



Review

Development of a luciferase based viral inhibition assay to evaluate vaccine induced CD8 T-cell responses



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ABSTRACT

Emergence of SIV and HIV specific CD8 T cells has been shown to correlate with control of *in vivo* replication. Poor correlation between IFN- γ ELISPOT responses and *in vivo* control of the virus has triggered the development of more relevant assays to assess functional HIV-1 specific CD8 T-cell responses for the evaluation and prioritization of new HIV-1 vaccine candidates. We previously established a viral inhibition assay (VIA) that measures the ability of vaccine-induced CD8 T-cell responses to inhibit viral replication in autologous CD4 T cells. In this assay, viral replication is determined by measuring p24 in the culture supernatant. Here we describe the development of a novel VIA, referred to as IMC LucR VIA that exploits replication-competent HIV-1 infectious molecular clones (IMCs) in which the complete proviral genome is strain-specific and which express the *Renilla* luciferase (LucR) gene to determine viral growth and inhibition. The introduction of the luciferase readout does provide significant improvement of the read out time. In addition to switching to the LucR read out, changes made to the overall protocol resulted in the miniaturization of the assay from a 48 to a 96-well plate format, which preserved sample and allowed for the introduction of replicates. The overall assay time was reduced from 13 to 8 days. The assay has a high degree of specificity, and the previously observed non-specific background inhibition in cells from HIV-1 negative volunteers has been reduced dramatically. Importantly, we observed an increase in positive responses, indicating an improvement in sensitivity compared to the original VIA. Currently, only a limited number of “whole-genome” IMC-LucR viruses are available and our efforts will focus on expanding the panel to better evaluate anti-viral breadth. Overall, we believe the IMC LucR VIA provides a platform to assess functional CD8 T-cell responses in large-scale clinical trial testing, which will enhance the ability to select the most promising HIV-1 vaccine candidates capable of controlling HIV-1 replication *in vivo*.

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1. Introduction

The emergence of specific CD8 T cells has been shown to correlate with *in vivo* control of HIV and SIV replication (Koup et al., 1994; Harrer et al., 1996; Goulder et al., 1997; Cohen et al., 2011). These observations suggest that a functional HIV-1 vaccine aimed at inducing a protective immune response should elicit an effective CD8 T-cell response. Therefore, standardizable assays that assess HIV-1 specific CD8 effector T-cell responses elicited by vaccine immunogens are important for evaluating HIV-1 vaccine candidates, especially in early phase clinical trials, as a means to help select the most promising candidates. The IFN- γ enzyme-linked immunospot (ELISPOT) assay is most commonly used to determine HIV-1 specific CD8 T-cell responses. However, the expression of cytokines such as IFN- γ , as measured in the ELISPOT assay, is an indirect measure of CD8 T-cell induced inhibition of HIV-1 replication. Moreover, the requirement of the ELISPOT assay for high levels of exogenous peptides limits evaluation of overall HIV-1 responses (Bennett et al., 2008; Valentine et al., 2008). Recent studies have revealed a poor correlation between IFN- γ ELISPOT responses and *in vivo* control of HIV-1 replication (Lieberman, 2004; Valentine et al., 2008; Gray et al., 2009). Together these observations illustrate the need for an *in vitro* assay that correlates better with HIV-1 specific effector CD8 T-cell responses *in vivo*.

We and others have previously developed a viral inhibition assay (VIA) that measured inhibition of HIV-1 in infected CD4 T cells in co-culture with autologous CD8 T cells, *in vitro*. (Gauduin et al., 1998; Loffredo et al., 2005; Fauce et al., 2007; Tsukamoto et al., 2007; Chen et al., 2009; Saez-Cirion et al., 2010; Spentzou et al., 2010; Akinsiku et al., 2011; Freel et al., 2012; Yamamoto et al., 2012). The observed inhibition of HIV-1 replication could be the result of HIV-1 specific major histocompatibility complex (MHC) dependent cytolytic CD8 T-cell activity (Toso et al., 1995; Yang et al., 1997; Migueles et al., 2008) as well as non-cytolytic mechanisms (Walker et al., 1991; Cocchi et al., 1995; Tomaras et al., 2000; Freel et al., 2012) or a combination of both. The VIA has the potential to

measure the overall viral suppression induced by a combination of both mechanisms, which results in a more relevant readout of HIV-1 specific CD8 T cell responses than the IFN- γ ELISPOT. In addition, using a panel of viruses in the VIA allows for the assessment of the breadth of suppression.

The VIA protocol varies between different laboratories although most commonly the amount of *de novo* produced HIV p24 or SIV p27 concentration in the culture supernatant of *in vitro* infected CD4 T-cells is determined by ELISA as a measurement of viral replication or inhibition in the presence of CD8 T-cells (Gauduin et al., 1998; Fauce et al., 2007; Tsukamoto et al., 2007; Chen et al., 2009; Spentzou et al., 2010; Yamamoto et al., 2012). Other methods to determine viral inhibition include p24 intracellular staining (ICS) (Loffredo et al., 2005; Saez-Cirion et al., 2010) or indirect measurements, such as luciferase expression after infection of TZM-bl cells with the VIA culture supernatants (Akinsiku et al., 2011; Freel et al., 2012).

Our initial efforts have focused on the development of a VIA that determines the p24 release in cell culture supernatant as a measure of HIV-1 replication, and this assay has proven valuable for testing samples from several HIV-1 vaccine trials (Spentzou et al., 2010; Hayes et al., 2013; Borthwick et al., 2013). However, we recognized certain limitations and thus pursued technological advances towards the development and optimization of a second generation VIA, referred to throughout the manuscript as the IMC LucR VIA. The goal was to reduce the number of cells needed, increase assay sensitivity and specificity, and decrease time and overall cost to perform the assay. The international AIDS Vaccine Initiative (IAVI) in collaboration with the Collaboration for AIDS Vaccine Discovery (CAVD) funded consortia, the Comprehensive T Cell Vaccine Immune Monitoring Consortium (CT-VIMC) and the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-VIMC) achieved this goal through the combination of our VIA assay platform (developed within IAVI) with enabling *Renilla reniformis* luciferase (LucR) reporter HIV-1 technology (developed within CHAVI). We previously described and validated a LucR reporter HIV-1 approach utilizing

an NL4-3 proviral backbone expressing LucR (NL-LucR.T2A), and encoding heterologous env gene sequences of choice (Edmonds et al., 2010; Montefiori et al., 2012). While utility of NL-LucR.T2A-based replicating reporter viruses (collectively referred to as Env-IMC-LucR) in the VIA has been demonstrated (Freel et al., 2010), this approach was limited by the isogenic nature of these viruses outside of *env*; thus, strain-specific differences in VIA responses mapped solely to the different heterologous envelope genes. In the work presented here, we thus utilized a novel LucR expressing, replication-competent infectious molecular clone (IMC) which encompasses the full-length strain-specific genome of the transmitted/founder (T/F) HIV-1, CH077.t, which we recently described (Ochsenbauer et al., 2012). Application of CH077.t-LucR, and similarly constructed relevant strains of reporter allow the comparison of VIA responses against all viral genes, therefore allowing a much broader comparison of CD8 T cell responses between different samples tested.

