Establishment of an *in vivo* Antibody-Production Platform Via Precise, RNA-Mediated Insertion of a Broadly Neutralizing anti-HIV Transgene

ADDITION THERAPEUTICS

Gates Foundation

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INTRODUCTION

PRINT (Precise RNA-mediated INsertion of Transgenes) is an all-RNA, targeted-insertion technology based on R2-mediated target-primed reverse transcription. PRINT uses lipid nanoparticles (LNPs) to deliver retrotransposase mRNA and template RNA, the latter of which is copied as DNA into a safe harbor site within the ribosomal DNA (rDNA) array (Fig. 1). This non-viral system circumvents the safety challenges associated with viral delivery and allows for continuous expression of biologics after a single administration. To demonstrate the therapeutic potential of this system, we applied PRINT to the endogenous production of the antibody-like molecule eCD4-Ig.

Broadly neutralizing antibodies (bnAbs) and antibody-like molecules targeting the HIV envelope (Env) are promising tools for HIV prevention and treatment, reduction of latent reservoirs, and curative strategies. The highly conserved CD4 receptor binding site (CD4bs) is essential for viral entry, and biologics that neutralize HIV through CD4bs binding have demonstrated great potency and breadth. The immunoadhesin eCD4-Ig is such an HIV-entry inhibitor: it neutralizes all clinically relevant HIV isolates by engaging both the CD4bs as well as the highly conserved CCR5 and CXCR4 co-receptor-binding sites. It is composed of two extracellular human CD4 domains, D1 and D2, a human IgG1 Fc, and a CCR5-mimetic peptide (Fig. 2). As such, the safe and durable expression of these therapeutics is an attractive strategy for controlling and potentially eliminating HIV infection. Here, we demonstrate successful eCD4-Ig PRINT leading to robust *in vitro* and *in vivo* secretion of functional eCD4-Ig, positioning PRINT as a potentially broadly applicable and powerful platform for the delivery of therapeutic biologics for HIV and further indications.

