Targeted Immuno-Antiretroviral HIV Therapeutic Approach to Provide Dual Protection and Boosts Cellular Immunity: A Proof-of-Concept Study

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Research

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Abstract

**Background:** Human immunodeficiency virus (HIV)-infected active and latent C-C motif chemokine receptor-5 (CCR5) expressing long-lived T-cells are the primary barrier to HIV/AIDS eradication. Broadly neutralizing antibodies and latency-reversing agents are the two most promising strategies emerging to achieve ‘functional cure’ against HIV infection. Antiretrovirals (ARVs) have shown to suppress plasma viral loads to non-detectable levels and other strategies have demonstrated a ‘functional cure’ against HIV infection is achievable. Strategies are effective at inducing direct or immune-mediated cell death of latent HIV+ T-cells but have shown respective limitations. We designed a novel targeted ARVs-loaded nanoformulation that combines a CCR5 monoclonal antibody and antiretroviral drugs (ARV) as a dual protection strategy to promote HIV ‘functional cure’. The modified CCR5 monoclonal antibody (xfR5 mAb) surface-coated dolutegravir (DTG) and tenofovir alafenamide (TAF) loaded nanoformulation (xfR5-D+T NPs)

**Results:** The nanoformulation was uniformly sized <250 nm, with 6.5 times enhanced antigen-binding affinity compared to naïve xfR5 mAb, and provided prolonged DTG and TAF intracellular retention. The multivalent and sustained drug release properties of xfR5-D+T NPs enhance the protective efficiency against HIV by approximately 12, 3, and 5 times compared to naïve xfR5 mAb, D+T NP alone, and xfR5 NPs, respectively. Further, the nanoformulation demonstrated high binding-affinity to CCR5 expressing CD4+ cells, monocytes, and other HIV prone/latent T-cells by 25, 2, and 2 times, respectively. Finally, during short-term pre-exposure prophylaxis, the xfR5-D+T NPs induced a protective immunophenotype, with boosted T-helper (Th), temporary memory (TM), and effector (E) sub-population. Treatment with xfR5-D+T NPs to HIV-infected T-cells induced a defensive/activated immunophenotype with boosted naïve, Th, central memory, TM, EM, E, and activated cytotoxic T-cells population.

**Conclusion:** This dual-action targeted nanoformulation could potentially become a multifactorial solution to achieve a “functional cure.”

Background

Antiretroviral therapy (ART) is the only treatment strategy for human immunodeficiency virus (HIV) infection. ART improves life-expectancy by effectively controlling plasma viral load (pVL). However, it is unable to eradicate the virus. Patients have to commit to continual life-long ART. Additionally, ART stoppage can reactivate latent virus. Therefore, alternative ways are under investigation to search for potential candidates to ‘functionally-cure’ HIV infection [1].

One of the essential targets of HIV research is the C-C motif chemokine receptor 5 (CCR5), a predominant co-receptor expressed on lymphocytes (such as CD4+ T-cells, latently infected cells, dendritic cells (DCs) and macrophages), responsible for HIV-1 cellular entrance [2]. Maraviroc, a CCR5 antagonist blocks HIV entry by docking on the CCR5 receptor on CCR5+ cells, is the first approved CCR5-antagonist for HIV-1 treatment [3]. The other promising approach is genome editing that disrupts CCR5 alleles in CD4+ T-cells by infusing an engineered zinc finger nuclease (ZFN) [4]. Ribozymes [5], transcription activator-like effector nuclease (TALENs) [6], short hairpin RNAs [7] and the clustered regularly interspaced short palindromic repeat-Cas 9 (CRISPR/Cas9) nuclease system [8], have also been investigated as alternative CCR5-targeted gene-editing techniques for curing HIV. However, the only gene-therapy approach to date that had conferred HIV cure is the use of CCR5 delta32 natural mutant genotype. It resists HIV-1 entry in three patients it has been used, i.e., the “Berlin Patient,” “London Patient,” and the “Düsseldorf patient” upon transplantation of stem cells from a CCR5 delta32 donor [9].
Sustained ART-free remission is a well-accepted and effective strategy to control HIV infection. The main focus of HIV research is to develop strategies that prolong protection and target latent HIV+ cells. Recent studies have demonstrated designing a long-acting (LA) ARV delivery system would boost the HIV treatment strategy [10–13]. The nanoformulated ARV delivery system has been shown to enhance drug-solubility, stability, biodistribution, pharmacokinetics, efficiency, and concurrent drug safety, due to reduced ARV associated side-effects [14–16]. Even though LA ARV nanoparticles (NPs) are promising to be a successful injectable LA antiretroviral, these systems still need to show a good tolerability profile. In the LATTE-2 trial, cabotegravir plus rilpivirine LA injection group reported higher grade 3–4 adverse events compared to the oral comparative treatment group [17]. The ECLAIR study of LA ARVs [18, 19] revealed a significantly prolonged sub-therapeutic tail of residual drugs which places patients at increased risk of contracting HIV and the possibility of resistance development [20]. The emergence of ARV resistance would limit future treatment options in those patients treated with LA ARV NPs. However, ‘targeting’ HIV prone cells is another strategy that is under investigation.

Broadly neutralizing antibodies (bNAbs) against HIV have emerged as a promising strategy to protect or to treat circulating HIV [21]. However, bNAbs for HIV is still a naïve field, as the search is on-going to find bNAbs that have good neutralization potency [22]. Eliminating the circulating HIV is not sufficient to achieve “functional cure”, due to the presence of latently infected cells. Furthermore, bNAbs still need optimization [23–25]. Therefore, bNAbs strategy has two limiting factors; plasma concentration and neutralization potency. Recently, studies have already confirmed the existence of resistant HIV-1 strains against bNAbs [26, 27], challenging bNAbs clinical potency.

Furthermore, combination ARV therapy (cART) is successful at providing meaningful life to high-risk individuals as well as HIV+ patients. In fact, combination of nucleoside reverse transcriptase inhibitors (NRTIs) and integrase strand transfer inhibitors (INSTIs)-based cART to treat HIV infection has become most common practice for last decade [28]. Recently, tenofovir alfanamide (TAF) has become a common NRTI component of various approved first-line HIV-1 treatments [29]. Among INSTIs, dolutegravir (DTG) is a second-generation HIV integrase inhibitor is among the most common ARV in consideration as cART regimen [30]. The major therapeutic success of cART has gradually transform the HIV/acquired immunodeficiency syndrome (AIDS) disease to a chronic manageable disease.

However, cART could suppress plasma viral load but has been unsuccessful in immune restoration. Recovery from the viral infection needs reestablishment of a strong immunity. Therefore, research against HIV-1, faces challenges related to immune reconstitution failure. Studies have revealed that the HIV infection induce terminal differentiation of effector (E) CD8+ T cells to the memory phenotypes that causes progressive E population reduction, immune exhaustion, and promote activation-induced cell death [31]. HIV-infection strongly compromises the immune system and immune impairment results in its inability to respond to viral and other pathogens; producing rapid progression to AIDS. Ideally, the immune reconstitution could be promoted by stimulating both humoral immune responses and cellular immune responses to prevent and control HIV infection. The adaptive immune system plays a critical role in protection against HIV-1 infection. Studies have shown that promoting T-cell-based immunity, specifically cytotoxic T lymphocyte (CTL) stimulation [32, 33], would promote effective production of neutralizing antibodies, and would synergistically protect against active and latent HIV-1 infection.

Our hypothesis is a combination of HIV therapeutic strategies, cART and antibody-mediated HIV-entry blockage, would be an effective strategy to suppress HIV viremia, and promote protective immune-response. The cumulative effect could potentially induce a “functional cure.” In this study, we propose an innovative approach to combine ART and CCR5 mAb in a novel single nanoformulation to achieve dual protection against HIV infection. To achieve this, a novel LA ARV loaded and anti-CCR5 mAbs surface decorated nanoparticles (NPs) have been formulated (Fig. 1).
Initial treatment from the CCR5 mAb, blocks HIV-1 entry in the CD4 + cells surface similar to maraviroc. Secondly, internalization of ARV NP complex maintains intracellular ARV, (Fig. 1). Our study demonstrates the novel CCR5 mAb decorated ARV nanoformulation act as a single delivery-system with dual-layers of defense that could prevent primary HIV-infection as well as potentially induce a protective immunophenotype, to target and suppress HIV latency. Therefore, a dual protective mechanism will prevent new HIV infection from naive and latent CCR5 + cell population, potentially achieving a "functional cure."