This manuscript describes the successful development of the IMC LucR VIA by implementing a fast, less labor intensive and more cost effective luciferase read out. Optimization of the assay allowed for miniaturization from a 48 well to a 96-well plate format, allowing for the introduction of replicates. The IMC LucR VIA exhibits high specificity and a significantly reduced non-specific background inhibition, enhancing the interpretation of the assay which is important for the implementation into HIV-1 vaccine trials.

2. Material and methods

2.1. Cells

TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent program (NIH ARRRP) (ARP5011), as contributed by John Kappes and Xiaoyun Wu. 293T/17 cells were obtained from the American type culture collection (ATCC) (CRL11268). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS) (Sigma, UK), penicillin (100 U/ml) (Sigma, UK) and streptomycin (100 U/ml) (Sigma, UK).

PBMCs were isolated by density centrifugation and frozen in 10% dimethylsulphoxide (DMSO) (Sigma, UK)/90% FBS and stored in vapor phase liquid nitrogen. PBMCs were cultured in RPMI 1640 containing 30 U/ml recombinant human IL-2 (Roche, Germany), penicillin (100 U/ml) and streptomycin (100 U/ml). All samples were collected under informed consent and studies were approved by the relevant local ethics committees. Samples in London were stored in a Human Tissue Act (HTA) registered Biorepository (License 12560). PBMCs from long term nonprogressors (LTNP) and elite controllers (EC) were kindly provided by Bruce Walker, Harvard, Boston, USA. HIV-1 positive PBMCs were provided by Dr. O. Anzala, Kenya AIDS Vaccine Initiative from patient with plasma viral load below 10,000 copies/ml and ART untreated for a minimum of six months. The HIV-1 sero-negative PBMCs were obtained from South African National Blood Transfusion Services, South Africa. The HIV-1 vaccine trial samples are of two clinical trials conducted by IAVI and/or collaborators

with vectors expressing a variety of HIV-1 genes (Jaoko et al., 2010; Borthwick et al., 2013).

2.2. Proviral plasmid construction

We have previously described the construction of NL-LucR.T2A and NL-LucR.T2A-CH077.ecto (Edmonds et al., 2010) in which the *R.reniformis* luciferase reporter gene (GenBank accession AF362549) was introduced into the HIV-1 lab strain NL4.3 genome in between the *env* and *nef* genes, followed by a T2A self-cleavage site.

We previously generated the clade B T/F IMC, CH077.t (Ochsenbauer et al., 2012), which represents the proviral genome sequence of the HIV-1 founder virus that established clinical infection in subject 700010077 from the CHAVI-001 Acute Infection Cohort (Salazar-Gonzalez et al., 2009). We introduced the LucR-T2A reporter gene cassette to generate CH077.t-LucR.T2A, essentially mirroring the molecular design for NL-LucR.T2A. Briefly, we identified suitable unique restriction sites upstream of the *env* gene (EcoRI, nt 5770) and downstream of *nef* (MluI, in the plasmid vector sequence adjacent to the end of the 3' LTR) and designed forward and reverse primers, respectively, just 5' and just 3' of these sites (Fwd.Eco; Rev.Mlu). We furthermore designed a reverse primer overlapping the 3' end of *env*, and extending the sequence to include an NheI and XbaI site (Rev. Nhe-Xba.env), essentially as described previously. A forward primer (Fwd.Nhe-Xba.nef) overlapping with *nef* and introducing the same sites 5' of the *nef* ATG was also designed; these reverse and forward primers overlapped in the sequence containing the NheI and XbaI sites. We then amplified the fragments including *env* and *nef*, respectively, using pCH077.t as template DNA, and subjected the amplicons to a fusion PCR reaction utilizing primers Fwd.Eco and Rev. Mlu. The resulting amplicon spanning from EcoRI through *env*, the Nhe-Xba linker, *nef*, the 3'LTR, to MluI was then TA-cloned into pCR4-TOPO cloning vector (Invitrogen). We then excised the LucR.T2A coding sequence from NL-LucR.T2A using the engineered NheI and XbaI restriction sites, and inserted it between the NheI and XbaI sites of the TA-cloned subgenomic fusion PCR amplicon cloning intermediate. After sequence confirmation, the entire EcoRI through MluI fragment encompassing the *env*-NheI-Kozak-LucR.T2A-XbaI-*nef* cassette was excised and ligated into EcoRI/MluI digested pCH077.t. The entire proviral sequence of maxi-prepped plasmid DNA of pCH077.t-LucR.T2A was confirmed by sequencing reaction and trace analysis with Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

2.3. Generation of virus stocks and determination of infectivity

All IMC virus stocks were generated by transfection of 293T/17 cells as previously described (Ozaki et al., 2012). In short $3\text{--}5 \times 10^6$ 293T/17 cells were seeded in a T75 culture flask the day before transfection. Twelve microgram of DNA in DMEM was transfected using FuGENE 6 (Promega, UK) according to manufacturer's instructions. After 4 to 6 h the transfection medium was replaced with fresh medium. The supernatant was harvested and filtered after 48 h and aliquots were stored at -80°C until use.

The TCID₅₀ of the generated virus stocks were determined as previously described (Ozaki et al., 2012). In short, 1×10^5 TZM-bl cells per well were infected with a serial dilution of the virus stock in quadruplicate, starting with a 1/10 dilution in the presence of 10 µg DEAE Dextran (Sigma, UK) per ml. Forty-eight hours later the firefly luciferase expressed by the TZM-bl cells was determined with Britelight Plus reagent (PerkinElmer, UK). The relative light units (RLU) were measured on an Infinite M200Pro plate reader (Tecan, UK). Alternatively, the virus stocks used to determine the replication kinetics (Fig. 1) were also titrated on the TZM-bl reporter cell line to determine infectious units/ml by enumeration of β-galactosidase (β-gal) stained colonies as described previously (Wei et al., 2002).

2.4. Replication kinetics determination

Defrosted PBMCs were cultured as previously described (Spentzou et al., 2010) for 7 days at a concentration of 1 to 1.5×10^6 cells/ml in RPMI 1640 containing 30 U/ml recombinant human IL-2, penicillin (100 U/ml) and streptomycin (100 U/ml). CD3/CD8 bi specific antibodies (0.5 µg/ml) (Wong and Colvin, 1987) were added to the culture medium to expand the CD4 T cell population. For each virus, 1.5×10^6 of pooled and expanded CD4 T cells from 5 different HIV-1 negative donors were infected with 0.1 MOI (as based on the TCID₅₀ determination) in a 24 well plate format. The CD4 T cells were pooled at 1:1 ratio based on the cell counts on the day of infection. After 4 h of incubation at 37 °C the

supernatant was replaced with fresh medium. Supernatant was collected every 3 to 4 days and stored at -80 °C and an equal volume of fresh medium was added to replenish the cultures. The concentration of p24 was determined by enzyme-linked immunosorbent assay (ELISA) (PerkinElmer, UK). The luciferase activity was determined by *Renilla* Glo luciferase assay system (Promega, UK) in accordance with manufacturer's instructions.