Figure 1: PRINT™ uses a natural retrotransposon mechanism

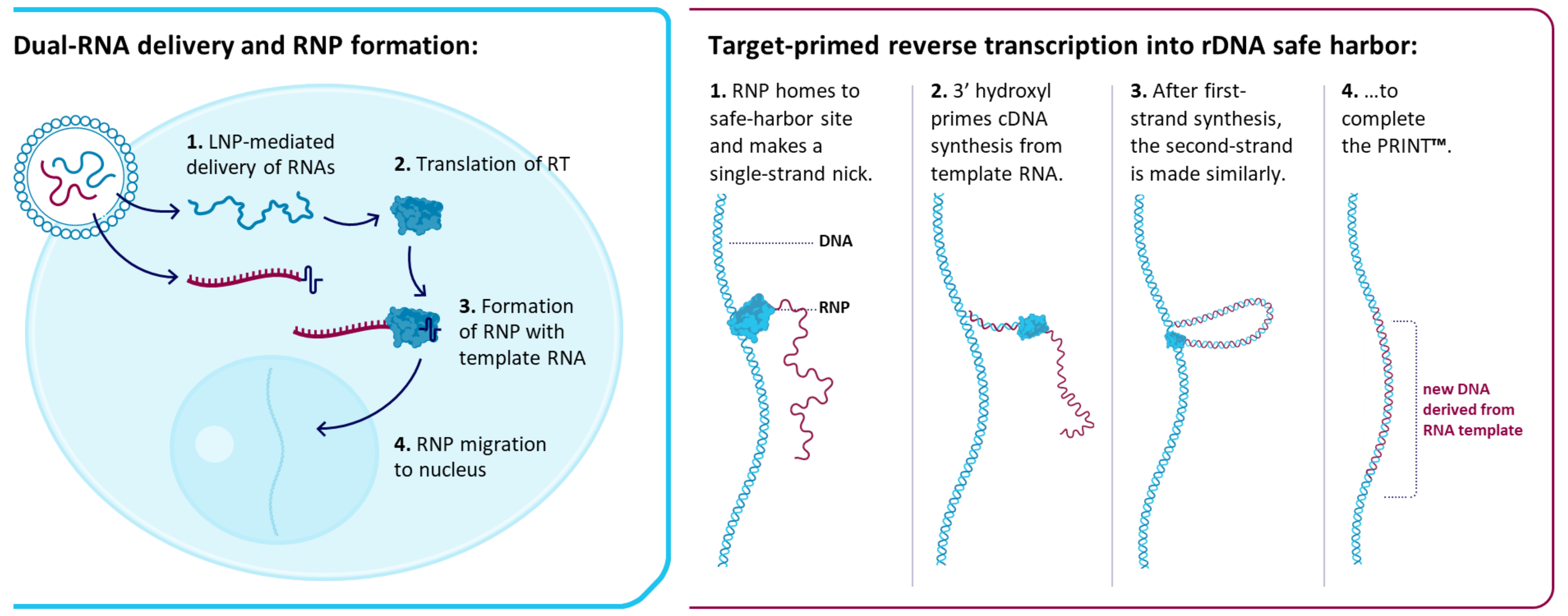
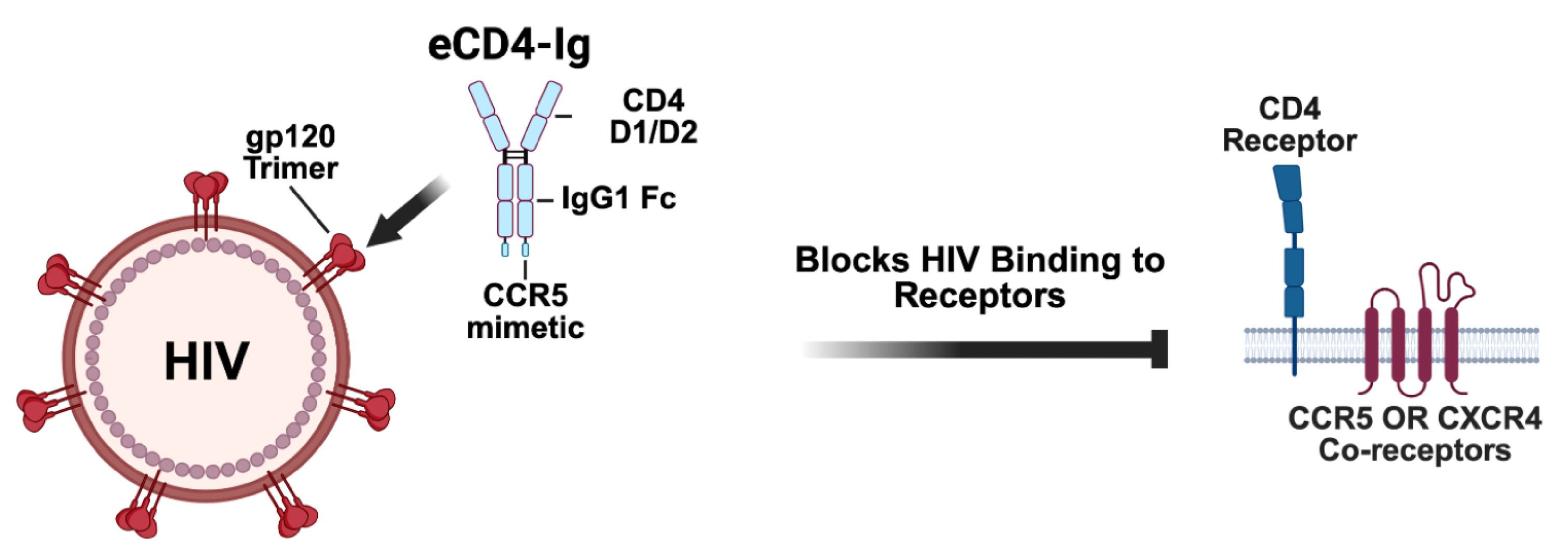

Dual-RNA delivery and RNP formation:
1. LNP-mediated delivery of RNAs
2. Translation of RT
3. Formation of RNP with template RNA
4. RNP migration to nucleus
Target-primed reverse transcription into rDNA safe harbor:
1. RNP homes to safe-harbor site and makes a single-strand nick.
2. 3' hydroxyl primes cDNA synthesis from template RNA.
3. After first-strand synthesis, the second-strand is made similarly.
4. ...to complete the PRINT™.
DNA
RNP
new DNA derived from RNA template

