAG®-TetFrC chimeric proteins could be detected by TetFrC specific antibodies in lentiviral preparations, an immunoprecipitation experiment was performed (Figure 4).

As a control for the starting sample equal amounts of protein from each vector were left unprecipitated but otherwise treated as for the immunoprecipitates (lane 1). As a positive control for immunoprecipitation, 293T cells were transiently transfected with each lentiviral chimeric expression vector (data not shown but expression of chimeric proteins demonstrated as for Figure 1) and lysed. Lysates were immunoprecipitated with Protein G-sepharose beads to which negative isotype control polyclonal human IgGs (lane 2) or polyclonal human immune serum anti-tetanus IgGs (lane 3) were stably cross-linked. In parallel, equal amounts of protein from LVTI and SI preparations were incubated with the same beads, namely Protein G-sepharose beads conjugated with either human IgGs (lane 4) or human immune serum anti-tetanus IgGs (lane 5). Proteins bound by the antibody-conjugated beads were subject to SDS-PAGE and western blotting with M2-HRP conjugated antibody probe to detect the FLAG®-epitope.

Molecular weight prediction from primary amino acid sequences gave expected average masses of 60 kDa for FLAG®-TetFrC-ICAM1 (LVTI) and 27 kDa for FLAG®-Streptavidin-ICAM1 (LVSI) (Expasy Compute pI/MW). In each unprecipitated lentiviral preparation, a band of consistent molecular weight with the appropriate chimeric construct was detected by anti-FLAG® antibody showing that each lentiviral vector preparation contained FLAG®-tagged proteins consistent in molecular weight with those predicted for the chimeric constructs. When lysates

from transfected cells known to be expressing the chimeras were immunoprecipitated with anti-tetanus antibodies from human sera, bands of expected molecular weight were also detected by M2 antibody probe that were not seen in the isotype control lanes.

Finally, when lentiviral preparations themselves were immunoprecipitated, FLAG proteins of the predicted molecular weights for the chimeric constructs were specifically pulled down with human anti-tetanus antibodies but not with isotype control antibodies. These results showed that the chimeric proteins are capable of being specifically bound by human anti-tetanus antibodies and that chimeric proteins are detectable in lentiviral preparations.

Lentiviral vectors with envelope associated chimeric proteins are susceptible to neutralisation with anti-tetanus antibodies

Immunoprecipitation showed that chimeric proteins could be bound by anti-tetanus antibodies in lentiviral vector suspensions. However this did not directly demonstrate that they were associated with the lentiviral envelope membrane due to the possible presence in the lentiviral preparations of exosomes and other cell debris. In order to test this and show that binding of chimeric proteins by anti-tetanus antibodies could produce functional effects, a neutralisation assay was performed. Initially neutralising antibodies were titrated from 10 μg to 200 μg on LVGFP and LVTI followed by infection of HT1080 cells (data not shown). With one batch of LVTI complete neutralisation was seen at the lowest dose while the other was maximally neutralised with a dose of 100 μg and so this dose was selected for subsequent experiments.

In Figure 5 HT1080 cells were separately transduced with 3 lentiviral vectors: LVTI (FLAG®-TetFrC -ICAM1) whose transfer plasmid expresses surface membrane extracellular FLAG®-TetFrC, LVSI (FLAG®-Streptavidin-ICAM1), a matched negative control virus where TetFrC domain is swapped for Streptavidin, and finally LVGFP, whose transfer plasmid expresses

cytoplasmic eGFP and would therefore not be expected to bear any surface membrane epitopes for anti-tetanus antibody neutralisation. In parallel, HT1080 cells were transduced with lentiviral preparations that had been pre-incubated for 30 min with 100 µg anti-tetanus antibodies from human sera or in addition, for LVTI viruses, with 100 µg isotype control human IgGs. 48 h post-infection, virus infectivity was assessed by measuring expression of chimeric proteins in target cells by FACS as described previously.

Representative plots from each infection showing gating on positive cells are shown in Figure 5a. Transduction by untreated LVTI and LVSI was lower than expected based on titrated MOI and the reasons for this are not yet known. However, treatment of LVTI TetFrC Trojan viruses with human serum anti-tetanus IgG causes a dramatic reduction in transduction and this is not seen with an isotype control human IgG or when viruses were produced using either an eGFP or Streptavidin-ICAM1 expression cassette.