2.5. Determination of *Renilla* Luciferase stability

The stability of the *R. reniformis* luciferase reporter gene was determined as previously described (Edmonds et al., 2010). Briefly, 1.5×10^6 pooled (5 different donors) CD4 T cells expanded by CD3/CD8 bi-specific antibodies (Wong and Colvin, 1987) as described above were infected with 0.1 MOI for each virus for a period of 13 days. Supernatant was collected at different time points after infection, cleared from cells by centrifugation, and stored at -80 °C. TZM-bl cells were plated at a concentration of 1×10^4 cells/well in a 96 well format and infected with different volumes of supernatant collected from the PBMC cultures (0.2, 1, 5 or 10 µl) in the presence of 10 µg/ml DEAE Dextran (Sigma, UK). After 4 h of the supernatants were replaced with fresh medium after an additional 18 h 5 µM reverse transcriptase inhibitor Nevirapine (Sigma, UK) was added to all cultures. After an additional 18 h of incubation the *Renilla* and firefly luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega, UK) in accordance with manufacturer's instructions.

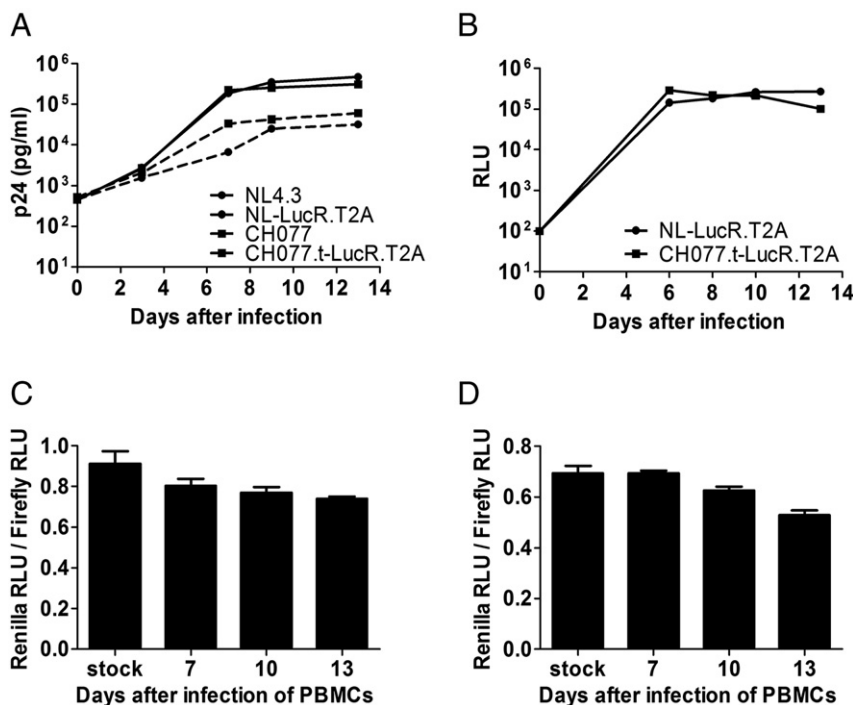


Fig. 1. Virus characterization. (A) Infection of HIV negative CD4 T cells with NL4.3, NL-LucR.T2A, CH077.t and CH077.t-LucR T2A. P24 concentrations were determined in the culture supernatant (ELISA). (B) Infection kinetics of HIV negative CD4 T cells with NL-LucR.T2A and CH077.t-LucR T2A using *Renilla* Luciferase activity in the cells as read-out at different time points during replication. (C & D) Determination of stability of *Renilla* luciferase expression relative to viral infectivity for luciferase containing IMCs. HIV negative CD4 T cells were infected with the luciferase containing virus and supernatant was collected at different time points (depicted on the X-axis). The supernatants were used to infect TZMbl cells. The ratio between virus-encoded *Renilla* and cell-encoded firefly luciferase (expressed by the TZMbl cells upon infection) is depicted. (C) NL-LucR.T2A (D) CH077.t-LucR T2A.

The ratio between *Renilla* Luciferase and *Firefly* luciferase was determined and compared to the ratio of the stock used to infect the CD4 T cells, to assess the stability of the *Renilla* Luciferase gene in the viral genome over time.

2.6. First generation VIA p24 and luciferase read out

Defrosted PBMCs were cultured as previously described (Spentzou et al., 2010) for 7 days at a concentration of $1\text{--}1.5 \times 10^6$ cells/ml in RPMI 1640 containing 30 U/ml recombinant human IL-2, penicillin (100 U/ml) and streptomycin (100 U/ml). CD3/CD4 or CD3/CD8 bi specific antibodies (0.5 µg/ml) (Wong and Colvin, 1987) were added to the culture medium to expand the CD8 or CD4 T-cell population, respectively. Fresh medium was added every 3 to 4 days. Half a million per well expanded CD4 T cell population were infected with IIB produced on PBMCs or CH077.t IMC produced by transfection, at an MOI of 0.01 in a 48 well plate. The CD4 T cells were cultured over a period of 13 days with or without autologous CD8 T cells at a 1:1 ratio. Half of the culture supernatant was replaced by fresh medium every 3 to 4 days. Viral replication was determined 13 days post-infection by p24 ELISA (PerkinElmer). The log inhibition was determined by subtracting the log₁₀ p24 concentration of the CD4 CD8 T cell co-culture from the log₁₀ p24 concentration determined for the culture of CD4 T cells alone. Alternatively, expanded CD4 T cells were infected with, NL-LucR.T2A and CH077.t-LucR.T2A at an MOI of 0.01. The cells were plated in triplicate wells at a concentration of 0.1×10^6 cells per well in a 96 well format. The CD4 T cells were cultured alone or in the presence of autologous CD8 T cells at a 1:1 ratio. After 13 days 100 µl supernatant was used to determine the p24 concentrations by ELISA (PerkinElmer) and the luciferase activity in the same well was determined by *Renilla*-Glo Luciferase assay system (Promega).

2.7. IMC LucR VIA

The CD8 and CD4 T cell population were generated and expanded over 7 days by the addition of bi-specific antibodies identical to the first generation VIA. On day 7 the CD4 cells were counted and infected with NL-LucR.T2A or CH077.t-LucR.T2A at an MOI of 0.01 (based on TZM-bl TCID₅₀) by spinoculation (1800 ×g, 2 h, RT) at a cell concentration of 10×10^6 cells/ml in RPMI 1640 containing 30 U/ml recombinant human IL-2, penicillin (100 U/ml) and streptomycin (100 U/ml). After the spinoculation fresh medium was added to dilute the cells to a concentration of 1×10^6 cells/ml. On the same day, fresh medium was added to the expanded CD8 T-cells. Both the infected CD4 and CD8 were incubated for an additional 3 days at 37 °C. Cells were then counted and 0.1×10^6 infected CD4 T cells per well were seeded in triplicate in a 96 well format with or without CD8 T cells at a 1:1 ratio, unless indicated otherwise. Cells were then incubated for an additional 5 days. Luciferase activity was determined with the *Renilla*-Glo Luciferase assay system (Promega) as a measure of viral infection. The log inhibition was determined by subtracting the log₁₀ relative light units (RLU) of the CD4 CD8 T cell co-culture from the log₁₀ RLU determined for the culture of CD4 T cells alone.