**Results**

*CCR5 targeted cARV loaded NPs characterization.*

By using standardized water-in-oil-in-water (w-o-w) interfacial polymer deposition method [13, 14], N–hydroxysuccinimide (NHS) functionalized DTG+TAF loaded NPs (D+T NP) were formulated (Figure 1). DTG and TAF were loaded at a 1:1 ratio in the xFR5-D+T NPs. Well-defined DTG+TAF NP (NHS-D+T NP) were formed as a result. Dynamic light scattering (DLS) size distribution analysis demonstrated D+T NPs size averaged 198.7 ± 10 nm with uniform size-distribution pattern (poly-dispersity index, PDI: 0.153 ± 0.008) (Table 1). The modified CCR5 mAb, (xFR5 mAb) were covalently conjugated on NHS-D+T NP (xFR5-D+T NP), by replacing NHS group. The xFR5 mAb binding on xFR5-D+T NP caused a slight increase in size to 212.6 ± 20.7 nm. The shape and surface properties evaluation by scanning electron microscopy reflected that NHS-D+T NPs obtained were uniform, and smooth-surfaced spherical particles (Figure 2A) and xFR5 mAbs surface conjugation does not cause any change in the particle's morphology (Figure 2B). The non-uniform NP morphology due to xFR5 mAb conjugation (Figure 2B) could be the cause of increased PDI, however still maintaining mono-disperse NP distribution (PDI: <0.3, [34]). The xFR5 mAb binding on D+T NP surface caused NP's surface charge suppression from -28.15 ± 1.9 mV to -17.77 ± 1.9. This reduction of the surface charge could be attributed to several factors such as charge masking by the ions from the PBS buffer or to xFR5 mAb surface decoration or both. The polymeric NPs encapsulation resulted in enhanced drug encapsulation efficiency (% EE) of 58.5 ± 5.2 % and 55.7 ± 3.9 % for DTG and TAF, respectively (Table 1).

The xFR5 mAb were conjugated on NHS-D+T NP by carbodiimide-crosslinking mechanism [35]. The xFR5 mAb binding on xFR5-D+T NPs quantified by bicinchoninic acid (BCA) protein assay, estimated ~ 3.7 ± 0.52 µg of xFR5 mAb per mg xFR5-D+T NPs (Table 1). The xFR5 mAb binding was further confirmed by FT-IR analysis, as shown in Figure 2C. The typical NHS ester bands at 1695-1818 cm⁻¹ (aromatic C=O stretching, green dashed-line box) and 967 cm⁻¹ (–CH wagging band, orange dashed-line box) of PEG-PLGA-NHS polymer, on NHS-Blank NPs and NHS-D+T NPs, confirmed free NHS functional group on the NP surface. The amide bond obtained when the primary amine group of the antibody by replacing the -NHS group of NHS-PEG on NP surface [36]. Thus, the disappearance of these -NHS band in xFR5-D+T NP proved the replacement of NHS ester group. Additionally, the shift of primary aliphatic amine NH stretching (3350 cm⁻¹ and 1420 cm⁻¹), and -CH stretching (2947 cm⁻¹) [37], along with primary amide I and II band presence (at 1640 and 1540 cm⁻¹ respectively) confirmed the covalent amide (-CONH-) bond formation. Whereas, other significant band C-O-C stretching (1000-1300 cm⁻¹) and broad O–H stretching band (3000-3500 cm⁻¹) confers xFR5-D+T NPs surface has open PLGA & PEG surfaces along with covalently bound xFR5 mAb. Therefore, xFR5 mAb is not densely packed on xFR5-D+T NP and have enough free space to avoid steric hindrance during targeted CCR5 receptor binding.

Overall, the enhanced electron density (Figure 2B) and BCA protein quantification (Table 1) validated binding of xFR5 mAbs on the xFR5-D+T NPs; whereas amide bond (NHC=O) conferred covalent bond between xFR5 mAb to PEG on NP surface (Figure 2C). Besides, the presence of amide bond, O–H stretching (3000-3500 cm⁻¹) conferred xFR5 mAbs
are surface decorated with an ample amount of free surface on NP with predominant PEG-PLGA composition. It is known that the onset of steric crowding compromises the binding avidity during multimeric interaction (38). Therefore, the spare xfR5 mAb coverage reduces the possibility of steric hindrance during multimeric xfR5 to CCR5-binding on the T-cells.

**Binding Affinity**

The antibody binding affinity was estimated by flow cytometric analysis, and the data are expressed as equilibrium dissociation constant, $K_d$. The binding affinity ($K_d$) of xfR5-D+T NP compared to naïve xfR5 mAb and wild-type CCR5 mAb on TZM-bl cells (CCR5+ CD4+ cell line) (Table 2), demonstrates xfR5 mAb displayed approximately 10-fold higher affinity (0.251 ± 0.15 nM) compared to wild-type CCR5 mAb (2.023 ± 0.655 nM). Simultaneous multiple xfR5 mAb within the xfR5-D+T NP (5.7 ± 2.7 nM) binding further improved the sensitivity by approximately 6.6 times (Supplementary Figure 1 and Table 2). Similarly, xfR5-D+T NPs (4.31 ± 1.47 nM) resulted in enhanced binding with the primary CD4+ T-cells, approximately 5 times lower $K_d$ value compared to xfR5 mAb alone (20.87 ± 10.65 nM). These results reveal that multipoint interaction of single xfR5-D+T NP via multimeric xfR5 mAb on NP leading to significant enhancement in the binding affinity compared to xfR5 mAb univalent interaction (Supplementary figure 1).

The binding affinity of xfR5-D+T NPs with other CCR5+ immune cell types such as cytotoxic T-cells (CTLs, CD8+), dendritic T-cells (CD2+) and monocytes (CD68+), were evaluated using a similar method (gating strategy, supplementary Figure 2). The comparative binding affinity study demonstrated xfR5-D+T NPs had an enhanced binding affinity with memory CD8+ T-cells, approximately 25 times higher affinity compared to naïve xfR5 mAb (Table 2). Whereas xfR5-D+T NP binding with CD2+ T-cells and CD68+ T-cells displayed slightly enhanced but non-significant difference in binding affinity compared to naïve xfR5 mAb.

**Intracellular drug kinetics**

The sustained release property of xfR5-D+T NP compared to D+T solution was evaluated by following the intracellular uptake/release and retention kinetics of DTG, tenofovir (TFV) and TFV-diphosphate (TFA-dp, active-metabolite) by non-compartmental analysis using Phoenix WinNonlin 8.1 software. The study result demonstrate that the maximum concentration ($C_{max}$) and the area under the concentration-time curve ($AUC_{all}$) of TAF upon xfR5-D+T NP treatment were 12.9 and 72.4 times higher than TAF solution treatment. DTG demonstrated comparatively higher $C_{max}$ and $AUC_{all}$ (110.9 and 254.5 times higher), respectively. Whereas, these parameters showed a non-significant difference for intracellular TFV and TFV-dp, between xfR5-D+T NP and D+T solution treatment. The rationale behind this non-significant difference in the active-drug, TFV and TFV-dp could be due to its dependence on cellular enzymes. The conversion of TAF ($\text{TFV} \rightarrow \text{TFV-dp}$ intracellularly, is restricted due to acquired steady state. Thus, in the absence of intracellular drug utilization, the cellular TFV and TFV-dp steady-state concentrations are maintained in both case of xfR5-D+T NP and D+T solution treatments. From the $C_{max}$ and $AUC_{all}$ data of TAF and DTG, it is evident that the nanoformulation enhances cellular uptake of ARV compared to the same drugs in solution. In terms of retention, NP formulation demonstrated 11.6 and 4.4 times higher TAF and DTG elimination half-life ($t_{1/2}$), than naïve drugs in solution, which is indicative of improved retention kinetics.

**Cytotoxicity and HIV Protection study**

A comparative cytotoxicity study performed on TZM-bl cells and primary peripheral blood mononuclear cells (PBMCs) (Table 4). The %viability has been evaluated based on untreated controls (normalized against untreated
control, Equation 2). Various studies have established that nanoencapsulation of ARV drugs reduces toxic effect compared to naïve drugs [13, 14, 38, 39]. Moreover, xfR5-D+T NP demonstrated 2-times higher 50% cytotoxic concentration (CC_{50}) value (2910 ± 134.9 nM) compared to xfR5 mAb (1464 ± 35.5 nM), in TZM-bl cells. However, in primary PBMCs, xfR5-D+T NP treatment demonstrated a non-significant change in CC_{50} compared to xfR5 mAb (Supplementary Table 1). Overall, in vitro cytotoxicity study results on TZM-bl cells and PBMCs suggest that nanoencapsulation doesn't pose toxicity and improves cell viability. The study revealed all the tested nanoformulations (xfR5-D+T NP, xfR5 NP, and D+T NP) were as safe as xfR5 mAb for cellular application. Upon cellular uptake PLGA breaks down into body metabolites, i.e. lactic and glycolic acid, by hydrolysis of ester bonds, which are then utilized by cellular Kreb cycle. Therefore, our studies also reflected that PLGA based CCR5 targeted ARV drugs loaded NP doesn't induce significant cytotoxicity.

The dual protection by xfR5-D+T NP due to multimeric xfR5 mAb blocking and prolonged ARV release is expected to improve the half-maximal inhibitory concentration (IC_{50}), compared to xfR5 mAb and D+T NPs alone. The HIV protection study in TZM-bl cells demonstrated xfR5-D+T NP (0.0352 ± 0.0086 nM) improved IC_{50} by 528 and 5.5 times compared to D+T NPs (0.195 ± 0.1 nM) and xfR5 mAb (18.53 ± 2.85 nM), respectively (Table 4). Similarly, in primary PBMCs, where only a fraction of cell expresses CCR5 surface receptor, xfR5-D+T NP treatment improved the protection against HIV-infection by 12, 5.5 and 3.4 times compared to naïve xfR5 mAb, xfR5 NP, and D+T NP, respectively (Table 4). Furthermore, xfR5-D+T NP reduced IC_{50} by 338 times (TZM-bl: 0.05471 ± 0.0103 nM) and 2-times (PBMCs: 53.34 ± 2.34 nM) compared to naïve xfR5 mAb. This result supports the hypothesis that multimeric mAb coated nanoformulation induces multi-valent interaction and thus enhances protection compared to xfR5 mAb alone (Supplementary Figure 1) [40, 41].

