RNase P Ribozyme Effectively Inhibits Human CC-Chemokine Receptor 5 Expression and Human Immunodeficiency Virus 1 Infection

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Simple Summary: Acquired immunodeficiency syndrome (AIDS), which is caused by human immunodeficiency virus (HIV), is a significant global public health concern. It is critical to generate new compounds and explore novel strategies against HIV infection to eliminate HIV-associated diseases such as AIDS. RNA enzymes, or ribozymes, represent a novel class of gene-targeting molecules with promising therapeutic potentials for the treatment of human diseases. In this report, we showed that an engineered ribozyme, called RNase P ribozyme, was able to shut down the expression of a cellular co-receptor for HIV and suppress HIV infection in human cells. Furthermore, our results suggest that the constructed anti-HIV ribozyme is highly specific, only inhibiting the expression of its target HIV co-receptor but not other co-receptors for HIV. These results demonstrate the utility of RNase P ribozyme as a gene-targeting agent for anti-HIV therapy and, furthermore, facilitate the development of new drugs and novel approaches for the treatment and prevention of HIV infection and AIDS.

Abstract: Developing novel antiviral agents and approaches is essential for the treatment against human and zoonotic viruses. We had previously produced RNase P-based ribozyme variants capable of efficiently cleaving mRNA in vitro. Here, engineered ribozymes were constructed from an RNase P ribozyme variant to target the mRNA encoding human CC-chemokine receptor 5 (CCR5), an HIV co-receptor. The constructed ribozyme efficiently cleaved the CCR5 mRNA in vitro. In cells expressing the engineered ribozyme, CCR5 expression diminished by more than 90% and the infection of HIV (R5 strain Ba-L) decreased by 200-fold. The ribozyme-expressing cells resistant to R5 strain Ba-L still supported the infection of HIV X4 strain IIIB due to its use of CXCR4 instead of CCR5 as the co-receptor. Thus, the ribozyme is specific against CCR5 but not CXCR4. This indicates that RNase P ribozyme is effective and specific against CCR5 to diminish HIV infection, and also displays the viability of developing engineered RNase P ribozymes against human and zoonotic viruses.

Keywords: RNase P; HIV; gene targeting; gene therapy; antisense

1. Introduction
Ribozymes derived from ribonuclease P (RNase P) catalytic RNAs are promising novel RNA-based gene interference agents [1,2]. By base-pairing with their mRNA target, RNase P-based ribozymes can cleave targeted mRNA substrates efficiently. For example, M1GS ribozyme was generated from catalytic M1 RNA of the RNase P holoenzyme in E. coli [1,2]. Previously, M1GS ribozymes were shown to be capable of cleaving the human cytomegalovirus (HCMV) mRNA sequence encoding the capsid scaffolding protein (CSP) in vitro [3]. We observed a noteworthy decline in HCMV CSP expression and viral replication in M1GS-expressing cells.

Novel therapeutic agents, including engineered ribozymes derived from hammerhead and hairpin ribozymes, have been tested for anti-HIV applications [4–6]. Few studies
have investigated the possibility of using RNase P ribozyme against human and zoonotic viruses including HIV. Macrophage tropic (M-tropic or R5) HIV strains (e.g., HIV Ba-L) use CC-chemokine receptor 5 (CCR5) as the foremost co-receptor of viral entry, while T cell tropic (T-tropic or X4) HIV strains (e.g., HIV IIIB) use CXCR4 as the major co-receptor [7,8]. Previous clinical [9,10] and gene targeting studies [11–14] indicated that even a partial reduction in CCR5 can yield benefits such as protection against HIV-1 infection or slower disease progression. As such, targeting CCR5 would be a promising avenue for the development of novel anti-HIV gene therapy.

In the present report, we engineered M1GS ribozymes to target CCR5 mRNA. Anti-CCR5 ribozymes were derived from an engineered M1 RNA previously chosen for cleaving mRNA substrates efficiently in vitro [15]. Our engineered ribozyme effectively diminished cellular CCR5 level and HIV infection. Our study also established the viability of developing RNase P ribozymes for gene-targeting anti-HIV treatment.

2. Materials and Methods
2.1. HIV and Cells
HIV and human cells used for HIV infection were either from Dr. Shibo Jiang or the NIH AIDS Research and Reference Reagents Program [16].

