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Replication competent molecular clones of HIV-1 expressing *Renilla* luciferase facilitate the analysis of antibody inhibition in PBMC

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ABSTRACT

Effective vaccine development for human immunodeficiency virus type 1 (HIV-1) will require assays that ascertain the capacity of vaccine immunogens to elicit neutralizing antibodies (NAb) to diverse HIV-1 strains. To facilitate NAb assessment in peripheral blood mononuclear cell (PBMC)-based assays, we developed an assay-adaptable platform based on a *Renilla* luciferase (LucR) expressing HIV-1 proviral backbone. LucR was inserted into pNL4-3 DNA, preserving all viral open reading frames. The proviral genome was engineered to facilitate expression of diverse HIV-1 *env* sequences, allowing analysis in an isogenic background. The resulting Env–IMC–LucR viruses are infectious, and LucR is stably expressed over multiple replications in PBMC. HIV-1 neutralization, targeting TZM-bl cells, was highly correlative comparing virus (LucR) and cell (firefly luciferase) readouts. In PBMC, NAb activity can be analyzed either within a single or multiple cycles of replication. These results represent advancement toward a standardizable PBMC-based neutralization assay for assessing HIV-1 vaccine immunogen efficacy.

Introduction

An efficacious vaccine against HIV-1 may require the elicitation of a potent, broadly neutralizing antibody (NAb) response (Baba et al., 2000; Haynes and Montefiori, 2006; Johnston and Fauci, 2007; Mascola et al., 2000; McMichael, 2006; Nishimura et al., 2003; Pantophlet and Burton, 2006; Phogat et al., 2007; Plotkin, 2008; Shibata et al., 1999). The ability to develop immunogens capable of eliciting such antibodies is integrally linked to a requirement for standardized, high-throughput *in vitro* assay measurements that reflect the *in vivo* potency and breadth of NAb responses elicited by natural infection or experimental vaccine immunogens (Fenyo et al., 2009; Mascola et al., 2005b; Montefiori et al., 2007; Polonis et al., 2008). It is not currently known which *in vitro* assay results may correlate with antibody protection from HIV-1 infection *in vivo*, and several assay formats will continue to have to be employed to assess antibody responses elicited by vaccine immunogens (Fenyo et al., 2009; Pantophlet and Burton, 2006; Polonis et al., 2008, 2009). With support from multi-institutional HIV/AIDS vaccine initiatives [NeutNet (Neutralization Network) Project, CAVD/CA-VIMC, Global HIV/AIDS Vaccine Enterprise (GHAVE), NIH Center for HIV/AIDS Vaccine Immunology (CHAVI), and International AIDS Vaccine initiative (IAVI)], great efforts are being invested in the discovery, standardization and implementation of new assay platforms for assessing breadth and potency of neutralizing antibodies (Fenyo et al., 2009; Mascola et al., 2005a; Montefiori et al., 2007; Montefiori, 2009; Polonis et al., 2008). A comprehensive review of the strengths and differences between the two most widely utilized neutralization assays, the PBMC- and the

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pseudovirion-based neutralization assays, was recently published by Polonis et al. (Polonis et al., 2008).

Improvements in PBMC assay performance are urgently needed. While considered to be more physiologically relevant, the PBMC assay is labor-intensive, expensive and not practical for high-throughput analysis (D'Souza et al., 1997; Gauduin et al., 1996). This assay also exhibits substantial variability owing in part to donor PBMC variability (Polonis et al., 2009), and to the extensive use of primary virus isolates which complicates standardization. Moreover, the assay has been dependent on measurements of HIV-1 p24 antigen production as the endpoint, requiring extensive washout of HIV-1-positive sera to avoid artifacts but also reducing the sensitivity of the assay. While HIV-1 antibody neutralization in a single infectious cycle can be measured in PBMC by using flow-cytometry, this approach still involves several complex handling steps (Darden et al., 2000; Mascola et al., 2002). Thus, current PBMC-based assays are not easily amendable to high-throughput and standardized analysis.

However, significant improvements in assay standardization and performance have been made by creating genetically engineered cell lines as host-cell targets that stably express defined levels of CD4, CCR5 and CXCR4 (Jones et al., 2007; Montefiori, 2005; Ochsenbauer-Jambor et al., 2006; Platt et al., 1998; Richman et al., 2003; Wei et al., 2002). In certain cell lines, reporter genes have been introduced that are responsive to HIV-1 infection. For example, the TZM-bl cell line (Wei et al., 2002) expresses firefly luciferase in response to Tat expression following HIV-1 infection with either replication competent or Env-pseudotyped viruses. TZM-bl cells enable sensitive, quantitative and high-throughput measurements of HIV-1 infection and inhibition with a linear dynamic range of several orders of magnitude (Montefiori, 2009; Wei et al., 2002), properties which contribute to their wide use as an easily transferable and reproducible method for assessing neutralizing antibody activity (Montefiori, 2009). Furthermore, it is necessary to screen vaccine sera against panels of genetically diverse viruses (Li et al., 2005; Li et al., 2006) to evaluate the breadth of antibody responses elicited by vaccine immunogens, and for this reason, pseudovirions have certain advantages. HIV-1 *env* genes can be easily cloned from plasma viral RNA or infected cells, and coexpressed by transfection with an *env*-minus viral backbone generating infectious, replication-defective, Env-pseudotyped virus-like particles (pseudovirions). In addition to the TZM-bl assay, another neutralization assay utilizing pseudovirions is based on a viral backbone that, in place of *env*, encodes the firefly luciferase gene which is expressed subsequent to infection of target cells. This assay is robust in cell lines (e.g. U87) that express CD4, CCR5 and CXCR4 (Binley et al., 2008; Petropoulos et al., 2000; Richman et al., 2003). However, when PBMC or other primary cells are used as targets of infection this pseudovirus approach is not sufficiently robust due to a weak luciferase signal-to-noise ratio, a limitation resulting in part from single-round infection (Montefiori, unpublished). Furthermore, while these assays offer certain advantages, pseudovirions which are produced in cell lines like 293T do not always resemble virus produced in primary cells (reviewed in (Ochsenbauer and Kappes, 2009)). Incorporation of unprocessed gp160 (Herrera et al., 2005), producer-cell dependent post-translational modification of Env with glycan (Willey et al., 1996) and incorporation of host-cell proteins into progeny virions (Bastiani et al., 1997; Fortin et al., 1997; Hioe et al., 2001; Rizzuto and Sodroski, 1997) may influence infectivity and neutralization sensitivity (Bastiani et al., 1997; Sawyer et al., 1994; Willey et al., 1996; Zhang et al., 1997).