3. Results

3.1. Virus characterization

Currently used VIA platforms have limitations with regard to cost, required sample volumes, time, sensitivity and specificity. To overcome these obstacles and develop a more efficient, sensitive and cost-effective VIA we introduced replication-competent *Renilla* luciferase reporter HIV-1 infectious molecular clones (IMC) to the VIA. Our rationale was that the introduction of the NL-LucR.T2A-based Env-IMC-LucR viruses has greatly advanced applications in the HIV-1 neutralizing antibody field which faces similar challenges (Edmonds et al., 2010; Brown et al., 2012; Montefiori et al., 2012; Pace et al., 2013), Env-IMC-LucR viruses have been used for proof of principle in CD8 VIA (Freel et al., 2010), which illustrates that VIA appropriate adaptation of the molecular approach can be achieved. We chose the IMC of clade B T/F HIV-1, CH077.t (Ochsenbauer et al., 2012), as a relevant HIV-1 strain to introduce the LucR reporter gene, and generated CH077.t-LucR.T2A. To determine the implications for the introduction of the reporter gene into the T/F IMC, we compared the replication curves of both LucR containing viruses with their parental the wild type viruses. P24 production in both LucR reporter viruses infected cultures was reduced compared to wild-type viruses after day three (Fig. 1A), indicating less efficient replication after the first round of infection, essentially as described (Edmonds et al., 2010). The LucR reporter viruses showed similar replication kinetics based on the p24 read out, which is confirmed by the replication curves based on the measurement of the LucR activity (Fig. 1B). The VIA involves several rounds of replication in CD4 T cells, therefore it was important to establish the stability of LucR gene expression over time since reduction of the LucR reporter activity due to elimination of the LucR gene from the proviral genome over time could negatively affect the interpretation of the IMC LucR VIA. Stability of the reporter gene was determined by infecting expanded CD4 T cells with the reporter viruses over a period of 13 days while supernatant was collected on day 7, 10 and 13. The supernatants were frozen at $-80\text{ }^{\circ}\text{C}$ until used to infect TZM-bl cells, which express cell-encoded *firefly* luciferase upon HIV-1 infection thereby providing a means to normalize LucR levels to relative infectivity. Therefore, the ratio between *Renilla* and *Firefly* luciferase at different time points can be compared to the original virus stock ratio, to determine stability and retention of the LucR in the proviral genomes after several cycles of replication. Both NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 1C and D, respectively) showed relatively stable RLU ratios even though a slight reduction of *Renilla* luciferase expression is observed at day 13, essentially as previously reported for NL-LucR.T2A (Edmonds et al., 2010) and illustrating that the LucR expression from both the NL-derived as well as the T/F IMC reporter virus is stable over the course of the IMC LucR VIA experiment. Even when CH077.t-LucR.T2A infected PBMC cultures were followed for 31 days, reduction of the *Renilla* Luciferase RLU to *Firefly* luciferase RLU ratio at the end point was only about 4-fold, and about 20% by day 12 and 18 (data not shown).

3.2. Correlation between the p24 and LucR read out

Comparison of the replication kinetics in CD4 T cells in the presence or absence of CD8 T cells was performed for the two reporter viruses in a panel of PBMCs obtained from HIV-1 negative, HIV-1 positive long term non-progressor (LTNP) and elite controller (EC) volunteers in the first generation VIA with the LucR read-out. Representative replication curves obtained using both the p24 and Luciferase read out are shown in Fig. 2A–D for the infection of cells from one HIV-1 positive LTNP and one HIV-1 negative donor with CH077.t-LucR.T2A. The replication curves in CD4 T cells derived from a HIV-1 positive LTNP in the absence or presence of the autologous CD8 T cells are similar between the p24 and luciferase read out (Fig. 2A

and B, respectively). Again, good concordance was observed when comparing the two read outs for the infection kinetics when using cells from a HIV-1 negative volunteer (Fig. 2C and D), where limited inhibition was observed in the presence of the autologous CD8 T cells. To further validate our observations we performed a correlation analysis between the log₁₀ p24 and log₁₀ LucR read out by combining results of four separate experiments performed in triplicate (Fig. 2E). Expanded CD4 T cells from LTNP, EC & HIV-1 negative donors were infected with NL-LucR.T2A or CH077.t-LucR.T2A and cultured in the presence or absence of autologous CD8 T cells. Both the p24 concentration in the culture supernatant and the LucR expression in the cultured cells were determined at multiple days within each experiment. A highly significant correlation was observed