2.2. Synthesis of M1GS RNA and Its Substrate
Constructs for M1 RNA, and its mutants, V858 (with mutations A_{86} → G_{86} and G_{316} → U_{316}) and C102 (with mutations A_{347}C_{348} → C_{347}U_{348}, C_{353}C_{354}G_{356} → G_{353}G_{354}A_{355} U_{356}) [15,17], were used to obtain the DNAs for M1-Rb, A-Rb, and I-Rb, respectively, by PCR with primers AP5 (5′-GGAATTCTAATACGACTCACTATAG-3′) and Rb-CCR-3 (5′-CCCGCTCGAGAAAAAATGGTGAGCCCGCTCGAGGAAATGGAATTGTG-3′). The DNA coding for C-Rb that targeted HCMV capsid scaffolding protein was derived from V858 by PCR with primers AP5 and M1AP3 (5′-CCCGCTCGAGAAAAAATGGTGAGCCCGCTCGAGGAAATGGAATTGTG-3′). We obtained the ccr5-38 coding sequence by PCR with primers AP5 and sCCR5-38 (5′-CGGGATCCGAGGCAGGAGGCGGGCTGCGATTTGGCTTCTCTATAGTGAGTCGTATTA-3′).

2.3. Analysis In Vitro
Ribozymes and ccr5-38 were synthesized by T7 RNA polymerase. The cleavage of [\textsuperscript{32}P]-labeled ccr5-38 by ribozymes was performed in the cleavage solution (100 mM MgCl\textsubscript{2}, 100 mM NH\textsubscript{4}Cl, and 50 mM Tris, pH 7.5) and analyzed with a phosphorimager [15]. The k_{obs} and (k_{cat}/K_{m})\textsuperscript{s} values were assessed [15,17]. M1GS and ccr5-38 were mixed in the binding solution (0.1% bromophenol blue, 0.1% xylene cyanol, 100 mM NH\textsubscript{4}Cl, 3% glycerol, 50 mM Tris, pH 7.5, 100 mM CaCl\textsubscript{2}), and the K_{d} values were assessed as described previously [15].

2.4. Ribozyme and Human Gene Expression in Human Cells
DNAs for M1GS molecules inserted into LXSN vector and driven by the U6 RNA promoter were introduced to PA317 cells [18,19]. Human PM1 cells were infected with culture supernatants, collected from transfected PA317 cells and cloned under the selection of 800 µg/mL neomycin (Thermo Fisher, Waltham, MA, USA). The expression of ribozymes, and actin, CXCR4, and CCR5 mRNAs was studied by Northern blot analyses, while the protein levels of actin, CXCR4, and CCR5 were studied by Western blot analyses with antibodies, as described previously [15,20]. We studied ribozyme cytotoxicity by assessing cell viability for 90 days with an MTT assay (Sigma, St Louis, MO, USA) [21].

2.5. Studies of the Anti-HIV Effect
Parental PM1 cells and ribozyme-expressing cells were mixed with HIV (MOI = 0.05). We collected culture media at specified times. The culture supernatants were examined for p24 expression in a p24 ELISA assay. Intracellular HIV RNA were quantified with qRT-
PCR, amplifying the HIV tat sequence. Different RNA specimens were isolated at 48–72 h post-infection, treated with DNase, and then reverse-transcribed [22]. The synthesized cDNA was mixed with the qPCR reagents and SYBR Green (1:50,000), and primers 5′-TA-5′ (5′-CATCCAGGAAGTCAGCCT-3′) and 3′-TA-3′ (5′-TTCTGCCCAGGAGATGC-3′) [22]. The qPCR experiments were run in a Bio-Rad iCycler. Similarly, actin mRNA levels were examined by qRT-PCR, as noted previously [23].

2.6. Statistical Analysis

We performed experiments in triplicate and repeated them three times. Data were analyzed using GraphPad Prism software. We carried out statistical analyses with the analysis of variance (ANOVA). Differences with \( p < 0.05 \) were considered significant statistically.