Further, the selectivity index (SI) which evaluates the therapeutic boundary has been evaluated demonstrating the potential of xfR5-D+T NPs (Table 4). In TZM-bl cells the xfR5-D+T NP improves SI value by 5.5 and 4.5 times compared to mAb NP or drug NP alone. However, in primary PBMCs, xfR5-D+T NP demonstrated 2.7, 2.4, and 11 times higher SI value compared to mAb NP, D+T NP, and naïve xfR5 mAb, respectively. These results demonstrate xfR5-D+T NP, effectively improved the therapeutic index of both individual therapeutic approaches.

**Immunophenotype during in vitro short-term PrEP and HIV-1 treatment study**

Immunophenotype performed on PBMCs isolated from healthy donors to evaluate the immunological potential of targeted ARV-loaded nanoformulation. The immune-differentiation pattern of T-cells during PrEP and HIV-1 treatment were evaluated by flow cytometry after treating uninfected PBMCs with xfR5-D+T NP or xfR5 mAb, along with respective controls. Supplementary figure 3 illustrates the gating strategy for the immunophenotypic study. Based on the expression level of CCR7, CD27 and CD45RO receptors on T-cells, five distinct T-cell subpopulations were determined, i.e., naïve cells (CCR7+ CD27+ CD45RO-), central memory cells (CM, CCR7+ CD27+ CD45RO+), transition memory cells (TM, CCR7- CD27+ CD45RO+), effector memory cells (EM, CCR7- CD27- CD45RO+), and effector cells (CCR7- CD27- CD45RO-). The immunophenotype of the T-cell subpopulations were determined over different time-points and conditions, on the day before phytohemagglutinin (PHA) activation (unstimulated T-cell population); after PHA-activation (one day after PHA); HIV infection (one day after HIV infection); as well as one and four days after xfR5-D+T NP or xfR5 mAb treatment (Figure 3).

The unstimulated PBMCs evaluation was composed of 18%, 30.3%, 1.4%, 60.7% and 36.8% of naïve, CM, TM, EM, and E T-cell subpopulations, respectively (Figure 3A). As expected, the PHA stimulation (mock infection) significantly changed the immunophenotype of T-cell sub-populations stimulating the naïve and E sub-population (45.7% and
EM population was significantly decreased (27.4%) during PHA stimulation. Emergence of activated effector cytotoxic T-lymphocytes (aCTLs, CD8+ CD69+) from minimal (0.66%) to 41.8% could be also be attributed to PHA-activation effect (Figure 4A). Further, we evaluated binding potency of xfR5-D+T NPs compared to other variables, i.e., xfR5 NPs, xfR5 mAb (supplementary figure 4). Over time binding study shows all the treatment variable shows non-significant change in the % cells binding with different immune cell type. Therefore, xfR5 bound NPs don’t preferentially binds with CD4+ cells but it binds with all type of that expresses CCR5 receptor.

The short-term HIV protection study (4 days) ex-vivo study, demonstrated xfR5-D+T NP significantly protected cells against HIV infection (Table 4), which in part could be attributed to a protective immunophenotype (Figure 3B). The xfR5-D+T NP resulted in increased CM, TM, and E sub-populations. However, compared to PHA-activated (untreated), the xfR5-D+T NP demonstrated a significant boost in TM and E sub-populations (Figure 3B). The aCTLs and TH population demonstrated a reciprocal effect (Figure 4B). The aCTL sub-population after initial spike (day 1) followed a decline. In contrast, TH population showed a gradual increase over time (day 4) (Figure 4A, B).

The ex-vivo HIV-1 infection and short-term treatment (4 days) study were performed to predict the immunophenotypic differentiation pattern that the novel treatment could produce (Figure 3C). The results demonstrated HIV infection, mainly influenced naïve and CM sub-population (Figure 3C). In the presence of HIV infection, the naïve sub-population after initial increased (day 1, post-infection, PI) reverts to basal levels (day 4, after HIV infection), and the CM population showed gradually increased population (day 4 PI). The xfR5-D+T NP treated population also showed enhanced naïve and CM sub-population during the initial active HIV infection stage (day 1 PI). In the long-term, however, the xfR5-D+T NP treated population demonstrated a reverse effect in the CM and TM sub-population. Prolong xfR5-D+T NP treatment (day 4, after treatment) of HIV-infected PBMCs (day 5 PI) displayed 2.4 times higher CM sub-population, and a 6-times higher TM sub-population boost, compared to initial HIV infection. Moreover, xfR5-D+T NP treatment also significantly increased in EM sub-population. The xfR5-D+T NP treatment resulted in significantly higher CM, EM, E, and TH sub-population compared to xfR5 mAb and untreated controls. These results demonstrate the multimeric interaction of xfR5-D+T NP could potentially improve the CM⇒TM⇒EM rate-limiting steps that would help in maintaining a high EM population during possible HIV challenge.

Discussion

Our primary aim is to investigate a potential alternative candidate that effectively endorse ‘functionally-cure’ in HIV+ patient. In this study, we have demonstrated a strategy that combine two different HIV-treatment approaches in a single nanoformulation with sustained release property. Our strategy combines CCR5-antagonist-based HIV-entry blocking strategy [3] and ARV-based HIV-1 protection strategy [42]. To achieve this, we formulated xfR5-D + T NPs, a CCR5 targeting ARV loaded nanoformulation with the potential to block HIV entrance and promote cellular immunity against HIV infection, specifically in memory CD4+ T-cells as they commit themselves to provide antiviral immunity as latent HIV-1 reservoir (34). Alongside memory CD4 + T-cells, the myeloid lineage, such as monocytes/macrophages, are believed to be other potential HIV-1 sanctuaries (35, 36). Our hypothesis is the novel CCR5 targeted ARV nanoformulation approach could combine different strategies to ensure dual protection against HIV to the CCR5+ cell types. First, CCR5 receptor docking on the CCR5+ cells will block HIV entrance to prevent HIV infection; and second, intracellular ARV released from endocytosed NP will providing protection against HIV-1 intracellularly to the naïve cell or latent HIV-infected cell population (Fig. 1). The nanoformulation will also boost anti-HIV immunity to contribute to the possible “functional cure” strategies (37).
To date the only effective CCR5 receptor associated blocking strategy that blocks HIV virus entry in lymphocytes and confirmed HIV-1 cure [43], is CCR5 delta32 natural mutant genotype [9]. Other strategies like maraviroc, ZFN, ribozymes, TALENs, short hairpin RNAs, and CRISPR/Cas9) nuclease, has shown to block HIV-1 entry, but are not yet successful in conferring HIV cure [43]. A study by Platt et al. reported about 7 × 10^2 and 2 × 10^3 CCR5 receptors/cell leads to HIV infection [44]. Consequently, at any given time a minimum 7 × 10^2 unbound CCR5 receptors are available for HIV infection. To block HIV infection at least 10^3 blocker molecules are needed to occupy 7 × 10^2 unbound CCR5 receptors/cell. The most effective CCR5 blocker molecule, maraviroc in vivo haven’t shown accumulate at a concentration around the cell. Additionally, another factor is that ligand/CCR5 endocytosis increased CCR5 receptor turn-over dynamics, causing increased CCR5 density as viral load increases [45]. Incomplete blockage due to insufficient number of molecules near CD4 + cells, and or increased CCR5 receptor density because of CCR5 receptor regulatory dynamics [46], is supposed to be major reason behind failure of CCR5 blocking strategies other than the CCR5 delta32 natural mutant genotype approach.