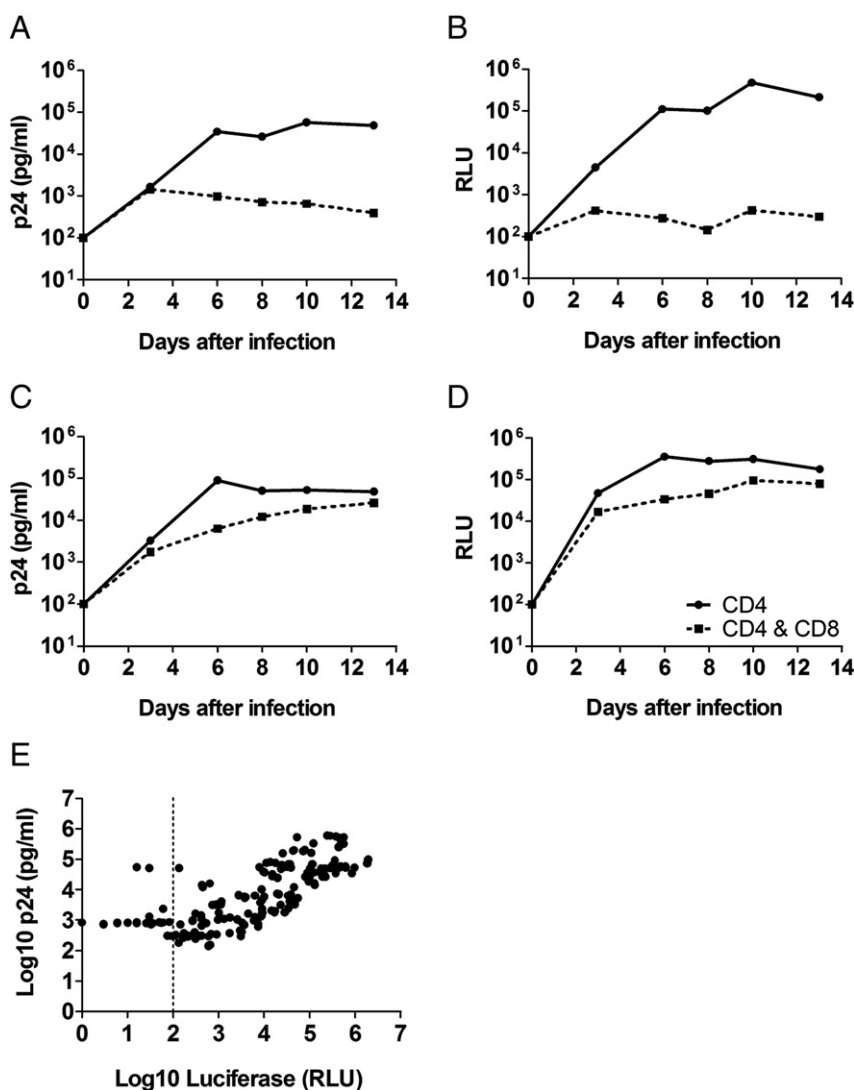


Fig. 2. Correlation between p24 and Luciferase read out. (A–D) An example of the replication curves based on the p24 and luciferase read out in the VIA when infecting with CH077.t-LucR T2A. (A) Replication in HIV-1 negative CD4 T cells, in the presence or absence of autologous CD8 T cells, based on p24 concentrations in the culture supernatant. (B) Replication in HIV-1 negative CD4 T cells, in the presence or absence of autologous CD8 T cells, based on luciferase read out. (C) Replication in HIV-1 positive CD4 T cells, in the presence or absence of autologous CD8 T cells, based on p24 concentrations in the culture supernatant. (D) Replication in HIV-1 positive CD4 T cells, in the presence or absence of autologous CD8 T cells, based on Luciferase read out. E. Correlation between p24 and Luciferase read out for both NL-LucR T2A and CH077.t-LucR T2A. The data is combined from 4 independent experiments with a total N of 172. The Pearson correlation was determined.

between the log₁₀ p24 and the log₁₀ LucR RLU. Altogether, our data support that changing the assay read-out from p24 to LucR yield similar results for the replicating kinetics of the viruses as well as the level of viral inhibition observed.

3.3. Optimization of the IMC LucR VIA

We proceeded to further optimize the VIA to fully realize the potential of the IMC LucR VIA. To increase infection of the CD4 T cells we introduced a spinoculation step followed by an incubation of 3 days before addition of expanded autologous CD8 T cells, thereby allowing for a persistent infection of the CD4 T cells prior to addition of the CD8 T cells. The luciferase activity is determined 5 days after addition of the CD8 T cells, resulting in a decrease of 5 days in overall assay time. In addition the assay was miniaturized to a 96 well format instead of a 48 well plate (as in the 1st generation). This resulted in fewer cells per well being used and therefore allowing for the introduction of more replicates.

We then investigated the effect of CD8:CD4 ratio in the IMC LucR VIA. The log inhibition was determined for different CD8 and CD4 T-cell ratios for a HIV-1 negative volunteer for NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 3A and B, respectively). Little to no inhibition was observed at the 1:1 or lower cell ratio for both viruses. However, non-specific inhibition increased proportionally with ratios over 1:1. In the HIV-1 positive PBMCs, the extent of the specific inhibition for NL-LucR.T2A (Fig. 3A) and CH077.t-LucR.T2A, (Fig. 3B)

decreased slightly when the CD8:CD4 T-cell ratio decreased, but the effect is much less pronounced than that observed for non-specific inhibition in the HIV-1 negative PBMCs.

To determine if the increase of non-specific inhibition associated with an increase of CD8 T cells is a reoccurring phenomenon, we tested another eight HIV-1 negative and eight HIV-1 positive donors (Fig. 3C and D respectively) and combined the results from both viruses. The results showed that the non-specific background inhibition was consistently and significantly higher ($P < 0.0001$, paired *T* test; increased by 0.8 log) when the CD8:CD4 ratio was increased (Fig. 3C). The same was observed for the specific inhibition using HIV-1 positive samples, although this effect was less pronounced ($P = 0.0005$, paired *T* test; increased by 0.4 log) (Fig. 3D). Determination of the coefficient of variation (CV) across the HIV-1 positive samples revealed a large difference in variability between the 1:1 and 10:1 cell ratio. The 1:1 cell ratio had a CV of 52.84% whereas the 10:1 cell ratio had a CV of 23.86%, illustrating a decrease in variability within this group indicating an undesirable decreased range and ability to distinguish the log inhibition differences between different volunteer PBMCs. In addition, the reduction of non-specific background inhibition in the VIA could have positive implication for the sensitivity of the assay. In summary, the optimization process allowed us to decrease assay time by 5 days, decrease number of cells used by miniaturization and select the optimal CD8:CD4 ratio of 1:1. All these changes were incorporated in the IMC LucR VIA.

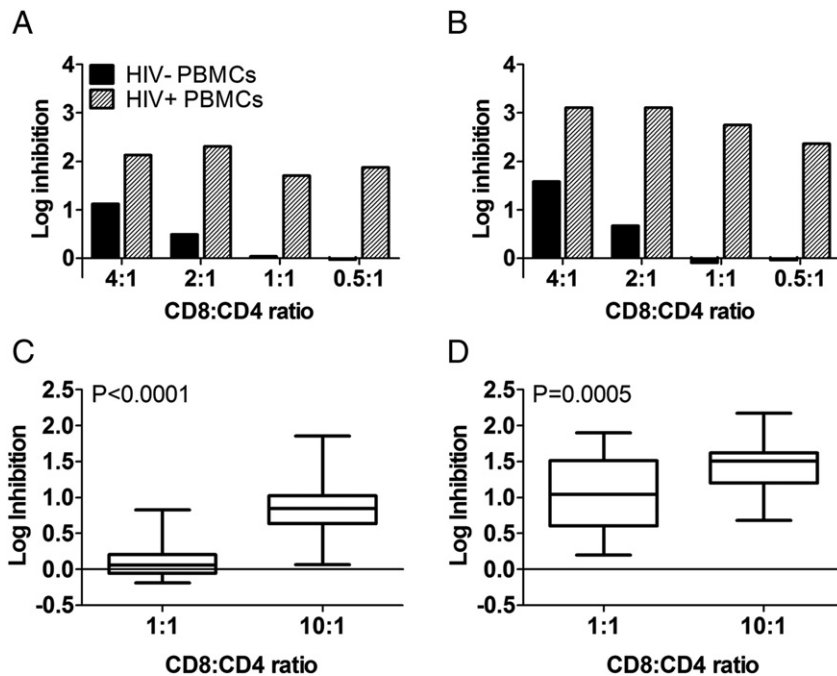


Fig. 3. The influence of CD8-CD4 T cell ratio on the level of non-specific inhibition in the IMC LucR VIA. (A & B) The experiment was performed in triplicate and the log inhibition was determined by subtracting the average RLU for infected CD4 cells + CD8 T cells from infected CD4 T cells. The black bars represent the PBMCs from a HIV-1 negative donor and the hashed bars represent the results when using PBMCs from a HIV-1 positive donor. (A) NL-LucR.T2A (B) CH077.t-LucR.T2A. The data is from a representative experiment, repeated 3 times. (C) The effect of CD8 & CD4 T cell ratio in the IMC LucR VIA on the non-specific background inhibition is shown. The data is determined on PBMCs from 9 different HIV-1 negative volunteers and the results for NL-LucR T2A and CH077.t-LucR T2A were combined. (D) The log inhibition was determined on PBMCs from 8 HIV-1 positive volunteers for different CD8 & CD4 T cell ratios. The results for both NL-LucR T2A and CH077.t-LucR T2A were combined. Both box and whiskers graphs represent the 95 percentile, the median is depicted by the line in the box and the maximum and minimum values are depicted as the whiskers. A paired *T*-test was performed using GraphPad Prism version 5 to determine significance.