3. Results

3.1. Cleaving CCR5 mRNA In Vitro by RNase P Ribozyme

The ribozymes were designed to target the CCR mRNA position 82 nucleotides downstream from its first AUG codon. This position may be exposed to ribozyme binding because of its proximity to the ribosome binding site and its accessibility for dimethyl sulphate modification in our experiments. Further sequence analyses revealed that this sequence shared no sequence homology [24,25] with those of other CC-chemokine receptors.

An active ribozyme, A-Rb, was constructed by linking to M1GS variant V-858 using a guide sequence complementary to the target CCR5 mRNA sequence. V-858 was a M1GS ribozyme variant selected to slice an mRNA efficiently in vitro, and contained two mutations (i.e., A\(_{86}\) → G\(_{86}\) and G\(_{316}\) → U\(_{316}\)) at the M1 RNA sequence [15]. Inactive ribozyme I-Rb was developed from A-Rb, containing several point mutations in the P4 active domain known to abolish M1 RNA catalytic activity [20]. The DNA sequences for these ribozymes were generated and used as templates for the in vitro synthesis of ribozymes A-Rb and I-Rb.

Kinetic analyses of the in vitro cleavage reaction of A-Rb and I-Rb with a 38-nucleotide-long CCR5 mRNA substrate, ccr5-38, revealed that A-Rb is highly active. Indeed, ribozyme A-Rb was 30 times better than M1-Rb, which was derived from the wildtype M1 RNA sequence, in cleaving the substrate ccr5-38 (Table 1). As expected, I-Rb was not catalytically active. However, I-Rb bound to substrate ccr5-38 as well as A-Rb because its binding affinity to ccr5-38, when assayed as the \( K_d \) values, was not different from that of A-Rb (Table 1). Thus, the antisense effect was represented by I-Rb in our experiments.

Table 1. Values of overall cleavage rate \([k_{cat}/K_m]^a\) and binding dissociation constant (\(K_d\)) in M1GS reactions with substrate ccr5-38. Experimental details are described in Materials and Methods.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>((k_{cat}/K_m)^a) (µM(^{-1})·min(^{-1}))</th>
<th>(K_d) (nM)</th>
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<tr>
<td>M1-Rb</td>
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<tr>
<td>A-Rb</td>
<td>6.5 ± 2.5</td>
<td>0.35 ± 0.09</td>
</tr>
<tr>
<td>I-Rb</td>
<td>&lt;5 × 10(^{-5})</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>C-Rb</td>
<td>&lt;5 × 10(^{-5})</td>
<td>ND</td>
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\(a\) ND = not determined.

A control ribozyme, C-Rb, was developed from A-Rb via linking ribozyme V-858, with a guide sequence aimed at the mRNA sequence coding for human cytomegalovirus (HCMV) capsid scaffolding protein. We observed no cleavage of substrate ccr5-38 by C-Rb in vitro (Table 1). Ribozyme C-Rb served as the control to assess whether the MGS ribozyme could affect the CCR5 mRNA with an incorrect guide sequence.

3.2. Anti-CCR5 Ribozyme Expression in Human Cells

DNAs for the M1GS ribozymes were inserted into the LXSN vector and downstream from the U6 RNA promoter [19,26,27]. Cell clones containing these vectors and with
ribozyme expression were derived from human PM1 cells [16], with ribozyme expression confirmed by Northern blot analyses using H1 RNA expression as a loading control (Figure 1). The constructed cell lines were identical in growth and viability for 90 days when compared to those introduced with only LXSN vector without M1GS sequence (Supplemental Figure S1), suggesting that RNase P ribozyme expression would induce no meaningful cytotoxicity.

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Figure 1. Northern blot analyses of RNase P ribozyme and H1 RNA expression in human cells. (A). We used total RNA samples (30 µg) extracted from PM1 cells with LXSN empty vector (P, lanes 1 and 5) and a cloned cell line producing C-Rb (lanes 2 and 6), I-Rb (lanes 3 and 7), and A-Rb (lanes 4 and 8). (B). Results are conveyed in % in comparison to those in PM1 cells producing C-Rb, and indicated as mean ± SD. NS, not significant. We performed experiments in triplicate and repeated them three times.