To overcome the above constrains, we strategically surface decorated xFR5-D + T NP with multiple xFR5 mAb to maximize CCR5 receptor blocking as well as proving hindrance to viral binding with the CCR5 receptors on T-cells (Fig. 1). Due to multivalent CCR5 interactions of xFR5-D + T NPs, each xFR5-D + T NPs could effectively block multiple CCR5 receptors, compared to univalent interaction of blocker molecules like maraviroc or mAbs with CCR5 per cell (Supplementary Fig. 1). The enhanced binding affinity of xFR5-D + T NP (Table 2) could be attributed to the multi-valent interaction strengthening the binding affinity between the target biomolecules on the NP and the receptors on cell-surface (39). Theoretical calculation has revealed that each mAb conjugated NP using the flexible PEG spacers, at a given time could maximally occupy ten mAb/receptor complex in T-cells (39), although, a single naïve mAb could only bind/block one CCR5 molecule per T-cell (32) (Supplementary Fig. 1). Therefore, based on this theoretical assumption, the xFR5-D + T NP practically resulted in 7-times higher binding affinity compared to xFR5 mAb on TZM-bl cells. The CCR5 co-receptor characteristically is expressed on CD4 + T-cells in peripheral blood (40). In addition to CD4 + T cells, the CCR5 chemokine receptor is also expressed on CTLs (CD8+) (41), dendritic cells (CD2+) (42) and monocytes/macrophages (CD68+) (43, 44), that also plays critical roles in HIV infection, propagation, and latency. The binding affinity of xFR5-D + T NP on other CCR5 + expressing cell types are essential to estimate the success of this targeted nanoformulation strategy. Our study demonstrates the multimeric binding potency of xFR5-D + T NP evaluation on primary HIV-1 susceptible cells type, especially memory Th (CD4+) and CTLs (CD8+) T-cell population showed ~ 5 and ~ 25 times higher binding affinity compared to naïve xFR5 mAb (Table 2). The enhanced CCR5 expression during infection or PHA-activation promotes migration of activated effector, and memory CTLs T-cells (45) Possibly enhanced CCR5 + expression on T-cells due to induction (by PHA-activation) of migrating phenotype possibly contributes to the substantial enhanced binding affinity of xFR5-D + T NP to CTLs. However, dendritic CD2 + populations and monocyte/macrophage CD68 + populations demonstrated slightly higher binding affinity for xFR5-D + T NP compared to naïve xFR5 mAb. This reason could be due to the low frequency of CCR5 + CD2 + the dendritic population in PBMCs (supplementary Fig. 2) (46). Whereas, phagocytic CCR5 + CD68 + cells, i.e., monocytes/macrophages population (47), in addition to CCR5 receptor-specific binding, the non-specific phagocytic uptake of xFR5-D + T NP and xFR5 contributes to binding affinity evaluation. Hence, affinity binding study reflects multimeric interaction by xFR5-D + T NP significantly improves CCR5 receptor blocking capacity, compared to other CCR5 blocking strategies.

Additionally, the assembly of NPs size (~ 212 nm, with weak surface negative charge) around target cells and strong cell surface interaction together pose strong stearic hindrance to compete out virion particle (sized ~ 145 nm [47]), thus minimizing their cellular binding probability (Fig. 1). Hence steric hinderance strategically also contributes
towards protection against viral entry. This twofold competitive hindrance by xfR5-D + T NPs, could overcome the incomplete blockage constrain of other reported CCR5 blocking strategies.

A study has shown viral interaction with CCR5 receptor promotes increases in surface CCR5 density on T-cell surface [48]. CCR5 receptor endocytosis and recycling rate increases compared to degradation [49]. Others reported that blockage in HIV/CCR5 entry delayed HIV disease progression as observed in long-term non-progressors populations [50]. Following the same direction, the NP interaction with target T-cell generates strong adhesive forces due to specific ligand–receptor binding, and non-specific bonds (i.e., van der Waals, electrostatic and steric interactions) increased membrane stiffness by physically nullifying or overshading the receptor-mediated endocytosis as membrane tension increases. Hence, NP mediated interference with the ligand-receptor endocytosis process, reportedly prolongs targeted NP retention [51]. This process could result in delayed CCR5 receptor turn-over rate, suppressing CCR5-receptor upregulation, similar to of long-term non-progressors [52]. Overall, target-specific nanoformulation have several unique properties that physically and mechanically can blocks HIV entry and delay or prolong protection against new HIV infection.

Even though, long-term non-progressors delays CCR5 receptor turn-over rate, the CCR5 receptor is eventually going to be endocytosed, leading to new receptors on the cell surface, returning to naïve surface where the virus will get new CCR5 receptors to bind to start the infection. Latent HIV + cells, when activated, will produce new virion components (reverse transcriptase, integrase and protease) to be packed into fresh virion particles to start new HIV infection. To counter that, our strategy is to introduce internal protection against new infection or during re-activation of latently infected cells. To achieve this, we loaded the targeted NPs with common NRTI (i.e., TAF or T) and integrase inhibitor (i.e., DTG or D) [28–30]. The xfR5-D + T NPs upon endosomal uptake causes sustained release of loaded ARVs in cell cytoplasm due to polymeric degradation. Therefore, any new HIV virions introducing new reverse-transcriptase or integrase will immediately be shut down by the intracellular NRTI and INSTI concentration (Fig. 1, schematic diagram). In fact, our result prove this is possible in our short HIV-1 protection assay. The xfR5-D + T NP dual protection, boosted IC50 value by 526 (TZM-bl cells), and 12 (PBMCs) times compared to xfR5 mAb (Table 4).

Further, it is well established (9, 10, 30, 31) and our study also demonstrates (Table 4), that nanoformulation of the ARV drugs promotes a wide therapeutic index compared to naive drug. Additionally, the early T cell activation study (supplementary Fig. 6C), also reflects, multimeric xfR5 binding by xfR5-D + T NPs and xfR5 NPs prolongs retention of early-activation phenotype of CD8 + T population both in presence and absence of HIV infection. Therefore, these studies emphasize, HIV protection efficacy of xfR5-D + T NP is associated with the combined CCR5 blocking as well as prolong D + T intracellular retention.

The CCR5 plays a role in the inflammatory response and stimulate T-cell functions. Therefore, blocking CCR5 receptor on T-cells are known to influence T-cell differentiation, especially after HIV-infection [48, 53]. In general terms, the T-cell differentiation progresses in the following path: Naïve cells ⇒ CM ⇒ TM ⇒ EM ⇒ E. During initial HIV-1 infection, the immune system promotes naïve T-cell differentiation to antigen-specific memory and E sub-population (48). The immunophenotype evaluation (Fig. 3) during the ex-vivo HIV protection study demonstrated xfR5-D + T NP treatment enhanced naïve T-cell, memory (CM and TM), and E sub-population and the effect was sustained over the entire study period (Fig. 3B). This suggests that xfR5-D + T NP for PrEP application could help the immune-system to be ready to promote fast clearance of the virions upon HIV challenge.

HIV-1 infection and ART induces different immunophenotypic profile. Studies have shown that HIV-1 infection induces naïve T-cells proliferation, whereas ART causes depletion (49). A similar result was observed upon treating HIV + PBMCs with xfR5-D + T NP and xfR5 mAb (Fig. 3C). The ex-vivo immunophenotype of HIV-infected PBMCs...
demonstrated an increase in the naïve sub-population (47, 48). Whereas, xfR5-D + T NP treatment-induced HIV-1 suppression (Table 4) and reverted the naïve sub-population to its basal level. Studies have shown HIV can integrate into both naïve and memory cells directly [54]. Therefore, even though the naïve cells expresses low CCR5 receptor, they are found to be susceptible to R5 viral infection (less efficiently than memory T-cells[54]) because R5 virus infection is not governed solely by the level of CCR5 expression, but rather by the balance between CCR5 expression and β-chemokine expression [54, 55]. However, other studies have shown that peripheral blood naïve CD4 T cells from are refractory to HIV infection [56–58]. Therefore, no clear concept of decline in naïve cell infection due to HIV infection has been shown. We observed declined in naïve T-cell population and increased CM sub-population (on day 4) and this could be due to differentiation induced population shifting from naïve ⇒ CM sub-population [59].

The memory T-cell population plays a vital role not only in promoting anti-HIV immunity but also in AIDS prognosis since these cells govern the functions of CTLs and B-cells, which in turn regulates cellular and humoral immunity against HIV (50). Persistent HIV-1 viremia is known to drive the differentiation CM ⇒ EM sub-population (51, 52), and the high EM sub-population may be responsible for the long-lasting and exhausted T-cell population in HIV + patients (52). The ART treatment partially restores high CM sub-population and reduces EM sub-population over time to rebalance the homeostasis disrupted during HIV infection (52). These experiments demonstrates that as treatment progresses, xfR5-D + T NP treated population demonstrates significant increased CM sub-population (day 4, after treatment), as well as atypical to ART treatment, also significantly boosted TM and EM sub-population. During active infection, targeted specific treatment using xfR5-D + T NP or naïve xfR5 mAb maintained high E and aCTL sub-population, to counter HIV infection. Upon viral suppression, E and aCTLs sub-population tend to decrease. Studies have shown that despite persistently low antigenemia, high Th and E sub-population is essential to control HIV replication in the presence or absence of ART to achieve “functional cure” (50, 53). However, long-term patients under ART-treatment results in the loss of effector function. We observed HIV targeted treatment prolongs high E and Th sub-population. Overall, the immunophenotypic study displayed the xfR5-D + T NP multivalent interaction results in enhanced and improves protective and defensive immunophenotype compared to naïve xfR5 mAb treatment as well as D + T NPs and xfR5 NPs (Supplementary Fig. 5). Additionally, the multivalent phenomenon also contributes to maintaining high TM and EM subpopulation. Therefore, the immunophenotypic differentiation study indicated that targeted cARV NP could also overcome the CM ⇒ TM ⇒ EM rate-limiting steps to promote high E and aCTL population maintenance during the HIV challenge.