Mean gene expression, as percentage FLAG®-positive, transduced cells, was calculated from a minimum of 3 wells for each combination of virus and antibodies and is shown in Figure 5b. Statistical analysis showed a significant ($P<0.0001$) decrease in transduced cells after ICAM1-TetFrC bearing viruses were treated with human serum anti-tetanus IgG but not isotype control IgG. As expected, human serum anti-tetanus IgG pretreatment did not produce a significant effect on the mean percentage cells transduced by LVGFP or LVSI (Streptavidin-ICAM1).

Discussion

Altering the host cell-range of lentiviral vectors through the introduction of cell-entry glycoproteins from other enveloped viruses is a long established practice [18]. In this study we have generated chimeric proteins designed to be expressed in the membrane of lentiviral vectors as they bud from host cells. We propose these so-called Trojan proteins as a means to prevent HIV viruses from avoiding effective immune responses by making HIV virions susceptible to immunity generated by clinically proven vaccination against another pathogen such as Tetanus. The results we have shown are restricted to *in vitro* models with replication incompetent lentiviruses but we hypothesize that Trojan proteins could be delivered as a gene therapy in HIV infected individuals.

One theoretical application for this is in so called "shock and kill" strategies, which have been proposed as a future method of sterilising cure for HIV infected patients on HAART drugs [7, 28]. Small molecules are used to reactivate HIV transcription in order to expose viral reservoirs to host immune responses. However reactivation alone has not been shown to be effective enough for patient immunity to clear the latently infected cells [7, 29]. We propose that delivery of Trojan genes to latently infected cells under the control of a Tat-responsive promoter would allow these antigens to be used during "shock and kill" therapy. We speculate that activation of HIV transcription by Latency Reversing Agents would cause cell surface expression of Trojan molecules on HIV producing cells. This would be predicted to have 2 main sequelae: firstly pre-existing anti-tetanus immunity could be used to target and destroy infected cells but also any emerging HIV virus would be neutralised by serum immunity. Future work will address the success of targeting of Trojan molecules to the surface of lab strain or patient HIV.

The Trojan expression cassette, under the control of an HIV responsive promoter, can be delivered to cells known to harbour latent HIV infection. Delivery of gene therapy, as a strategy for HIV treatment has experienced a surge of interest after the "Berlin Patient" report showed that infusion of CCR5-negative cells could provide long-term protection from HIV re-emergence in an HIV-positive individual [30, 31]. In addition, the CRISPR/Cas9 system has been tested for HIV co-receptor knockdown to protect cells from infection but also as a means to target and destroy HIV genomes [32, 33]. (Add refs Bialek and Kaminski)

Clearly gene therapy strategies such as the Trojan expression we have proposed, as well as the gene editing strategies discussed above face challenges of therapeutic delivery (Saayman 2016). The cellular targets of HIV are well defined and the key reservoirs for HIV latency have been identified as resting memory T cells and cells of the myeloid lineage, with involvement of CNS cells being more controversial (reviewed in Kulpa 2015, Melkova 2016, and Joseph 2015). Historically Lentiviral vectors have been posited as ideal vectors for treating HIV and have been shown to infect relevant target cells (Mautino 2002). In more recent times, Lentiviral vectors have been directly tested in HIV blocking strategies using RNAi and gene editing by CRIPSR (Chung 2014, Kaminski 2016, Choi 2016). Though we have used a VSVg pseudotyped lentiviral vector expression system in our *in vitro* model, technologies to improve the delivery of lentiviral vectors through pseudotyping and cell-type specific retargeting are in development (Levy 2015, Kaikkonen 2009, Uhlig 2015). Furthermore, the Trojan Chimeras genes could foreseeably be delivered by other gene therapy .vectors such as AAV, which have already been tested for use in gene editing strategies for HIV (Sather *et al*. 2015).

With the expression of the Trojan cassette being stimulated in cells containing reactivated HIV, we predict that the newly replicated HIV released would be coated with the Tetanus antigen. Pre-treatment with anti-tetanus vaccination and passive immunisation with anti-Tetanus human

antibodies would be a way to block released virus and potentially clear the latent cellular reservoirs due to expression of Tetanus Toxoid epitopes.