3.3. Ribozyme-Mediated Inhibition of Human CCR5 Expression

CCR5 and CXCR4 mRNA expression was measured by Northern blot analyses using actin mRNA as an internal control (Figure 2). CCR5 mRNA level decreased by 95 ± 5% (average of three experiments) in cells with active ribozyme A-Rb (Figure 2, lane 8). In contrast, the CCR5 mRNA level decreased by less than 10% in cells with inactive ribozyme I-Rb and control ribozyme C-Rb (Figure 2, lanes 6 and 7). Thus, the ribozyme-mediated cleavage of CCR5 mRNA resulted in a substantial reduction in CCR5 mRNA expression in A-Rb-expressing cells. CXCR4 mRNA expression exhibited no significant changes among all these cells (Figure 2).
A-Rb is specific against CCR5 but not CXCR4. Without ribozymes (P), and indicated as mean ± SD. ** p < 0.05. NS, not significant. We performed experiments in triplicate and repeated them three times.

Figure 2. Northern blot analyses of CCR5 (lanes 5–8), CXCR4 (lanes 9–12), and actin mRNA expression (lanes 1–4). (A). We used total RNA samples (40 µg) isolated from PM1 cells without ribozyme (P) and with C-Rb, I-Rb, and A-Rb. (B). Results are conveyed in % in comparison to the control, PM1 cells without ribozymes (P), and indicated as mean ± SD. ** p < 0.05. NS, not significant. We performed experiments in triplicate and repeated them three times.

CCR5 and CXCR4 protein levels were assessed by Western blot analysis with antibodies recognizing CCR5 and CXCR4 using actin protein as an internal control (Figure 3). The expression of CCR5 decreased by 94 ± 5% in cells that expressed A-Rb, and by less than 10% in cells that expressed I-Rb, and C-Rb. CXCR4 protein expression exhibited no significant changes among all these cells (Figure 3). These results suggest that ribozyme A-Rb is specific against CCR5 but not CXCR4.

Figure 3. Western blot analyses of CCR5 (lanes 5–8), CXCR4 (lanes 9–12), and actin protein expression (lanes 1–4). (A). We used protein samples (30 µg) isolated from PM1 cells without ribozyme (P) and with C-Rb, I-Rb, and A-Rb. (B). Results are conveyed in % in comparison to the control, PM1 cells without ribozymes (P), and indicated as mean ± SD. ** p < 0.05. NS, not significant. We performed experiments in triplicate and repeated them three times.
3.4. Anti-HIV Effects of M1GS in Cells

Decreased expression of CCR5 could lead to enhanced cellular protection against an M-tropic HIV strain (e.g., HIV_{Ba-L}) infection due to the usage of CCR5 as a co-receptor [28,29]. HIV_{Ba-L} was introduced to ribozyme-expressing cells with subsequent harvest. Experiments with qRT-PCR amplifying the HIV tat sequence were used to quantify intracellular HIV RNA, using actin mRNA expression as an internal control. The levels of total HIV-1 intracellular RNA decreased by 92% ± 7% in cells expressing A-Rb, and by less than 10% in I-Rb and C-Rb (Figure 4).

Progression quantification of HIV_{Ba-L} infection was assayed via viral p24 level present in the supernatant (Figure 5). HIV_{Ba-L} replication substantially diminished in cells expressing A-Rb but not in those with I-Rb or C-Rb during the infection. At 12 days post-infection, the levels of HIV_{Ba-L} p24 diminished by 200-fold in A-Rb expressing cells, while no reduction was found in those expressing I-Rb or C-Rb compared to PM1 cells with ribozymes (Figure 5). While I-Rb exhibited no catalytic activity, both it and A-Rb bound to the CCR5 mRNA sequence (Table 1). Thus, CCR5 mRNA cleavage by A-Rb in A-Rb-expressing cells resulted in a decrease in HIV infection.