**Conclusion**

In summary, we formulated a dual-action targeted nanoformulation, i.e., xfR5-D + T NP. The clinically relevant ex-vivo system (primary PBMCs) experiments demonstrated xfR5-D + T NP induced CCR5 blocking and intracellular ARV drug release which provides two-levels of protection against fresh HIV-1 infection or in latently infected HIV + cells. Further, xfR5-D + T NP treatment also promotes reprogramming of the immune repertoire function of memory T-cells and could help to reconstitute anti-HIV immunity. Based on this study, we conclude that xfR5-D + T NP combines the advantages of ART and augmentation of anti-HIV immunity reconstitution could be a promising multifactorial strategy to target the HIV latent reservoir to achieve a “functional cure.”

**Materials And Methods**

Acid terminated PLGA (75:25, Mn = 4000–15000 Da), polyvinyl alcohol (PVA), dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate (KH2PO4), dichloromethane (DCM), IL-2 and methanol were all purchased from Sigma-Aldrich (St. Louis, MO). Poly(lactide-co-glycolide)-block-poly(ethylene glycol)-succinimidyl ester (PLGA-PEG-
NHS) and Methoxy Poly(ethylene glycol)-b-Poly(lactic-co-glycolic acid; PEG-PLGA (5,000:15,000 Da, 75:25 LA:GA) was purchased from Akina, Inc. (IN, USA). Pluronic F127, the stabilizer was obtained from D-BASF (Edinburgh, UK).

The ARV drugs, i.e., DTG (98% purity) was purchased from BioChemPartner co., Ltd., (China), whereas TAF (100% purity) was a generous gift from Gilead Sciences Inc. (CA, USA), under MTA agreement. Internal standards i.e. tenofovir-d6 (TFV-d6), TAF-d5, TFV-dp-d6 and dolutegravir-d4 (DTG-d4) were purchased from Toronto Research Chemicals Inc. (ON, Canada).

Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine medium, Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (HiDMEM) and 100 antibiotic-antimycotic (AA) were purchased from ThermoFisher Scientific (OK, USA), whereas fetal bovine serum (FBS) was from VWR International (PA, USA). All the chemicals were used as received.

Primary cells and cell lines

The TZM-bl cell line obtained from the National Institutes of Health (NIH) acquired immunodeficiency syndrome (AIDS) reagent program is a JC53-bl (clone 13)/HeLa cell line that are phenotypic similar to HIV-1 infecting cell type (stably overexpresses CD4 and CCR5 receptor). TZM-bl cells were maintained in complete DMEM medium (HiDMEM medium supplemented with 10% FBS and 1× AA), as standard protocol [13, 14]. Whereas, peripheral blood mononuclear cells (PBMCs) were purchased from AllCells® (Alameda, CA, USA) and maintained in complete RPMI (RPMI 1640 medium supplemented with 10% FBS, 1× AA and 50 U/ml IL-2 (Sigma-Aldrich; MO, USA)). The XF-CCR5 28/27 43E2AA hybridoma (xfR5 mAb producing cell line), was maintained in RPMI supplemented with 10% FBS and 1× AA [60].

HIV strain

HIV-1_ada virus obtained from the NIH AIDS research program and further propagated by the following published standardized method [61]. The TCID_{50} evaluated by standardized p24 ELISA assay using ZeptoMetrix® HIV Type 1 p24 Antigen ELISA kit (NY, USA) on PBMCs received from healthy donors and following the manufacturer's protocol.

Production, purification, and characterization of xfR5 mAb

The modified high affinity xfR5 mAb were produced and isolated from a Hybridoma cell line, i.e., XF27/28/CCR5 43E2-AA (PTA-4054; ATCC repository) [60], by following published method with modifications as described below [62]. Briefly, XF-CCR5 28/27 43E2AA hybridoma cells were seeded (at 10^6/mL concentration) and maintained in antibody production inducing media, i.e., RPMI medium with 1× AA, for several days until 50% cells were found to be compromised (cell death). The supernatant with soluble xfR5 mAb was harvested by pelleting out dead cells and debris. The soluble xfR5 mAb from the supernatant was isolated using HiTrap™ Protein-A HP prepacked column (GE Healthcare; NJ, USA) following standard manufacturer’s protocol. The purity and concentration of the xfR5 mAbs were determined respectively by the SDS-PAGE method and BCA assay using Pierce™ BCA Protein Assay Kit, following manufacturers’ protocol. The xfR5 binding was evaluated based on the standard curve (linear regression analysis) from a known concentration of IgG4 isotype control mAb, as xfR5 is recombinant human CCR5 mAb with IgG4 isotype backbone [60].

D+T NPs formulation, xfR5 mAbs conjugation, and characterization
The targeted nanoformulation was fabricated by following multiple steps. First, NHS functionalized D+T loaded nanoformulation was obtained by following the modified oil-in-water emulsions phase inversion method [13, 14]. Briefly, in the DCM organic phase PLGA, PLGA-PEG-NHS, PEG-PLGA, PF127 along with TAF and DTG have dissolved at 1:1:2:2:4:4 ratios, TAF (at comparative ratio 2) in PBS were added dropwise under constant stirring condition. The water-in-oil (w-o) emulsion was sonicated as described below and then added dropwise to the three times higher volume of 1% PVA solution (aqueous phase) under high-speed stirring conditions. The above w-o-w emulsion was immediately probe-sonicated for 5 mins on ice (setting: 90% Amplitude; pulse 0.9 cycle/bursts) with the help of UP100H ultrasonic processor (Hielscher Inc. Mount Holly, NJ, USA). The organic phase from the o-w emulsion was completely evaporated overnight (O/N). The NHS functionalized D+T NPs were desiccated by lyophilization using Millrock LD85 lyophilizer (Kingston, NY, USA) to eliminate the aqueous phase. The complete formulation method was carried out under the hood to maintain sterility during fabrication.

The NHS functionalized D+T NPs, xfR5 mAb was conjugated using its amine group to amine-reactive NHS esters of D+T NPs [63]. Briefly, NHS ester-D+T NPs were dissociated in PBS (pH 7.4) as the NHS esters (4-5 h half-life of at pH 7.4). The xfR5 mAb maintained in PBS to have protonated amine groups of xfR5 mAb. The xfR5 mAb were added to NHS ester-D+T NPs at 1:10 weight ratio under constant stirring at room temperature (RT), and the reaction proceeded for 2 hours at RT. Immediately, after the reaction, the unbound xfR5 mAb was washed off by dialysis using Float-A-Lyzer™ G2 Dialysis Devices (Thermo Fisher Scientific; NH, USA) in cold PBS supplemented with 10mM hydroxylamine to quench any non-reacted NHS groups present on the xfR5-D+T NPs surface. Followed by three consecutive cold PBS buffer-exchanges, xfR5-D+T NPs were collected and stored at 4°C. The concentration of xfR5 mAb conjugated on the xfR5-D+T NPs surface was evaluated by the BCA assay. To avoid background issues, PBS, as well as D+T NPs, were run parallel during BCA assay, and the obtained values were considered background.

The physicochemical properties of the D+T NPs were evaluated based on dynamic light scattering (DLS), fourier transform infrared (FT-IR) spectroscopy, and scanning electron microscopy. The size, surface charge, and polydispersity index (PDI) of the D+T NPs were determined by a ZetaPlus Zeta Potential Analyzer instrument (Brookhaven Instruments Corporation; NY, USA) following standardized methodology [13, 14]. The DLS analysis determined the D+T NPs size and polydispersity index (PDI), i.e., size homogeneity and size-distribution pattern. The zeta potential analysis identified the surface charge density on the D+T NPs. The D+T NP’s surface NHS functionalization and xfR5 mAb binding via amide bond were evaluated based on FT-IR spectroscopic analysis following a previously published method [64]. Briefly, the spectra of each sample in powder form were collected in the range 600–4000 cm⁻¹ using 25 scans at a resolution of 4 cm⁻¹ with % transmittance intensity mode and Happ-Genzel function apodization under IRPrestige-21 Fourier transform infrared spectrometer (FT-IR) instrument (Shimadzu; MD, USA) and by using LabSolutions IR software (Shimadzu; MD, USA) the data were analyzed. By scanning electron microscopy, the morphology and shape of the D+T NPs were evaluated [65]. Briefly, D+T NPs were deposited on Whatman® Nuclepore Track-Etch Membrane (~50 nm pore size) and air-dried for one day at RT under a chemical hood. The air-dried NPs membrane was sputter-coated with a thin layer (~3-5 nm thick) of chromium and imaged under a Hitachi S-4700 field-emission SEM (New York, NY, USA).