In this study, we describe an approach that combines the advantages of PBMC-based assays with those of replication competent molecular clones of reporter viruses. We introduce a recombinant HIV-1 proviral backbone that preserves all viral open reading frames (orfs), is replication competent, stably expresses *Renilla reniformis* luciferase (LucR) and allows different *env* sequences to be shuttled in and expressed *in cis*. This approach facilitates the construction of reference panels encoding *env* sequences from genetically diverse strains of HIV-1, including recently described transmitted/founder viruses (Keele et al., 2008; Salazar-Gonzalez et al., 2008, 2009). The response of the LucR readout to NAb is nearly identical to that of firefly luciferase when measured in the TZM-bl assay. Using PBMC as host-cell targets, the Env–IMC–LucR viruses enable sensitive, quantifiable assessment of infection and NAb activity, which can be measured either within a single cycle or after multiple rounds of virus replication. The robust and simplified assay read out enables analysis of large sample numbers and, thus, the approach represents a significant advancement towards the establishment of standardized high-throughput PBMC-based neutralization assays.

Results

Generation of a replication competent Renilla luciferase-expressing HIV-1 proviral DNA backbone

Since our objective was to create a versatile approach for sensitive and quantitative analysis of HIV-1 infection and the inhibition thereof in primary cells, we constructed a reporter HIV-1 proviral DNA backbone, pNL–LucR.T2A, which is replication competent, encodes all viral open reading frames and stably expresses a luciferase reporter gene over multiple rounds of virus replication. The sea pansy *Renilla reniformis* luciferase (LucR) gene was selected as a reporter since it comprises fewer nucleotides (935 vs. 1652 with firefly luciferase), a feature that we hypothesized would favor its retention within the genome during virus replication. LucR was inserted into the genome at the position of *nef* and linked in-frame at the 5′ end of *nef* with a "self-cleaving" T2A sequence (54 nucleotides) (Fig. 1) (Szymczak et al., 2004). During translation, the T2A sequence causes a ribosomal skip that impairs normal amino acid peptide bond formation between the penultimate glycine and the C-terminal proline (Fig. 1) (Donnelly et al., 2001a,b). The ribosomal skip releases the newly synthesized polypeptide as translation of the downstream sequence continues.

The principal purpose for constructing the pNL–LucR.T2A reporter proviral DNA backbone was to facilitate the analysis of HIV-1 inhibitory molecules, particularly neutralizing antibodies, against panels of *env* containing reference viruses in primary cells. Therefore, a molecular strategy was devised to best accommodate the expression of genetically diverse Envs within the pNL–LucR.T2A

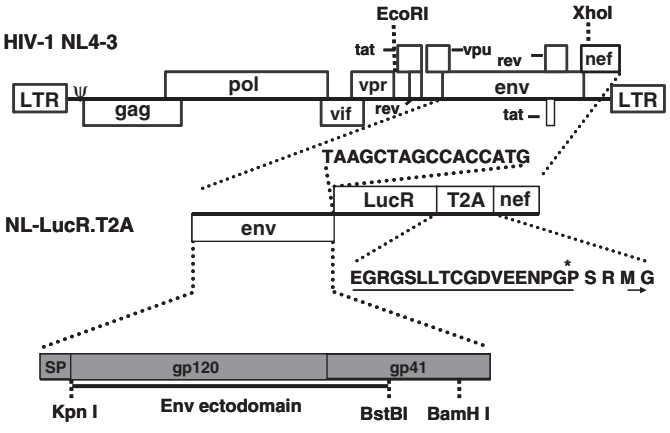

Fig. 1. Schematic representation of the insertion of the *Renilla* luciferase gene into the NL4-3 viral backbone and the "shuttling in" of heterologous *env* ectodomain sequences. The LucR gene and a T2A peptide (amino acid sequence underlined) were fused in-frame and inserted between the NL4-3 *env* and *nef* genes. * indicates the co-translational cleavage point between the penultimate and last amino acid of T2A and the arrow indicates the start codon of Nef. The nt sequence is depicted for the junction between the *env* TAA and LucR gene ATG. Furthermore, the "shuttling in" of heterologous *env* ectodomain sequences between the viral KpnI site (NL4-3 nt 6343) and the introduced silent BstBI site (NL4-3 nt 8301) in the membrane-spanning domain is depicted.