3.5. Strain-Specific Anti-HIV Effects of M1GS Ribozymes

PM1 cells simultaneously express CCR5, a co-receptor for the M-tropic HIV strains (e.g., HIV_{Ba-L}), and CXCR4, a co-receptor for the T-tropic strains (e.g., HIV_{IIIB}) [16,28]. Due to this aspect, the presence of constructed ribozymes with a high specificity against CCR5 expression should not impede PM1 cells from supporting infection from T-tropic strains, since the ribozymes would not impact other proteins such as CXCR4. Here, we used T-tropic HIV_{IIIB} strain and M-tropic HIV_{Ba-L} strain to infect cells, examining HIV production via p24 levels in culture supernatants (Figure 6). On day 15 post-infection, A-Rb expressing cells exhibited a 200-fold decrease in p24 level when they were infected with HIV_{Ba-L} (Figure 5, Figure 6). However, these cells were susceptible to HIV_{IIIB} as we found no difference in HIV p24 levels in the IIIB-infected cells with A-Rb, I-Rb, and C-Rb (Figure 6). Thus, the A-Rb mediated inhibition of viral replication is specific because the anti-CCR5 ribozyme-expressing cells could support infection of the X4 strain IIIB despite protective measures against R5 strain Ba-L. Ribozyme-mediated cleavage of target mRNA appeared
to be critical for decreasing HIV_{Ba-L} infection, because both HIV_{Ba-L} and HIV_{IIIb} strains efficiently infected and replicated in cells expressing the “inactive” ribozyme I-Rb, which displayed similar binding affinity to CCR5 mRNA as A-Rb but had no cleavage events.

**Figure 5.** HIV_{Ba-L} growth in PM1 cells without (P) or with C-Rb, I-Rb, and A-Rb. 5 \times 10^5 cells were infected with HIV-1_{Ba-L} (MOI = 0.02–0.1). A p24 antigen assay was used to quantify HIV production. Results are indicated as mean ± SD. **p < 0.05. We performed experiments in triplicate and repeated them three times.

**Figure 6.** Supernatant HIV-1 p24 protein levels in cells having R5 strain HIV_{Ba-L} (A) or X4 strain HIV_{IIIb} at 15 days post-infection (B). A p24 antigen assay was used to quantify p24 expression in supernatants isolated from cultures of cells without ribozymes (P) or with C-Rb, I-Rb, and A-Rb. Results are indicated as mean ± SD. **p < 0.05. NS, not significant. We performed experiments in triplicate and repeated them three times.
4. Discussion

RNA-based gene interference strategies embody novel therapeutic agents for clinical applications [9,10,30–33]. The RNase P ribozyme used in our study is unique as it was derived from a variant of the catalytic M1 RNA [1,2]. This variant, V-858, was generated by in vitro selection of M1GS ribozyme mutants displaying efficient cleavage of target mRNA in vitro [15].

Here, M1GS ribozymes A-Rb and I-Rb that target cellular CCR5 mRNA were derived from variant V-858. Active ribozyme A-Rb efficiently cleaved CCR5 mRNA sequences in vitro with 30-fold greater efficiency than the wild-type ribozyme M1-Rb. V-858 has two point mutations (i.e., $A_{86} \rightarrow G_{86}$ and $G_{316} \rightarrow U_{316}$) in the M1 RNA sequence. How these mutations increase the ribozyme cleavage activity has not been studied. Further studies on these issues may offer insight into the mechanism facilitating the efficient cleavage by M1GS ribozymes against their target mRNAs.

In human cells, A-Rb induced decreases in both CCR5 mRNA and protein expression by more than 90%, and led to a more than 200-fold reduction in HIV infection. On the contrary, we only observed a minor CCR5 expression decrease and HIV infection inhibition in cells expressing the inactive ribozyme I-Rb or control ribozyme C-Rb. C-Rb targeted an unrelated mRNA, and I-Rb was able to bind to the CCR5 mRNA sequence similarly to A-Rb but was catalytically inactive. Thus, targeted cleavage by A-Rb results in CCR5 expression decrease and HIV infection inhibition, but not an antisense effect or other nonspecific effects of the ribozymes.

The results imply the RNase P ribozyme to be specific. To start, we observed no substantial distinction in cellular viability and growth between parental cells and ribozyme-expressing cells when they were allowed to grow for three months. Thus, ribozyme expression did not lead to substantial cytotoxicity. Second, the anti-HIV effect by ribozyme A-Rb was due to its induced CCR5 expression reduction. CCR5 expression and HIV infection decreased only in cells expressing A-Rb but not in those expressing I-Rb or C-Rb. There was a correlation between the decreased CCR5 levels and the extent of the HIV infection reduction. Third, ribozyme A-Rb only diminished CCR5 expression but had no effect on the expression of other targets including H1 RNA, actin, and CXCR4 (e.g., Figures 2 and 3). The A-Rb-expressing cells were also still able to support the infection of Tropic HIV IIIB strain despite resistance against infection of the M-tropic HIV Ba-L strain. Thus, the ribozyme specifically affects its mRNA target (i.e., CCR5) but not those of other targets (e.g., CXCR4).