Initially 4 chimeric proteins were constructed and were shown to be expressed transiently on the surface of cells used for lentiviral packaging. The constructs were then used as transfer plasmids in the production of VSVg pseudo-typed lentiviral vectors. Viral titres were variable and the titres for LVSV containing the FLAG®-Streptavidin-VSVg chimeric protein were lowest. The reasons for this trend are unknown as VSVg has been commonly used as a transmembrane carrier for lentiviral pseudo-types [18, 19]. It is possible that the chimeric VSVg transmembrane region is competing with the VSVg pseudo-type protein for recruitment to the viral surface resulting in reduced infectivity of the virus. However, the LVTV (FLAG®-TetFrC-VSVg) titres were not significantly lower statistically than viruses carrying FLAG®-Streptavidin-ICAM1 proteins.

Streptavidin on the viral surface might also cause steric hindrance of virus assembly or infectivity; long cytoplasmic tails of pseudo-types from measles viruses were shown to be detrimental to lentiviral titres [34]. In a study investigating the alteration of the lentiviral surface for redirection of infectivity using Streptavidin-VSVg and gp64 pseudo-types, Kaikkonen *et al*. [20] found that ratio of pseudo-type to Streptavidin-VSVg plasmids was critical to viral titres; therefore it may be that altering the plasmid recipe may be sufficient to improve the low titres seen in our study.

We also showed that Trojan lentiviral constructs could be used to transduce human cell lines and lead to surface expression of TetFrC antigen. In the absence of selection, in both cell types and with both viruses there was TetFrC surface-expression in a subset of cells at two months post-transfection, though longer term expression was not tested. A broad range of MFI

was seen particularly in the 293T wells but was not unexpected given the adherent nature of the cells and the dilution method used.

Transduced PM1 wells at two months post-infection showed a narrow peak of expression but also contained some dim and FLAG®-negative cells; a gradual loss of expression of chimeric antigens from daughter cells cannot be excluded. Further sorting and screening may identify true stable clones with more restricted ranges of MFI.

The 293T lines so generated have the potential to be used as cell factories for further production of the Trojan-altered viruses, for example with eGFP transgene cassettes. PM1 T cell lines express the CD4 receptor and co-receptors CCR5 and CXCR4 necessary for infection by macrophage and T cell trophic (R5 and X4) strains of HIV. PM1 T cells expressing the Trojan antigen can be used to test whether HIV lab strains or primary isolates would be coated with TetFrC protein and therefore be neutralised by anti-Tetanus antibodies [35]. Such T cell lines can also be used to test the potential for HIV mutational escape from the Trojan strategy [32].

The key to generating effective antibodies against HIV envelope protein by vaccination remains elusive. Our results have demonstrated that other immunogenic proteins can be delivered to the surface envelope of lentiviruses and that this can make them susceptible to neutralisation by antibodies against a different pathogen. In figure 5 we used the equivalent of 2.3 IU/mL of international standard human tetanus immunoglobulin for neutralisation; units in this antibody are based on *in vivo* neutralisation assays in mice. However, some batches of Trojan virus were completely neutralised with 10 fold less antibody (data not shown). Different amounts of debris in lentiviral vectors prepared by ultracentrifugation without density cushions may be a possible cause of this experimental variation. 0.01 IU/mL is considered to be protective

against tetanus infection in human sera [36]. The concentration of antibody needed to neutralise HIV in the context of our proposed Trojan therapy would require further analysis.

The effect on lentiviral titres and stability of altering the viral envelope in this way requires further investigation since we noted lower transduction than predicted based on original titration, in particular with LVSI, in our neutralisation studies. Some error may be inherent to the titration method used but it is possible that alteration of the envelope may have consequences for the stability of viral vectors during storage and thawing.

We have demonstrated that Lentiviral vector mediated delivery can be used to deliver Trojan proteins for expression on the surface of the T cell line PM1. Clearly, the effectiveness of lentiviral Trojan delivery to primary patient T cells requires testing. Uncontrolled expression of Trojan proteins on T cells may be undesirable but lysis of non-HIV-infected cells due to anti-Trojan immunity has the potential to be controlled by making Trojan protein expression dependent on HIV transcription [37, 38]. Testing Trojan protein expression in T cell lines may also reveal whether, in addition to neutralising cell-free virus, antigen expression on the host cell surface may cause anti-host cell immune responses against Tetanus epitopes.