Figure 2: eCD4-Ig Structure and Function

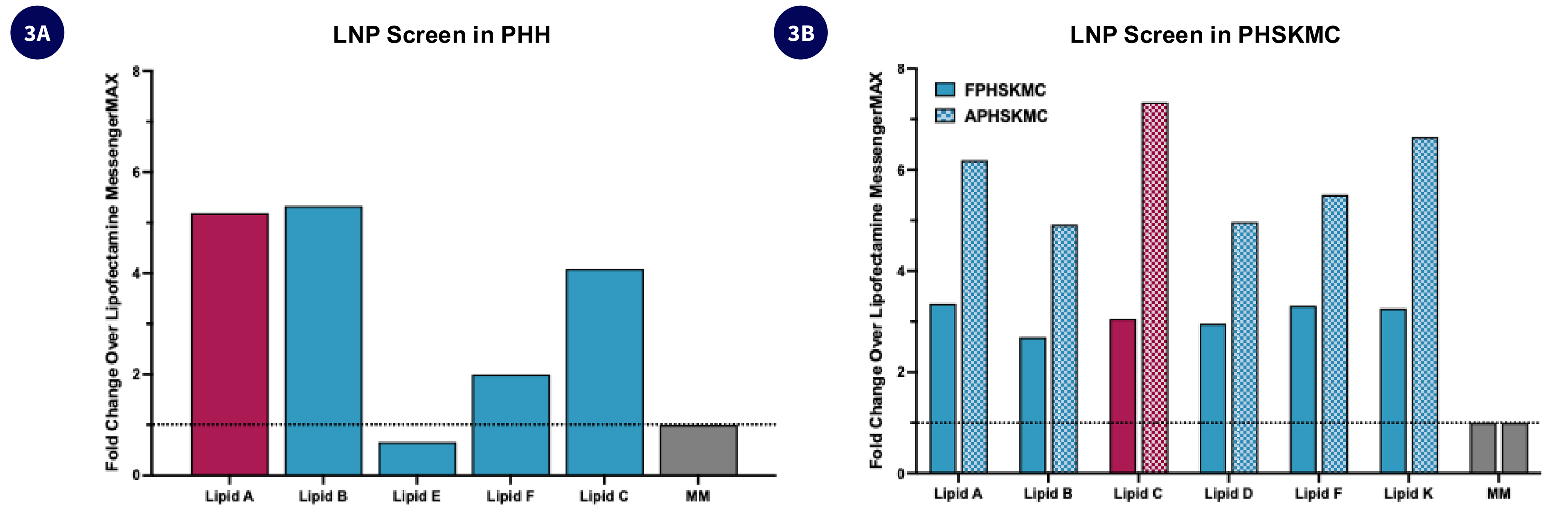


METHODS

Primary human hepatocytes (PHHs) and fetal and adult human skeletal-muscle cells (FPHSKMCs and APHSKMCs, respectively) were used to evaluate the *in vitro* efficiency of eCD4-Ig PRINTing. Lipid nanoparticle (LNP) formulations and subsequent PRINT reagent screens were performed in both primary human cell types to optimize PRINT reagent delivery and maximize eCD4-Ig secretion. Selected PRINT-eCD4-Ig candidates were subsequently assessed in rats. Secreted eCD4-Ig levels were quantified using an in-house MSD-based gp120 binding assay. The neutralizing activity of collected secreted eCD4-Ig was assessed using the widely employed TZM-bl infectivity assay with HXB2 (CXCR4-tropic) and YU-2 (CCR5-tropic) pseudovirus.