The % drug entrapment efficiency (%EE) of DTG and TAF in D+T NPs and xfR5-D+T NPs were evaluated by high-performance liquid chromatography (HPLC) instrument by following published methodology [38, 39, 66]. Briefly, 1 mg of D+T NPs dissociated in 50 µL DMSO and mobile phase (25mM KH₂PO₄ 45%; ACN 55%) added to get 10% DMSO final concentration in the injection volume (20 µL). For the standard curve evaluation, the same procedure was followed to prepare the D+T standard solutions (with each drug concentration from 0.5 to 0.0019 mg/mL). The chromatography separation was performed under a HPLC instrument (Shimadzu Scientific Instruments; MD, USA)
equipped with SIL-20AC auto-sampler, LC-20AB pumps, and SPD-20A UV/Visible detector, using Phenomenex® C-18 (150×4.6 mm, particle size 5 μm) column (Torrance, CA, USA), under isocratic elution process with 0.5 mL/min mobile phase flow rate, temperature: 25°C; and detection at 260 nm (retention time of 4 mins for TAF and 6.3 mins for DTG). The quantification of the drug was determined by evaluating the peak area under the curve (AUC) analysis at their respective retention time. The amount of TAF and DTG loaded in the D+T NPs was analyzed based on the standard curve construction (linear correlation, $r^2 \geq 0.99$) respective from TAF, and DTG standard concentration ranges from 0.5 mg/mL to 0.0019 mg/mL. The HPLC instrument illustrated inter-day and intra-day variability of <10%. The % encapsulation efficiency (%EE) of each drug in the D+T NPs batch was estimated by equation 1, respectively. The data presented as mean± standard error of the mean (SEM) of three D+T NPs batches (n=3).

$$\%\text{EE} = \frac{\text{Amount of drug entrapped in NPs (mg)}}{\text{Amount of drug used for encapsulation (mg)}} \times 100$$

Equation 1

**Antibody binding and binding affinity evaluation**

To establish and estimate the binding affinity of isolated xfR5 mAb and xfR5-D+T NPs in comparison to wild-type CCR5 mAb, Cy3 conjugated to xfR5 mAb by Cy3 NHS Ester Mono-Reactive CyDye (GE Healthcare; PA, USA), following manufacturer’s protocol. The Cy3 dye to xfR5 mAb binding ratio was evaluated based on regression analysis of respective standards (i.e., 0.5 to 0.00625 mg/mL) data obtained from UV/vis spectroscopy and BCA assay. The 3:1 dye to xfR5 mAb ratio Cy3 conjugated xfR5 mAb (Cy3-xfR5 mAb) batches considered for further studies. To study binding affinity by flow-cytometry, Cy3-xfR5 mAb conjugated D+T NPs (Cy3-xfR5-D+T NPs) formulation were fabricated and characterized, similarly as described for xfR5-D+T NPs. By using the standardized formulation, three independent batches were obtained and evaluated for further studies.

For binding affinity of Cy3-xfR5 mAb and Cy3-xfR5-D+T NPs, TZM-bl cells ($10^5$/well) and phytohemagglutinin (PHA, at 5 µg/mL) activated PBMCs (10^6 cells/well) were treated with wild-type Cy3-CCR5 mAb (rabbit anti-human mAb; Bioss Inc.; MA, USA), Cy3-xfR5 mAb and Cy3-xfR5-D+T NPs at different concentrations (20, 10, 1, 0.1, 0.1 µg/mL of xfR5 concentration) O/N at 37°C and 5% CO₂ atmosphere. Reportedly, PHA is known to induce reactivation of latent HIV-infected primary T-cells [42] consistently, therefore, to achieve latent HIV-infected primary T-cells phenotype (promote CD2+ T-cells), PHA-activated PBMCs were used. The treatment was washed-off by washing thrice with 1% BSA in PBS (PBA) solution by centrifugation (220 g at 4°C). As HIV-1 primarily infects CD4 T-cells, therefore, evaluate binding affinity of xfR5 mAb and xfR5-D+T NPs compared to wild type anti-CCR5 mAbs, all the above-treated cells were incubated with anti-CD4 AlexaFluor700 mAb (Table 5) for 20mins at RT (at 1:100 dilution) and washed thrice with PBA. Similarly, to evaluate binding affinity with the latent population (CD2+ T-cells) and monocytes (CD68+ T-cells), PBMCs were treated as described above. The treated cells incubated for 20 mins with anti-CD68 APC mAb and anti-CD2 Pacific Blue (Table 5). The above marker antibody bound treated cells were fixed for 20 min with 4% PFA at 4°C and washed again twice with PBA. The binding of Cy3-xfR5 mAb and Cy3-xfR5-D+T NPs to respective T-cell type was detected and evaluated respectively by the BD LSRII flow cytometer instrument (BD Biosciences; San Jose, CA, USA) and Flowjo software v10 (BD, Franklin Lakes, NJ, USA). The supplementary figure 1 details the complete gating strategies. Each experiment was performed on three healthy independent donors PBMCs. The binding affinity was calculated based on Michaelis-Menten’s non-linear fitting analysis of mean ± SEM (standard errors of means).

**Immunophenotype study**
Immunophenotype variation upon xfR5-D+T NPs treatment compared to xfR5 mAb in uninfected (mimicking PrEP condition) and HIV-1ADA-infected PHA-activated PBMCs was evaluated by flow cytometry. Briefly, PBMCs (10^5 cells/well) were treated respectively with xfR5 mAb and xfR5-D+T NPs (at 20 µg/mL of xfR5 concentration) for 96 h at 37°C and 5% CO_2 atmosphere. As the control and to compare activated PBMCs immunophenotype, PHA-activated PBMCs (10^5 cells/well) were maintained alongside for 96 h [67]. The immunophenotypic study during HIV challenge, the respective cells were treated on day 0. On day 1, treated cells were challenged with HIV-1ADA virus (MOI: 0.1) for 16 h, followed by wash-off and the cells were maintained in fresh medium for 4 days. For HIV-infection condition, the cells were infected on day 0 with HIV-1ADA virus as mentioned above, after washing off the PHA stimulation. After washing off HIV infection, cells were treated respectively as mentioned above for 4 days. On the respective days (day 0, day 1 and day 4), all cells were washed thrice with cold PBA solution by centrifugation (220xg at 4°C) and incubated with marker mAbs against T-lymphocytes (CD3), helper T-cells (CD4), cytotoxic T-cells (CD8), memory T-cells (CD45RO), transition T-cells (CCR7), activated T-cells (CD69), intermediate memory T-cells (CD27), and HIV latently infected T-cells (CD2) markers (as detailed in Table 5), for 20 mins at RT (at 1:100 dilution). The treated cells were washed with PBA, fixed for 20 min with 4% PFA at 4°C, and rewashed thrice with PBA. The immunophenotype of the marker treated cells were evaluated by flow cytometry. Three independent studies have been performed on three healthy donor's PBMCs. The data presented as mean ± SEM obtained from three independent donors.

**Intracellular kinetics Experiments**

The intracellular uptake and retention kinetics of D+T NPs and D+T solution were evaluated by LC-MS/MS analysis following a standardized method [13, 38, 68]. Briefly, TZM-bl cells (10^4 cells/well) seeded in the 24-well plate with the complete HiDMEM medium. Following O/N cell adherence, respective cells group were treated with D+T NPs and D+T solution at 10 µg/mL concentration of each drug, i.e., DTG and TAF. For uptake experiments, at respective time-points (i.e., 1, 6, 18, and 24 h), the treated cells were then washed twice with warm PBS and detached by Trypsin-EDTA (25%; Thermo Scientific, OK, USA), washed twice with PBS. One set of untreated detached cells were counted at each time-point to determine cell count at respective time-point. The cells were air-dried under a biosafety cabinet. The air-dried samples were then lysed with 70% methanol and stored at -80°C until analysis. Drug-retention experiments, the adhered TZM-bl cells were treated with xfR5-D+T NP and D+T solution, respectively, for 24 h and washed thrice with warm 1×PBS. The washed treated cells were in fresh complete HiDMEM medium until respective time-points (i.e., 1, 6, 24, and 72 h after wash, that corresponds to 25, 30, 48, and 96 h, time-point respectively after treatment). At these time-point, the cells were rewashed with PBS, detached, lysed, and stored following the same method as explained above. The samples were analyzed using the LC-MS/MS method described in the section below.

For the intracellular DTG, TAF, TFV, and TFV-dp drug-kinetics evaluation by LC-MS/MS instrument, the respective cell lysates were centrifuged (14000 rpm for 5 mins at 4°C) and the supernatant was collected. To an aliquot of 100 µL supernatant, 300 µL of internal standard spiking solution (10 ng/mL each of DTG-d4, TAF-d5, TFV-d6, and 100 nmol/mL of TFV-dp-d6 in ACN) was added, and vortexed. The samples were then dried at 45°C under the stream of nitrogen and reconstituted with 100 µL 50% acetonitrile. The drug and metabolites were quantified from the same sample using LC-MS/MS instrument.

For TAF, TFV, and DTG estimation, the similar conditions that were previously published by our group were used with minor modification [68]. One µL of the processed sample was injected on to LC-MS/MS operated in positive mode. The chromatographic separation was carried-out using the Restek Pinnacle DB Biph column (2.1 mm × 50 mm, 5
µm) with 0.5% formic acid in water and 0.1% formic acid in ACN (48:52 v/v) mobile phase. The calibration range for all the analytes was 0.01 to 50 ng.