To our knowledge, diverting neutralising immunity against one pathogen onto another is an entirely novel concept at this time, though suicide gene therapies have been suggested for HIV and other diseases. For example, the conditional expression of a thymidine kinase in T cells has recently been reported to cause cytotoxicity upon ganciclovir treatment in HIV infected cells and a similar gene therapy using thymidine kinase induced drug sensitivity has been tested for prostate cancer [39, 40]. In our experiments we have used TetFrC as a model antigen with known human serum neutralising antibodies but it may be possible and desirable to use other or

perhaps multiple antigens to reduce the potential for mutational escape and/or reductions in responses due to HIV-mediated damage to immune responses [41, 42].

In addition, the ability to display such Trojan proteins on the lentiviral envelope has other possible applications for pseudo-typing of lentiviral vectors. For example, a modified TetFrC has been proposed for use to direct neurotropism of viral vectors [43]. Furthermore, ICAM1 as a transmembrane anchor may offer new ways targeting lentiviral vectors to specific cell types. Our expression cassette design offers the possibility for interchangeability of transmembrane and extracellular domains.

Conclusions

We have generated novel chimeric proteins designed to coat lentiviruses with antigens from other pathogens to which neutralising memory immunity is present in vaccinated human populations. We predict that altering HIV particle surface will redirect these immune responses to neutralise HIV. Overall, the foundation data in this paper show that the Trojan chimeric molecules for neutralisation of lentiviral particles are functional and merit further investigation.

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Author Disclosure Statement

No competing financial interests exist

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613

Figure Legends

Fig. 1. Schematic structure of lentiviral vector and chimeric constructs. Top diagram shows the lentiviral eGFP transgene transfer vector pRRLsc_CEW used for control lentivirus production in this study. Domain structure of chimeric transgenes TI, SI, TV and SV. The TI and SI constructs consist of a gp64 signal peptide (light grey rectangle, 21 amino acids (aa)), followed by FLAG®-epitope (black rectangle, 9 aa) tagged TetFrC (451 aa) or Streptavidin (StrAv, 159 aa) extracellular domain fused to the Transmembrane and Cytoplasmic domains (T/C) of VSVg (dark grey rectangle, 72 aa). In the TV and SV constructs FLAG- tagged TetFrC or Streptavidin extracellular domains are fused to ICAM1 signal peptide (light grey striped rectangle, 26 aa) and T/C (black striped rectangle, 64 aa).

Fig. 2. Transient transfection of lentiviral vector plasmids carrying Trojan constructs. (a) Lentiviral Transfer plasmids TI, SI, TV and SV were transiently transfected into 293T cells using Lipofectamine®. On d3 post-transfection cells were stained with isotype control (mIgG1, grey fill plot), anti-FLAG® (black line) or anti-TetFrC (dashed line) antibodies followed by secondary antibody conjugated to Alexa Fluor® 647 and analysed by FACS. (b) Expression levels of chimeric proteins above background from mock transfected cells stained in the same way were quantified 48 h post-transfection of 293T cells using Calcium Phosphate (n=3 per plasmid). Representative FACS histograms are shown. Chart shows mean of 3 wells for % positive cells above background (black bars) and Median Fluorescence Intensity (MFI, grey bars). Error bars are ± SD of the mean. Lines with asterisks indicate significant differences between means (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$).

Fig. 3. Gene expression from cells infected by lentiviruses bearing chimeric transfer cassettes. (a and c) 293T cells (b and d) PM1 T cells were infected with LVTI and LVSI. On day 3 post-infection untransduced (grey fill) or transduced (black line) cells were stained with anti-FLAG® antibody and fluorescent-conjugated secondary and analysed by FACS. After dilution cloning and growth, 293T (c) and PM1 (d) cell colonies were stained with anti-FLAG® antibody and secondary antibody followed by FACS. Percentages are FLAG positive cells above background. PM1 colonies SI.6 and TI.20 were further analysed for median fluorescence intensity of FLAG positive cells (lower value).