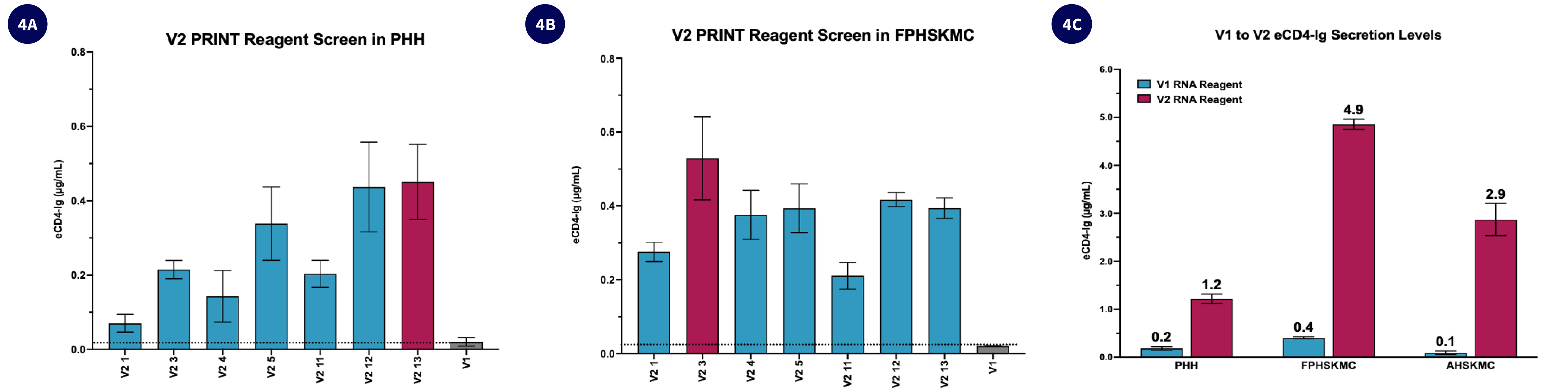
RESULTS

Figure 3: LNP-mediated eCD4-Ig PRINTing in primary hepatocytes and muscle cells



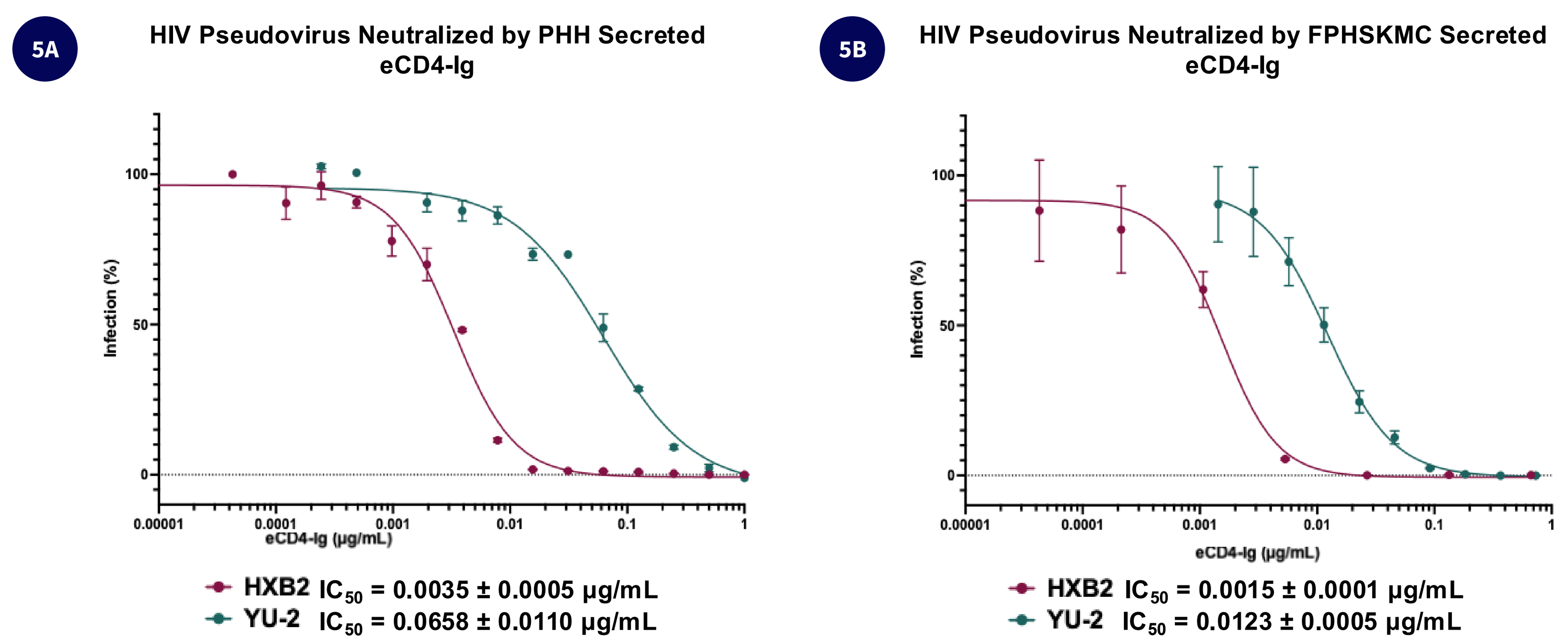
Lipid nanoparticle delivery optimization improves transgene PRINTing *in vitro*. Screening of LNP formulations in primary human hepatocytes (Fig. 3A) and muscle cells (Fig. 3B) show different optimal formulations to boost secretion over commercially available Lipofectamine MessengerMAX (MM) (in grey) for first generation PRINT reagents (V1 in Fig. 4). Numerous LNPs were tested, top 5 candidates are shown, and lead formulations Lipid A in PHH and Lipid C in PHSKMC are highlighted in red.