For the quantification of TFV-dp, Phenomenex Kinetex C18 (75×4.6 mm, 2.6µm) column was used with an isocratic mobile phase (10mM ammonium acetate pH 10.5: ACN (70:30) at a flow rate of 0.25 mL/min. The dynamic calibration range was from 0.01 to 100 ng. The LC-MS/MS system consisting of an Exion HPLC system (Applied Biosystems, CA, USA) coupled with AB Sciex 5500 Q Trap with an electrospray ionization (ESI) source (Applied Biosystems, CA, USA) was used in positive ionization mode. The retention time of TFV-dp was 2.1 min, and the runtime for each sample was 3.5 min. The average inter-day and intra-day variability were < 15%, which corresponds to the FDA bioanalytical guidelines [69].

**In vitro cytotoxicity Experiments**

The comparative *in vitro* cytotoxicity of D+T NP vs. D+T solution was evaluated using TZM-bl cell line and CellTiter-Glo® luminescent assay method, as described previously [70]. Briefly, the TZM-bl cells (10^4 cells/well) in complete DMEM medium and PBMCs (10^5 cells/well) in complete RPMI medium, were treated in triplicate respectively with D+T NP or D+T solution, at different concentrations (20, 10, 1, 0.1, 0.01 µg/mL each drug concentration) for 96 h. Similarly, the 5% DMSO treated cells and 1×PBS (treatment equal volume) treated cells were the positive and negative control, respectively. The cytotoxicity was evaluated by the CellTiter-Glo® luminescent-cell viability assay kit (Promega; WI, USA) following manufacturer protocol. The luminescence intensity read from the Synergy II multi-mode reader with Gen5TM software (BioTek; VT, USA) and the percentage cytotoxicity (% cytotoxicity) values by subtracting the % normalized viability (against the untreated negative control group) from 100. The experiment was carried out on three independent batches of D+T NPs and D+T solution. The result represents the mean ± SEM of three independent batches studies. The untreated control cells were considered 100% viable. The % cytotoxicity was evaluated by following equation 2:

\[
\text{% Cytotoxicity} = \frac{\text{Treated}}{\text{Positive control}} \times 100
\]

**Equation 2**

**In vitro protection Experiments**

The comparative *in vitro* prophylaxis (PrEP), i.e., protection study between D+T NP vs. D+T solution against HIV-1\textsubscript{NL4-3}, was performed on TZM-bl cells, and peripheral blood mononuclear cells (PBMCs) was evaluated by following standardized method [13, 70]. Briefly, TZM-bl cells (10^4 cells/well) and PBMCs (10^5 cells/well) were seeded in 96-well plate and were treated with different concentrations of D+T (20, 10, 1, 0.1, 0.01 µg/mL each drug concentration) either as D+T NP or as D+T solution. Whereas untreated/uninfected cells and untreated/infected cells were considered negative and positive controls, respectively. After 24 h of treatment, the TZM-bl cells were infected with HIV-1\textsubscript{NL4-3} virus (multiplicity of infection, MOI: 1) for 8 h, whereas PBMCs received HIV-1\textsubscript{ADA} virus (MOI: 0.1) for 16 h. At respective time-point, the inoculated and control cells were washed with warm PBS (thrice). The TZM-bl cells and PBMCs were then maintained respectively in fresh complete HiDMEM medium and complete RPMI for 96 h. The HIV-1 infectivity was evaluated by the luminescence intensity following Steady-Glo® luciferase assay (Promega; WI, USA), following company specified methodology. The luminescence intensity based on relative luminescence units (RLU) due to HIV-1 infection, was read on the Synergy HT Multi-Mode Microplate Reader (BioTeck; VT, USA). The % HIV-1 infection was calculated by following equation 3:
For TZM-bl cells, data collected from three independent experiments performed with three different batches of D+T NP and D+T solution (each performed in duplicate). For PBMCs, the data obtained after treating three different healthy donor’s PBMCs (each performed in triplicate) at independent time. Finally, the selectivity index (SI), was evaluated by following equation 4:

$$SI = \frac{CC_{50}}{IC_{50}}$$  \hspace{1cm} \text{Equation 4}$$

Where, ‘CC_{50}’ (cytotoxic concentration at 50%) and ‘IC_{50}’ (50% inhibitory concentration), was evaluated from the above described in vitro cytotoxicity and protection study.

**Statistical Analysis**

All study results presented are expressed as mean ± SEM of the obtained data from at least three independent experiments. The CC\textsubscript{50} value was determined by non-regression curve fitting based on log (DTG or TAF concentration) vs. normalized luminescent (three-parameter logistic fits) of cytotoxicity response curves. Whereas, the IC\textsubscript{50} value was evaluated by fitting the non-regression inhibitory curve of log [DTG] vs. normalized TZM-bl luminescence (three-parameter logistic fits) luminance values. Analysis of variance (ANOVA) method was used to determine significant differences between treated (D+T NP and D+T solution) vs. control groups at p-value ≤ 0.05. All the statistical analysis presented was determined by GraphPad Prism 7 software (La Jolla, CA, USA).

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**Abbreviations**

CCR5 C-C motif chemokine receptor-5

HIV/AIDS Human immunodeficiency virus/Acquired Immunodeficiency Disease Syndrome

ARVs antiretrovirals

ARV antiretroviral

xfR5 mAb modified CCR5 monoclonal antibody

DTG dolutegravir

TAF tenofovir alafenamide

XfR5-D+T NPs DTG+TAF loaded nanoformulation

Th T-helper cell
TM temporary memory T-cell
E effector T-cell
EM effector memory T-cell
CM central memory T-cell
ART Antiretroviral therapy
pVL plasma HIV viral load
DCs dendritic cells
ZFN zinc finger nuclease
TALENs Transcription activator-like effector nuclease
RNA ribonucleic acid
CRISPR/Cas9 Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9
LA Long-acting
bNAbs broadly neutralizing antibodies
cART combination antiretroviral therapy
NRTIs nucleoside reverse transcriptase inhibitors
INSTIs integrase strand transfer inhibitors
CTL cytotoxic T lymphocytes
NHS N-hydroxysuccinimide
DLS dynamic light scattering
PBS phosphate-buffered saline
FT-IR Fourier-transform infrared spectroscopy
PLGA poly(lactic-co-glycolid acid)
PEG poly(ethylene glycol)
TZM-bl HeLa cell derivative engineered to contain Tat-responsive reporter gene for firefly luciferase
TFV tenofovir
TFV-dp tenofovir diphosphate
PBMCs peripheral blood mononuclear cells
SI selectivity index
PHA phytohemagglutinin
aCTL activated cytotoxic T-lymphocytes
DMEM Dulbecco's modified eagle medium
HiDMEM high glucose medium
AA antibiotic-antimycotic
FBS fetal bovine serum
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
BCA Bicinchoninic acid
DCM Dichloromethane
RPMI Roswell Park Memorial Institute
RT Room temperature
PDI Polydispersity index
%EE Percent entrapment efficiency
HPLC high performance liquid chromatography
DMSO dimethylsulfoxide
LC-MS/MS liquid chromatography tandem mass spectrometry
ACN acetonitrile
ESI electrospray ionization
PrEP preexposure prophylaxis
MOI multiplicity of infection
CC<sub>50</sub> cytotoxic concentration at 50%
IC<sub>50</sub> 50% inhibitory concentration

Declarations

Ethics and consent to participate: Not applicable
Consent to publish: Yes
Availability of data and materials: Upon request

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45. Desmetz C, Lin YL, Mettling C, Portalès P, Rabesandratana H, Clot J, Corbeau P: The strength of the chemotactic response to a CCR5 binding chemokine is determined by the level of cell surface CCR5 density. Immunology


**Tables**
Table 1
Physicochemical characteristics of xfR5-D + T NPs.

<table>
<thead>
<tr>
<th>Type</th>
<th>Size (nm)</th>
<th>Surface charge (mV)</th>
<th>Polydispersity Index (PDI)</th>
<th>%Drug entrapment efficiency (% EE)</th>
<th>xfR5 mAb bound per mg D + T NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS-D + T NP</td>
<td>198.7 ± 10</td>
<td>-28.15 ± 1.9</td>
<td>0.153 ± 0.008</td>
<td>DTG: 58.8 ± 10</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TAF: 60.5 ± 10</td>
<td></td>
</tr>
<tr>
<td>xfR5-D + T NP</td>
<td>212.6 ± 20.7</td>
<td>-17.77 ± 1.9</td>
<td>0.203 ± 0.0175</td>
<td>N/A</td>
<td>3.7 ± 0.52 µg</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM, obtained from three independent batches of NHS-D + T NPs or xfR5-D + T NPs (n = 3); N/A = not applicable

Table 2
Comparative binding affinity (K_d) of xfR5-D + T NPs vs. xfR5 mAb with TZM-bl cells (CD4 + CCR5 + cell line), primary CD4 + T-cells (T_h cells), and with other HIV-1 prone and CCR5 receptor-expressing immune cells (primary CD8+, CD68+, and CD2 + T-cells).

<table>
<thead>
<tr>
<th>Type</th>
<th>Binding Affinity (K_d, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TZM-bl cells</td>
</tr>
<tr>
<td>xfR5-D + T NPs</td>
<td>0.038 ± 0.020</td>
</tr>
<tr>
<td>xfR5 mAbs</td>
<td>0.251 ± 0.15</td>
</tr>
<tr>
<td>Wild-type anti-CCR5 mAb</td>
<td>2.023 ± 0.655</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM obtained from three healthy independent donors; ND = not determined.