Fig. 4. Immunoprecipitation of chimeric proteins in lentiviral preparations with human anti-Tetanus antibodies. Lentiviral preparations made using chimeric transfer cassettes were left unprecipitated (U, lane 1), immunoprecipitated with negative control human IgG (hIgG, lane 4) or human anti-tetanus IgG (hαTetIgG, lane 5). For molecular weight controls, lysates were prepared from 293T cells transfected with chimeric constructs and cell lysates were immunoprecipitated with negative control human IgG (hIgG, lane 2) or human anti-tetanus IgG (hαTetIgG, lane 3). PGNase F treated lysates and immunoprecipitates were separated by SDS-PAGE and western blots were probed with M2-HRP antibody.

Fig. 5. The effect of anti-Tetanus antibodies on lentiviruses bearing Trojan chimeric proteins. Lentiviruses were pretreated with PBS, isotype control hIgG antibody or anti-Tetanus serum polyclonal IgG antibody. HT1080 cells were then infected for 18h before virus was removed. 48h post-infection cells were analysed by FACS for expression of surface FLAG®

epitope. (a) Representative FACS plots with percentage positive cells above background given in top right corner**.** (b) Mean % transduced cells above background was calculated. Lentivirus was left untreated (PBS, black columns), or pretreated with human anti-Tetanus serum polyclonal IgG antibody (white columns), or with isotype control hIgG antibody (grey column). Error bars are ± SD of the mean. Lines with asterisks indicate significant differences between means (ns= non-significant, **** $P \leq 0.0001$).

Tables

Table 1: Titre Transducing Units/mL in HT1080 cells

LVGFP	LVTV	LVTI	LVSV	LVSI
1.20E+09	1.10E+07	7.10E+07	1.83E+05	6.60E+06
1.60E+08	4.20E+07	9.80E+08	2.40E+06	5.00E+06
6.30E+08	2.26E+06	5.70E+07	Not detected	2.40E+06
2.50E+08	1.32E+06	3.78E+07		

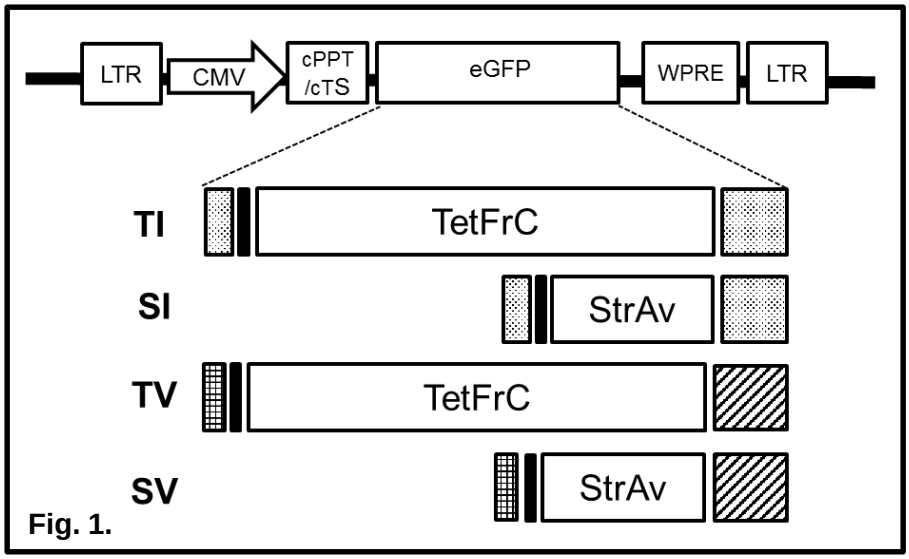
LTR
CMV
cPPT
/cTS
eGFP
WPRE
LTR
TI
TetFrC
SI
StrAv
TV
TetFrC
SV
StrAv

Fig. 1.