Figure 4: Lead eCD4-Ig PRINT reagents improve eCD4-Ig secretion in primary hepatocytes and muscle cells



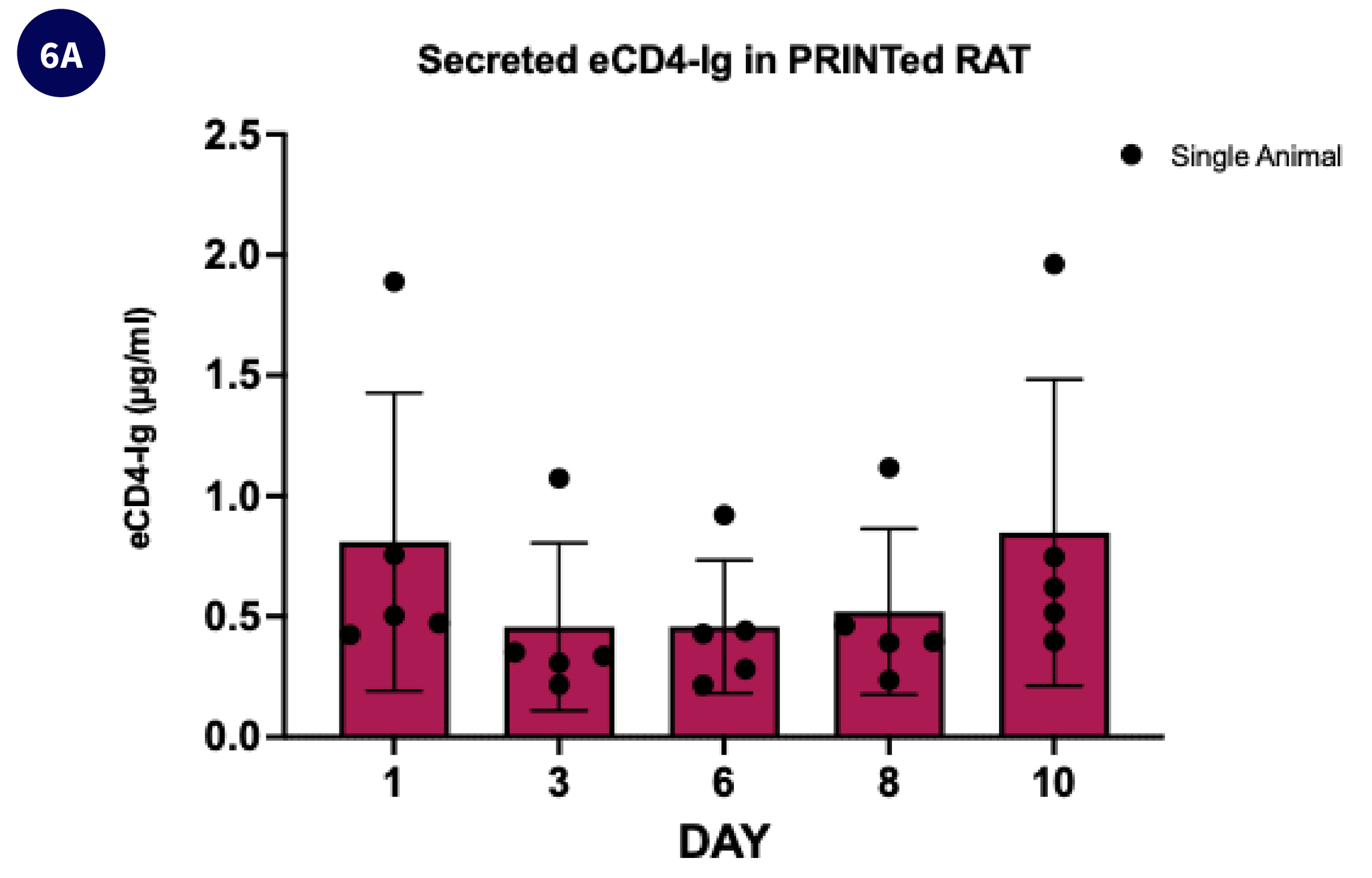
Iterative PRINT reagent engineering boosts PRINTed eCD4-Ig secretion in both PHH and PHSKMC. Second generation PRINT reagents (V2) result in increased eCD4-Ig secretion in both primary human hepatocytes (Fig. 4A) and muscle cells (Fig. 4B) in comparison to first generation PRINT reagents (V1 in grey). Select candidates are shown from the screening campaign (Fig. 4A and 4B) with the top candidate highlighted in red. Top V2 PRINT reagents combined with top LNP formulations lead to > 5-fold improvements in eCD4-Ig secretion over V1 (Fig. 4C).