3.4. Correlation between first generation VIA and IMC LucR VIA using samples from HIV-1 vaccine trials

The results obtained with the first generation VIA utilizing the p24 read out system (Spentzou et al., 2010) were compared to those from the newly developed IMC LucR VIA using PBMCs from several HIV-1 vaccine trials. The log inhibitions for the first generation VIA and the IMC LucR VIA were determined and compared. We compared IIB to NL-LucR.T2A and CH077 to CH077.t-LucR.T2A for the first generation VIA and the IMC LucR VIA, respectively. NL 4.3 is derived from IIB and therefore the viruses share a high degree of sequence homology (98% homology when comparing sequenced IIB to NL 4.3 (AF324493), data not shown). The results of the assay comparison for both viruses using pre-immunization PBMCs from twenty eight vaccine trial volunteers revealed a significant reduction ($P < 0.0001$, Wilcoxon matched pairs test) of the non-specific background inhibition in the IMC LucR VIA when compared to the first generation (Fig. 4A). These results allowed us to determine the assay cutoff for the IMC LucR VIA at 0.6 log inhibition, as calculated by the mean log inhibition plus 3 standard deviations, (represented as a dashed line in Fig. 4A). In

contrast, a higher cut-off of 1.4 log inhibition was calculated for the first generation in line with the previously observed value of 1.5 log inhibition (represented as a dashed line in Fig. 4A) (Jaoko et al., 2010; Spentzou et al., 2010; unpublished data). A comprehensive comparison of the first generation VIA and the IMC LucR VIA was performed using sixty-seven pre- and post vaccination PBMCs from forty volunteers participating in two different HIV-1 vaccine trials (Fig. 4B) (Jaoko et al., 2010; N Borthwick et al. manuscript in preparation). Results obtained for both virus combined showed a significant correlation ($P < 0.0001$, Pearson correlation) between the two assays. Two PBMC samples showed inhibition in the first generation VIA but not in the IMC LucR VIA. On the contrary, seventeen samples did not inhibit in the first generation VIA but did in the IMC LucR VIA, which could indicate an increased sensitivity of the IMC LucR VIA, but additional screening need to be performed to confirm this.

Importantly, the responses of placebo treated volunteers as well as all pre-immunization samples were below the specific cutoff for both VIAs indicating a high specificity of both VIAs. In both the first generation VIA and the IMC LucR VIA there were more responses to IIB and NL-LucR.T2A than to CH077 and CH077.t-LucR.T2A. Positive responses to CH077

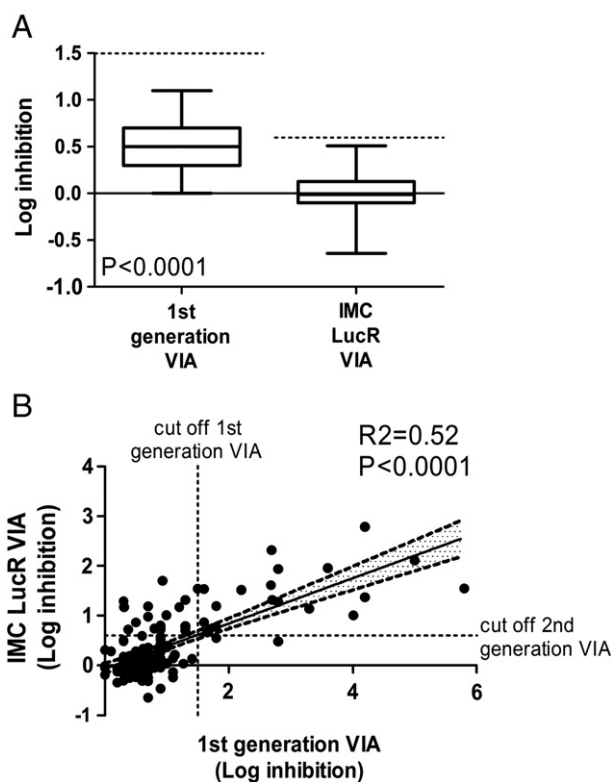


Fig. 4. Comparison of the non-specific background inhibition between the first generation VIA and the IMC LucR VIA. IIB was compared to NL-LucR T2A and CH077 was compared to CH077.t-LucR T2A for the first generation VIA and the IMC LucR VIA, respectively. (A) The PBMCs used were all from pre-immunization time points and the results from NL-LucR T2A and CH077.t-LucR T2A were combined, resulting in an N of 56 (N = 28 for each virus). A paired *T* test was performed using GraphPad Prism version 5 to determine significance. The box and whiskers graph represent the 95 percentile, the median is depicted by the line in the box and the maximum and minimum values are depicted as the whiskers. The cutoff for each assay are depicted as a dotted line. (B) The correlation was determined between the first generation VIA and the IMC LucR VIA for both virus comparisons combined. The data is comprised of data from 40 patients and 68 different time points, varying from before or after immunization. The vertical and horizontal dotted lines represent the cutoff of the first generation VIA and the IMC LucR VIA, respectively. A Pearson correlation was performed using GraphPad Prism version 5. Depicted in the graph by the dashed line is the standard error of the linear regression.

and CH077.t-LucR.T2A in general correspond to positive VIA responses to IIIB and NL-LucR.T2A, respectively.

3.5. Specificity and interassay variability of the IMC LucR VIA

Interassay variability was determined on eight different HIV-1 positive PBMCs and nine HIV-1 negative PBMCs infected with NL-LucR.T2A or CH077.t-LucR.T2A in four to six independent experiments (depending on the sample) performed in triplicate by two different operators (Fig. 5). Comparison of the maximum infection of HIV-1 positive CD4 T cells for NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 5A and B, respectively) revealed a high consistency of results between experiments and operators. The same was observed when determining the maximum of infection in HIV-1 negative CD4 T cells for NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 5C and D, respectively). The maximum infection was donor dependent since it was consistent across individual experiments and operators. The intradonor coefficient of variations of the log₁₀ RLU varied from 2.33% to 14.75% with a mean of 7.90% for the HIV-1 positive cells for both viruses combined. The same range was observed for the HIV-1 negative cells for both viruses combined, which varied from 2.21% to 12.94% with a mean of 7.17%. Overall these results demonstrate a low variability of infection between separate experiments and operators, which is of note since this is a complex primary cell culture based assay with multiple steps which could potentially introduce variability. In addition, results indicated that there is no difference in infection rates

between HIV-1 positive and negative samples. The HIV-1 positive samples used in these experiments are from patients with plasma viral loads below 10,000 copies/ml, indicating that the CD4 T cell population in these patients is not compromised, thereby having limited effect on the infection efficiencies of these cells. It is also important to keep in mind that the VIA is primarily developed to determine HIV-1 specific T cell responses in HIV-1 vaccinated individuals not in HIV-1 infected individuals, even though the assay could also be used to determine correlates of protection by comparing HIV-1 infected and uninfected individuals.