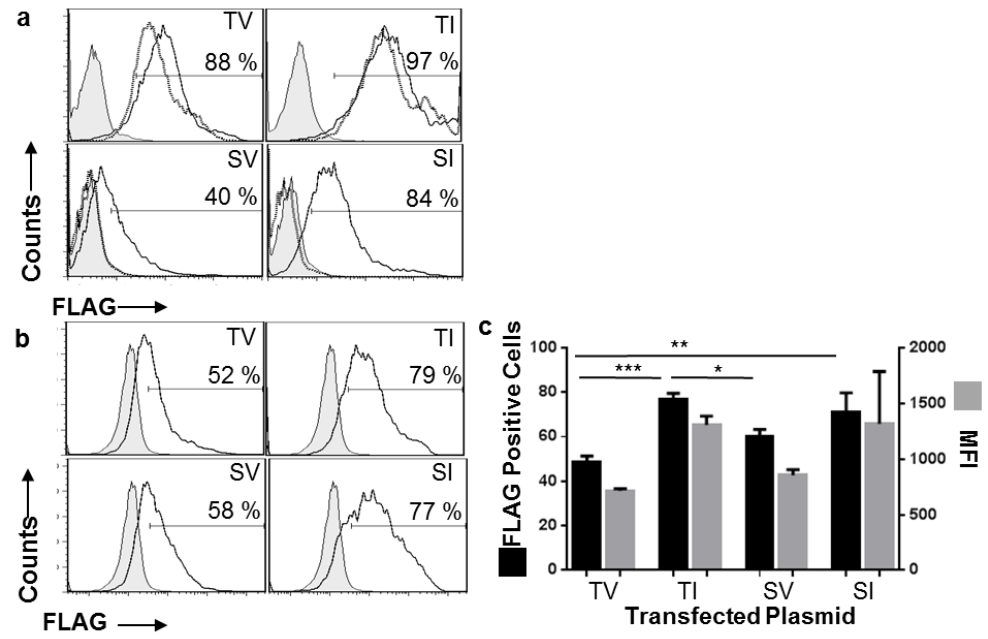
a
TV
88 %
TI
97 %
SV
40 %
SI
84 %
Counts
FLAG
b
TV
52 %
TI
79 %
SV
58 %
SI
77 %
Counts
FLAG
c
FLAG Positive Cells
MFI
**
***
*
100
80
60
40
20
0
2000
1500
1000
500
0
TV
TI
SV
SI
Transfected Plasmid

Fig. 2.

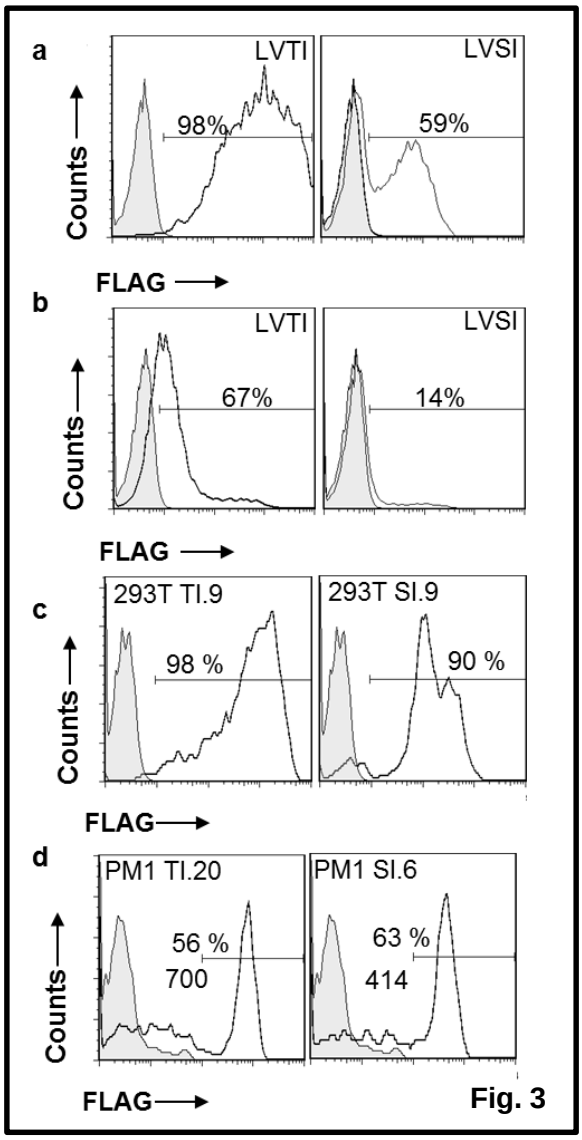
a
LVTI
LVSI
98%
59%
Counts
FLAG
b
LVTI
LVSI
67%
14%
Counts
FLAG
c
293T TI.9
293T SI.9
98 %
90 %
Counts
FLAG
d
PM1 TI.20
PM1 SI.6
56 %
700
63 %
414
Counts
FLAG