Figure 5: Secreted eCD4-Ig neutralizes HIV pseudovirus



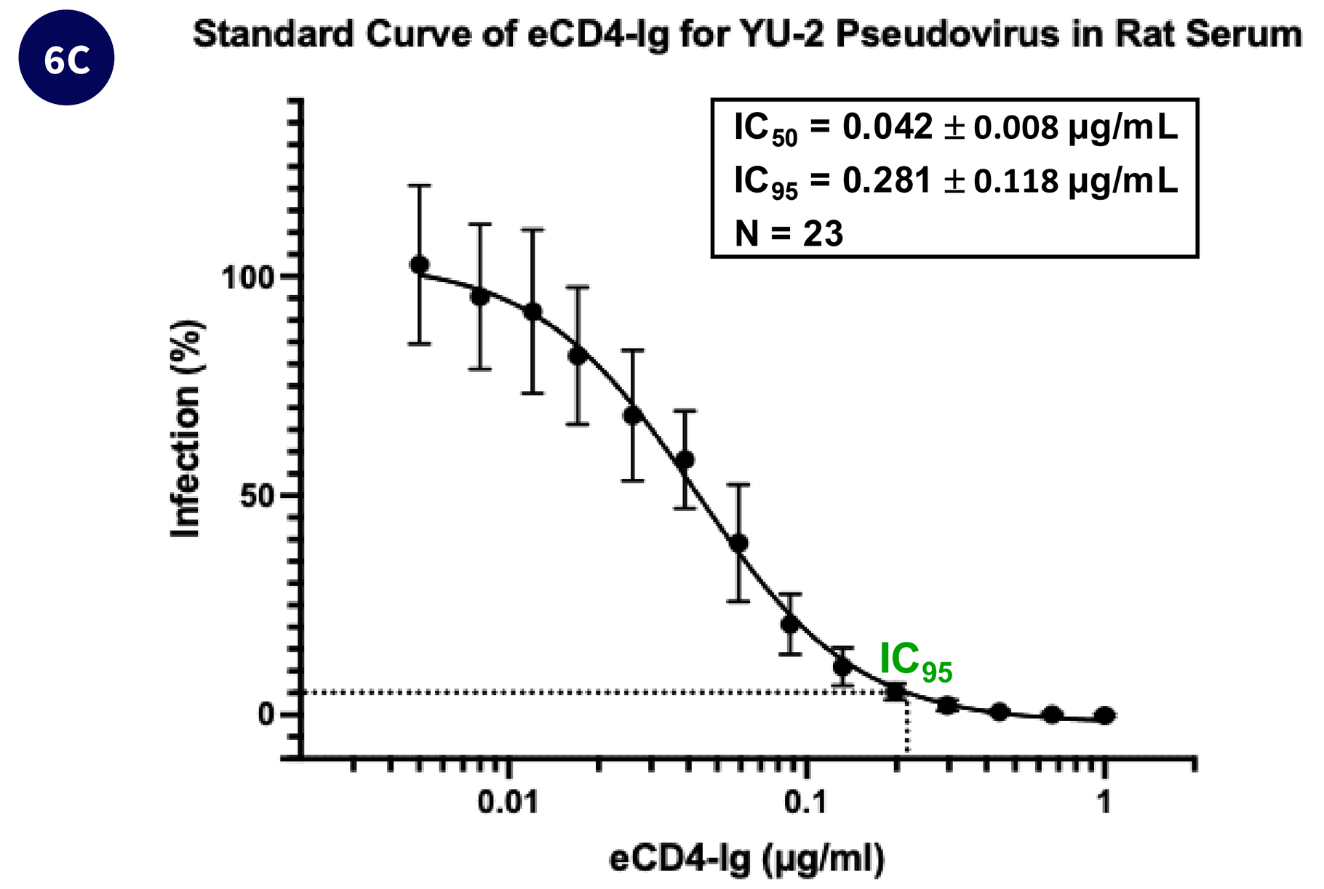
***In vitro* PRINTed eCD4-Ig neutralizes HIV pseudovirus.** eCD4-Ig secreted by PHH (Fig 5A) and FPHSKMC (Fig 5B) neutralizes HIV pseudovirus HXB2 (IC_{50}: 0.0035 and 0.0015 µg/mL, respectively) and YU-2 (IC_{50}: 0.0658 and 0.0123 µg/mL, respectively. These IC_{50} values are comparable to that of recombinant eCD4-Ig protein and reports from the literature (Fig. 6C and Fetzer, I., *et. al.*, *J. Virol.*, 2018; Yang, E., *et. al.*, *PLoS One*, 2018).