Subsequently we determined the CD8 T-cell induced log inhibition within the same experiments described above. CD8 T cells from HIV-1 positive PBMCs for NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 6A and B, respectively) show a range in inhibition. The intradonor coefficient of variation for these samples varied from 21.45% to 62.14% with a mean of 40.80% for both viruses combined. In addition, the CVs for each individual IMC were not statistically different. The log inhibition was also determined for PBMCs from nine HIV-1 negative volunteers for NL-LucR.T2A and CH077.t-LucR.T2A (Fig. 6C and D, respectively). The log inhibitions fell below the previously established cut-off of 0.6 log inhibition for all except one experiment with PBMCs from donor 17 for NL-LucR.T2A and in another experiment for CH077.t-LucR.T2A, illustrating that the observed false positives were not the results of an error in one specific experiment. The intradonor coefficient of variability was not determined because most means were around zero, which would result in high CVs that do not represent relevant

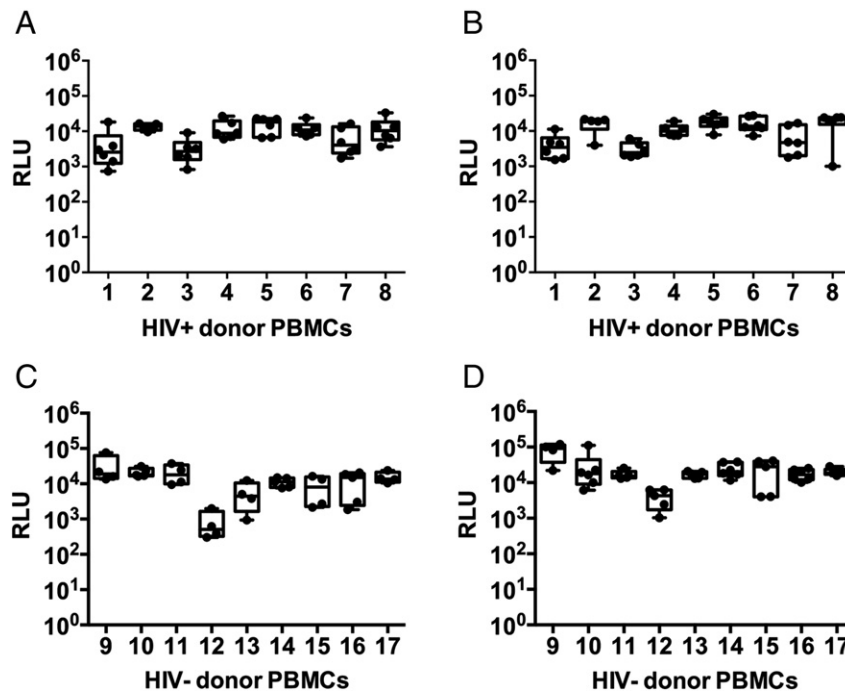


Fig. 5. Assay variability for the infection of CD4 T cells of the IMC LucR VIA. The rate of infection of CD4 T cells in 4 to 6 independent experiments performed by two different operators is depicted for each donor. Eight HIV-1 positive and nine HIV-1 negative donor PBMCs were tested (A) Cells derived from HIV-1 positive individuals infected with NL-LucR T2A. (B) Cells derived from HIV-1 positive individuals infected with CH077.t-LucR T2A. (C) Cells derived from HIV-1 negative individuals infected with NL-LucR T2A. (D) Cells derived from HIV-1 negative individuals infected with CH077.t-LucR T2A. The box and whiskers graphs represent the 95 percentile, the median is depicted by the line in the box and the maximum and minimum values are depicted as the whiskers. In addition to the box and whiskers all individual data points are shown as dots.

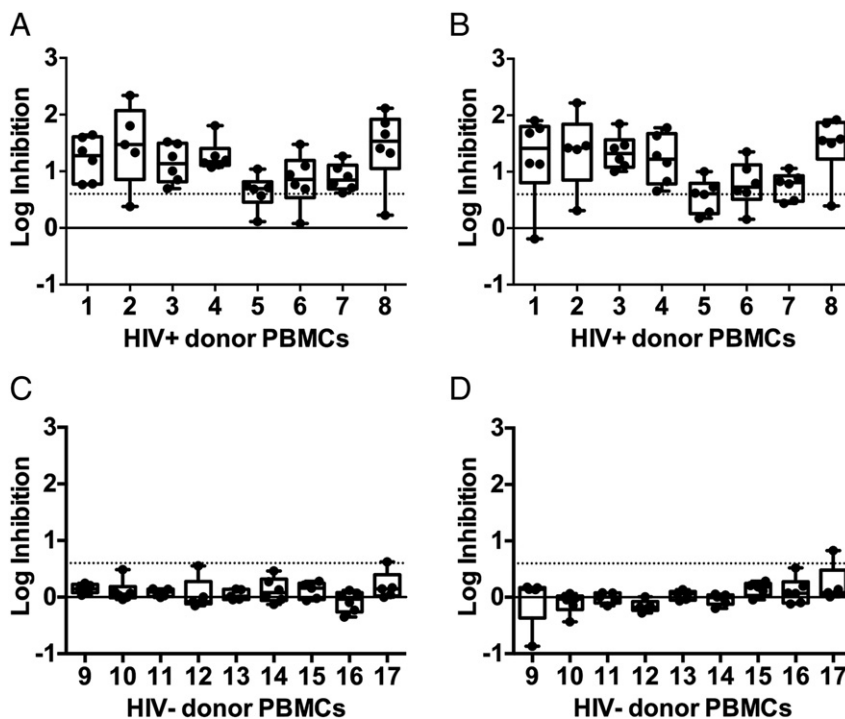


Fig. 6. Assay specificity and interassay variability for the log inhibition of the IMC LucR VIA. The log inhibition of 4 to 6 independent experiments performed by two different operators is depicted for each donor. The log inhibition was determined by subtracting the average RLU of the triplicates in each experiment for infected CD4 cells + CD8 T cells from infected CD4 T cells alone. Eight HIV-1 positive and nine HIV-1 negative donor PBMCs were tested. (A) Cells derived from HIV-1 positive individuals infected with NL-LucR T2A. (B) Cells derived from HIV-1 positive individuals infected with CH077.t-LucR T2A. (C) Cells derived from HIV-1 negative individuals infected with NL-LucR T2A. (D) Cells derived from HIV-1 negative individuals infected with CH077.t-LucR T2A. The assay cutoff is depicted as a dotted line in each graph. The box and whiskers graphs represent the 95 percentile, the median is depicted by the line in the box and the maximum and minimum values are depicted as the whiskers. In addition to the box and whiskers all individual data points are shown as dots.

variability. No correlation was observed between the rate of infections (RLU) and log inhibition when comparing HIV-1 positive donor cells for both viruses combined as well as when combining both viruses and both HIV-1 positive and HIV-1 negative donor cells (data not shown), indicating that the variability observed in the log inhibition is not caused by the variability in infection. Together these data show that the IMC LucR VIA has a high specificity that allows for the reliable and reproducible identification of negative *versus* positive responses in the IMC LucR VIA.