Fig. 3

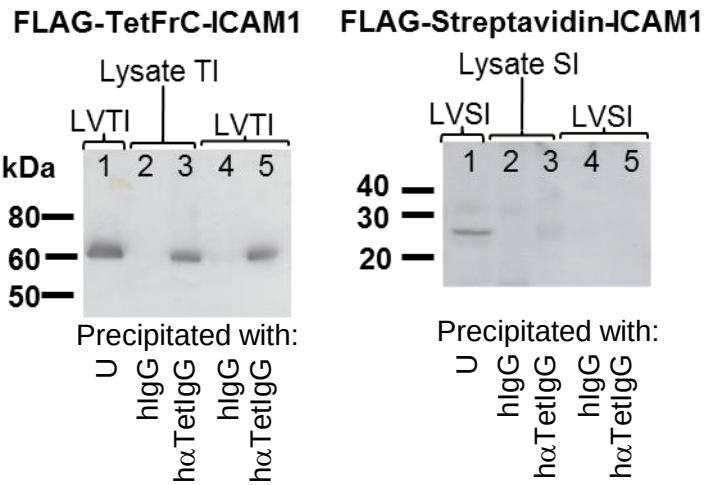
FLAG-TetFrC-ICAM1
Lysate TI
LVTI
LVTI
kDa
1 2 3 4 5
80
60
50
Precipitated with:
U
hIgG
hαTetIgG
hIgG
hαTetIgG
FLAG-Streptavidin-ICAM1
Lysate SI
LVSI
LVSI
1 2 3 4 5
40
30
20
Precipitated with:
U
hIgG
hαTetIgG
hIgG
hαTetIgG

Fig. 4.

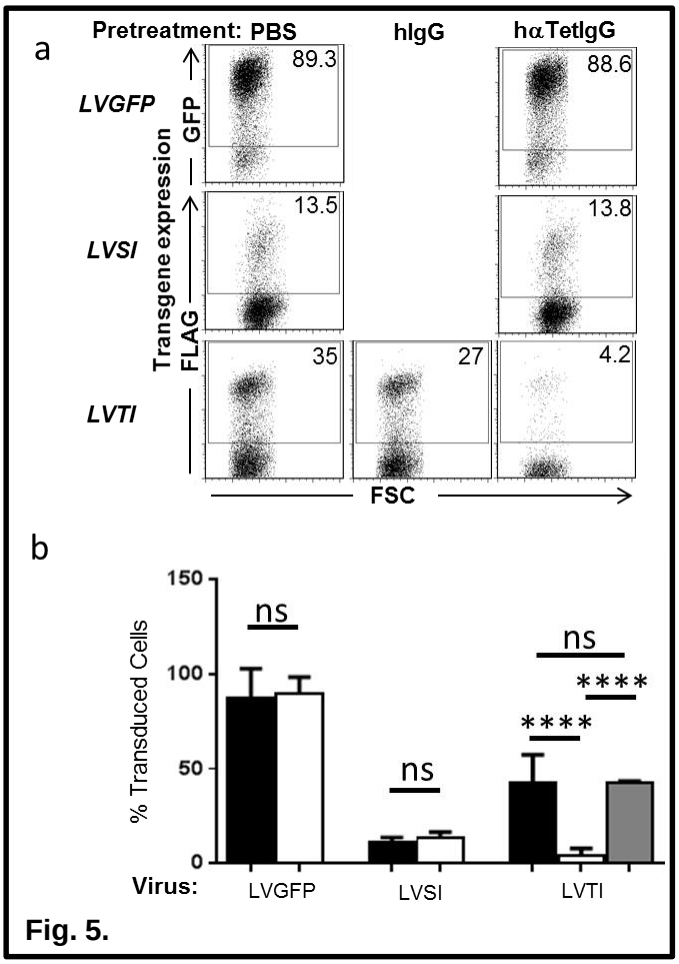
a
Pretreatment: PBS
hIgG
hαTetIgG
LVGFP
LVSI
LVTI
GFP
FLAG
Transgene expression
89.3
88.6
13.5
13.8
35
27
4.2
FSC
b
% Transduced Cells
150
100
50
0
ns
ns
ns
****
****
Virus:
LVGFP
LVSI
LVTI

Fig. 5.