Table 3
Comparative intracellular kinetics study of DTG, TAF, active drug (TFV) and its metabolite (TFV-dp), upon xfR5-D + T NPs or D + T solution treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>NP</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TAF</td>
<td>TFV</td>
</tr>
<tr>
<td>C_max</td>
<td>(pmole/10^6 cells)</td>
<td>18.1 ± 3.3</td>
<td>1250.2 ± 269.1</td>
</tr>
<tr>
<td>AUC_all</td>
<td>h* (pmole/10^6 cells)</td>
<td>825.4 ± 119.5</td>
<td>52384.8 ± 4613.2</td>
</tr>
<tr>
<td>t_1/2</td>
<td>h</td>
<td>75.5</td>
<td>21.6</td>
</tr>
</tbody>
</table>

C_max = maximum concentration; AUC_all = area under the curve; t_1/2 = half-life of the drug dose; Data presented as mean ± SEM of three independent experiments (n = 3), with each experiment with three repeats/variable.
Table 4
Comparative cytotoxicity (CC$_{50}$), protection (IC$_{50}$), and selectivity index (SI, CC$_{50}$/IC$_{50}$) study of xfR5-D + T NPs vs. xfR5 mAb on TZM-bl cells and primary CD4 + T-cells.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment Type</th>
<th>CC$_{50}$ (nM)</th>
<th>IC$_{50}$ (nM)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZM-bl</td>
<td>xfR5-D + T NPs</td>
<td>2910 ± 134.9</td>
<td>0.0352 ± 0.0086</td>
<td>82670</td>
</tr>
<tr>
<td></td>
<td>xfR5 NPs</td>
<td>998.7 ± 122.2</td>
<td>0.05471 ± 0.0103</td>
<td>18254</td>
</tr>
<tr>
<td></td>
<td>D + T NPs</td>
<td>3095 ± 102.3</td>
<td>0.195 ± 0.16</td>
<td>15872</td>
</tr>
<tr>
<td></td>
<td>xfR5 mAb</td>
<td>1464 ± 35.5</td>
<td>18.53 ± 2.85</td>
<td>79</td>
</tr>
<tr>
<td>PBMCs</td>
<td>xfR5 D + T NPs</td>
<td>690.6 ± 114.7</td>
<td>9.77 ± 2.03</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>xfR5 NPs</td>
<td>1428 ± 83.5</td>
<td>53.34 ± 2.34</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>D + T NPs</td>
<td>1009 ± 88.7</td>
<td>33.95 ± 1.91</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>xfR5 mAb</td>
<td>816.7 ± 93</td>
<td>115.9 ± 1.61</td>
<td>7</td>
</tr>
</tbody>
</table>

Each data represented as mean ± SEM (n = 3, healthy independent donors); CC$_{50}$ = 50% cytotoxic concentration; IC$_{50}$ = half-maximal inhibitory concentration; SI: Selectivity Index

Table 5
Detailed information about different T lymphocyte phenotypic markers used for the immunophenotypic study.

<table>
<thead>
<tr>
<th>T-lymphocyte target</th>
<th>Marker</th>
<th>Fluorescence dye conjugated</th>
<th>Monoclonal antibody type</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>T lymphocytes</td>
<td>CD3</td>
<td>Alexa Flour 594</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>helper T-cells</td>
<td>CD4</td>
<td>PerCP-Cy5.5</td>
<td>Mouse anti-human</td>
<td>TONBO biosciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alexa Flour 700</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Cytotoxic T-cells</td>
<td>CD8</td>
<td>PE/Cy7</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD68</td>
<td>APC</td>
<td>Mouse anti-human</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>HIV target on T-cell</td>
<td>xfR5</td>
<td>Cy3</td>
<td>Modified anti-human</td>
<td>ATCC</td>
</tr>
<tr>
<td>Transition T-cells</td>
<td>CCR7</td>
<td>CCR7-APC eFlour 780</td>
<td>Mouse anti-human</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Memory T-cells</td>
<td>CD45RO</td>
<td>Pacific Blue</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Activated T-cells</td>
<td>CD69</td>
<td>Alexa Flour 488</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>HIV maker on T-cells</td>
<td>CCR5</td>
<td>APC</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Intermediate memory T-cells</td>
<td>CD27</td>
<td>Alexa Flour 700</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
<tr>
<td>DC or latently infected T-cells</td>
<td>CD2</td>
<td>Pacific Blue</td>
<td>Mouse anti-human</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

Figures
Schematic diagram explaining the dual-action strategy of targeted cARV-nanoformulation. A) First level of protection: xfr5 D+T NP bound on the T-cell surface via CCR5 receptor (xfr5-D+T NP/CCR5 complex) blocks HIV interaction with CCR5 on T-cell surface by two approaches, i.e., first, by blocking HIV from CCR5 binding and the second, providing steric hindrance to HIV virions due to nanoformulation crowding on T-cell surface (red block arrows) [71]. B) The xfr5 D+T NP/CCR5 complex triggers the CCR5 trafficking pathway due to anti-CCR5 mAb binding with the CCR5+ T-cells [72], leading to internalization of xfr5 D+T NP. Within the endosome, the low pH induces degradation of polymeric NPs, leading to the release of DTG (INSTI) and TAF (NRTI) into the cytoplasm. The second level of protection: the presence of an intercellular pool of TFV di-phosphates and DTG to prevent (C) naive cell HIV infection. (D) The latently HIV+ cells, free intracellular high-affinity DTG (INSTI), will bind with fresh integrase enzymes produced during the reactivation stage, resulting in a non-functional INSTI/integrase complex. Therefore, fresh virons thus produced will be with non-functional INSTIs. Therefore, fresh viron will not be able to integrate viral DNA in the host genome.
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Figure 2

Representative scanning electron microscopic image of NHS-D+T NP (A) and xfR5 D+T NP (B). C) Representative FT-IR spectrum obtained from PEG-PLGA-NHS polymer, NHS functionalized NPs (NHS-Blank NPs), NHS functionalized D+T NPs (NHS-D+T NPs), xfR5 D+T NPs, and xfR5 mAbs. The significant functional group bands are presented in various color boxes (Orange: $-\text{CH wag and rocking vibration band (840-900 cm}^{-1})$; Purple: C-O-C stretching bands (1095-1198 cm$^{-1}$); Red: amide bond; Green: C=O stretching band (1695-1818 cm$^{-1}$); Red: $-\text{CON-}$ stretching based on reported FT-IR spectral studies [73, 74]. Inset graph enlargement image (C) shows NH- band shifting from 1450 cm$^{-1}$ to 1420 cm$^{-1}$ indicating COO-NC bond conversion to amide bond ($-\text{CONH-}$); and presence of Amide I and II at 1540 cm$^{-1}$ and 1640 cm$^{-1}$ respectively.
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**Figure 3**

T-cell differentiation phenotype under (A) untreated, (B) protection against HIV challenge (HIV prophylaxis), and (C), HIV infected T-cell treatment (HIV treatment) condition. A) The immunophenotypic differentiation (after day 1 and day 4) of untreated-nonactivated PBMCs were compared with PHA-activated PBMCs. B) Immunophenotyping
pattern evaluation after cell treatment followed by HIV challenge condition. The immunophenotypic differentiation of untreated PBMCs were compared to xfR5 mAbs and xfR5 D+T NPs were compared on day 1 (after 1 day of treatment) vs day 4 (after HIV-1ADA virus challenge). (C) Immunophenotyping pattern evaluation of HIV infected followed by treatment condition. The immunophenotypic differentiation of untreated+infected PBMCs were compared to infected PBMCs treated with xfR5 mAbs or xfR5 D+T NPs were compared on day 1 vs day 4 after 1 day of treatment. The differentiation pattern was evaluated following naïve, CM, TM, EM, and E sub-population as depicted in x-axis; whereas the y-axis represents respective marker % positive cells. The data presented as mean ± SEM of three independent experiments on three healthy donors (n=3). The significance was represented by the asterisk (*) symbol, where, '*', '**', '***' and '****' corresponding to P values <0.05, <0.01, <0.001 and <0.0001, respectively.
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Figure 4

The effect on effector (E T-cell) phenotype upon treatment (with xfR5-D+T NP and xfR5 mAbs) compared to untreated conditions during PrEP and HIV-1 infection. (A) Comparative effect on aCTL (CD8+ E T-cell) and Th (CD4+...
E T-cell) phenotype under unstimulated and stimulated (PHA-activated). (B) Under HIV challenge condition: aCTL (left graph) and Th (right graph) population on day 1 (black bar) and day 4 (gray bar). (C) The treatment effect in the presence of HIV-1 infection, on aCTL (left graph) and Th (right graph) on day 1, and day 4. Each data set is representing mean ± SEM of three independent experiments on PBMCs obtained from 3 healthy donors (n=3). The gating strategy for this study has been explained in Supplementary Figure 3. The significance represented as the asterisk (*) symbol, where, ‘*’, and ‘****’ corresponding to P values <0.05. and <0.0001, respectively.

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Supplementary Files

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- SupplementaryMaterialsccd.docx
- SupplementaryMaterialsccd.docx