Figure 6: PRINTing and secretion of eCD4-Ig in rats



6B Neutralization of YU-2 HIV Pseudovirus by Day 10 Secreted eCD4-Ig from PRINTed Rats

Rat #	eCD4-Ig, µg/mL	Infection, %	Above IC_{95}?
R1	1.96	<0.00	Yes
R2	0.75	<0.00	Yes
R3	0.52	<0.00	Yes
R4	0.62	<0.00	Yes
R5	0.39	2.44	At
TBS (Control)	0.00	>100.00	No



Secretion of eCD4-Ig in PRINTed rats is robust and neutralizes HIV pseudovirus. PRINTed rats secreted up to 2.0 µg/mL eCD4-Ig (Fig. 6A) that is at or above the IC_{95} for the neutralization of YU-2 pseudovirus (Fig. 6B). The table in Fig. 6B shows the measured protein serum levels and percent infection for secreted eCD4-Ig collected at Day 10 post PRINT administration. A representative standard curve for the neutralization of YU-2 pseudovirus by eCD4-Ig in rat serum is shown in Fig.6C.

CONCLUSION

We demonstrate robust secretion of bioactive eCD4-Ig following both *in vitro* and *in vivo* eCD4-Ig PRINT. *In vitro*, PRINTed primary human hepatocytes (PHH) and primary human skeletal-muscle cells (PHSKMC) produce high levels of eCD4-Ig. The secreted protein effectively neutralizes both CXCR4-tropic (HXB2) and CCR5-tropic (YU-2) HIV pseudoviruses with potency comparable to recombinant eCD4-Ig. Consistent with these findings, *in vivo* PRINT in rats yields sustained eCD4-Ig levels exceeding the IC95 required for YU-2 pseudovirus neutralization. Collectively, these results support the potential of a single PRINT administration to enable continuous eCD4-Ig expression for the prevention, control, and possible eradication of HIV infection. Moreover, these data support the translation of eCD4-Ig PRINT to non-human primates, eventual clinical application in humans, and the broader expansion of PRINT-delivered biologics to additional indications.

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