4. Discussion

Poor correlation between IFN- γ ELISPOT responses and *in vivo* control of HIV-1 has triggered the development of the VIA by several laboratories. The currently used VIA platforms have limitations with regard to cost, required sample volumes and assay time. To overcome these obstacles we have developed a more efficient, sensitive and cost-effective VIA by the introduction of replication-competent *Renilla* luciferase reporter HIV-1 IMC to the VIA. Most importantly, we can reproducibly identify negative *versus* positive responses in the IMC LucR VIA, which is of great importance for the assessment of functional CD8 T-cell responses in large-scale clinical trial testing, which will enhance the ability to select the most promising HIV-1 vaccine candidates capable of controlling HIV-1 replication *in vivo*.

We significantly reduced the non-specific background inhibition in the IMC LucR VIA, however we were unable to abolish it completely, which is most likely due to assay variability and previously described non-cytolytic activity of CD8 T cells, such as the production of β chemokines MIP-1 α , MIP-1 β and RANTES (Cocchi et al., 1995; Berger, 1997) as well as IL16 (Baier et al., 1995) and SDF-1 (Bleul et al., 1996; Oberlin et al., 1996). We use expanded CD4 and CD8 T cells which allow us to test a large number of viruses and time points with a limited amount of PBMCs. We achieved a high purity of target and effector cells (88–97%) (Spentzou et al., 2010), but nonetheless a low percentage of alternative cells was still present and could be responsible for the observed non-specific inhibition. The inhibition observed in the first generation VIA was shown to be predominately MHC class I dependent (Spentzou et al., 2010), thereby indicating that an HIV-1 specific cytolytic mechanism is likely responsible. The highly significant correlation between the first generation VIA and IMC LucR VIA implies that the same mechanism of inhibition is measured in the IMC LucR VIA.

We observed donor dependent variability of susceptibility to infection in Fig. 5, but found it to be independent of HIV-1 infection status of the donor. Variation of HIV-1 susceptibility of different PBMC donors has been previously observed (Kaslow et al., 2005) and is most likely due to differences in donor genetic background. The maximum infection achieved could influence the final read out of the VIA by reducing the overall range of the assay and thereby reducing the log

inhibition. However, we do not observe correlation between the maximum infection and the log inhibition in the IMC LucR VIA. Nevertheless, additional research is needed to determine the minimum infection required within this assay. Of note, in the context of antibody neutralization assays, no correlation was observed between the susceptibility of 75 donor PBMC to infection, and neutralization sensitivity (Edmonds et al., 2010).

The presence of the luciferase gene in the T/F CH077.t genome, resulted in decreased virus replication compared to wild type virus, the extent of which was similar to that observed for NL-LucR.T2A, as shown here and previously (Edmonds et al., 2010). The expression of the Nef protein is not compromised as shown by Western blot analysis for NL-LucR.T2A (Edmonds et al., 2010), therefore we are able to determine Nef epitope dependent CD8 T-cell responses. However, the *lucR.T2A.nef* configuration generates a Nef protein with a modified N-terminus. Thus, Nef is likely not myristylated. However since it retains the basic residues for membrane association in the N-terminal domain, its overall function is likely only modestly impaired (Welker et al., 1998; Benthall et al., 2006), and thus, we would argue that the modified N-terminus does not preclude meaningful analyses using the VIA approaches described above. Further research is ongoing to determine the extent of *nef* function, or loss thereof, of these LucR IMCs. In addition, since we envision application of LucR-IMC to approaches which will depend on fully functional Nef, we are investigating alternative molecular approaches which offer wild-type *nef* expression while retaining the advantages already realized for antibody neutralization and CD8 T cell virus inhibition assays.

When comparing the first generation VIA and the IMC LucR VIA we used IIIB and NL-LucR.T2A, respectively. NL4.3 is derived from IIIB and sequence analysis revealed a 97% sequence homology when comparing the sequence of the IIIB stock to NL4.3 (AF324493) (data not shown). The major difference is that IIIB does not express the *nef* gene due to a frame shift, where NL4.3 has an open *nef* reading frame, and thus, NL-LucR.T2 does express Nef, with the modification discussed above. Overall we believe the genetic differences to be sufficiently small to allow us to compare results obtained from these two viruses in the first generation VIA and IMC LucR VIA, and the significant correlation between the data supports this conclusion.

We demonstrated relevance of the IMC LucR VIA for clinical trial sample analysis by testing cells obtained pre and post HIV-1 vaccination. The pre immunization samples showed inhibition levels below the cut-off value, while among the post immunization samples some showed viral inhibition. In addition we tested an array of HIV-1 positive, LTNP and HIV-1 negative samples. As expected, none of the HIV-1 negative samples inhibited viral replication above the cut-off.

We describe the optimization of the IMC LucR VIA based on the use of two “full length” LucR IMCs. Both NL-LucR.T2A and CH077.t-LucR.T2A are clade B viral isolates. An expansion and diversification of the viral panel to include more HIV-1 clades is necessary to assess the breadth of the HIV-1 vaccine induced CD8 T cell responses. An emphasis on recent T/F viruses, which are more susceptible to CD8 T cell antiviral responses than viruses isolated later in infection (Freel et al., 2012), is recommended as it would be a better representation of the

vaccine targets. When testing samples from clinical trials, we observed that NL-LucR.T2A was inhibited more frequently than CH077.t-LucR.T2A, although the magnitude of the responses did not seem to vary (data not shown). The samples used were from trials of vaccine candidates based on clade B HIV-1 lab strains HIV-1, which may explain the higher response rate for the lab strain NL-LucR.T2A than to the T/F virus CH077.t-LucR.T2A. However, when we looked at samples from HIV-1 positive individuals very similar maximum infections and log inhibitions were observed for both viruses. It is possible that in the context of a chronic infection the CD8 T-cell response is broader. In addition, these individuals are most likely infected with a non-clade B HIV-1, so the response to clade B viruses also indicates a broad CD8 T cell response, explaining the very similar inhibition pattern between the two viruses for these specific donors.

In summary, we have successfully developed and optimized the IMC LucR VIA using *Renilla* luciferase-expressing IMCs containing the full length viral genome. This novel VIA allows for the determination of HIV-1 specific CD8 T-cell responses from HIV-1 vaccine trial volunteers by a rapid and cost effective luciferase read out. Most importantly we have shown that the IMC LucR VIA has a high degree of specificity, a reduction of non-specific background inhibition, and an increase in positive responses, indicating an increase in sensitivity compared to the first generation VIA. Overall, the IMC LucR VIA is a novel VIA that can be implemented in large-scale clinical trial testing to assess functional HIV-1 specific CD8 T cell responses, which will enhance the ability to select the most promising HIV-1 vaccine candidates capable of controlling HIV-1 replication *in vivo*.

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