Several potential issues may need to be addressed to develop M1GS RNAs for practical applications. First, M1GS ribozymes need to have specific delivery into target tissues and cell types. Efforts have been made to generate novel viral expression vectors such as lentiviral expression vectors. Furthermore, progress has been made to develop cell type- and tissue-specific expression systems. These studies should help to address the ribozyme delivery and expression in vivo. Second, more active ribozymes are needed to increase their cleavage and targeting activity. RNase P ribozyme variants with enhanced cleavage activity have been engineered by different methods including evolution in vitro approaches [1,2]. In this study, we showed that a ribozyme variant with two novel point mutations exhibited about 30-fold higher cleavage activity than that of the wildtype M1RNA sequence. The generation of M1GS ribozymes with better cleavage activity would enable the improvement of these gene-targeting molecules for applications.

Additional studies are needed to explore M1GS RNAs for anti-HIV therapy. For example, it is necessary to confirm if M1GS RNA (e.g., A-Rb) is also effective in reducing viral infection in primary cells known to be infected by HIV in vivo, such as the activated and resting CD4+ T cells [5,6]. We need to demonstrate if M1GS ribozyme is active in cleaving its target mRNA (i.e., CCR5 mRNA) and shutting down the CCR5 expression in these cells. Further efforts may also be needed to investigate how to deliver the ribozyme expression cassettes to these important HIV reservoir cells, and how to express M1GS
ribozymes at a high level in these cells. Lentiviral vectors may represent a promising class of vectors used for the delivery of MIGS into HIV reservoir cells.

Several clinical studies using ribozymes for anti-HIV therapy have been carried out using a gene therapy approach by expressing ribozymes via retroviral and lentiviral vectors in CD4+ cells or CD34+ hematopoietic stem cells, which differentiate into hematopoietic lineage cells including CD4+ T cells [33–36]. These studies indicated that the gene-delivered ribozymes are safe and feasible for anti-HIV therapy [37]. However, these studies encountered several obstacles including limited efficacy of the ribozymes and their suboptimal kinetics, low ribozyme expression due to chromatin silencing of the integrated expression cassettes, and poor engraftment of transduced cells [4–6]. Future challenges also include the minimization of immune toxicity and stimulation associated with ribozymes, and the large-scale manufacturing of these MIGS-containing lentiviral vectors to be used for investigating the anti-HIV activity of MIGS RNAs in clinical studies. Collectively, these studies would reveal whether MIGS RNAs can serve as anti-HIV therapeutics.

Anti-CCR5 RNA-based methods (e.g., ribozymes and RNAi) have led to CCR5 expression decrease and HIV infection reduction [11–14]. A potential issue associated with treatment against HIV infection is the resistant mutant emergence [38–40]. The anti-CCR5 RNase P ribozyme-based approach focuses on a cellular gene, targeting the entry step of HIV [41,42], and therefore should minimize the generation of viral mutations. Of note, an inhibition of p24 expression of about 200-fold may not be sufficient to abolish HIV infection completely in vivo. A combinatorial approach, such as the use of ribozymes and RNAi together, is a better anti-HIV strategy [9,10,43]. Moreover, better MIGS ribozymes with enhanced activity will be produced by genetic engineering and in vitro evolution process [44,45]. These studies will help to develop RNase P ribozymes for gene-targeting applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/zoonoticdis3020009/s1. Figure S1: Viability of the parental PM1 cells (PM1) and PM1 cells with empty LXSN vector (P) or PM1 cells expressing ribozymes C-Rb, I-Rb, and A-Rb after culturing for 90 days.

Author Contributions: Conceptualization, B.Y., Y.-C.C. and F.L.; methodology, B.Y., Y.L., Y.-C.C. and F.L.; validation, B.Y., Y.L., Y.-C.C. and F.L.; formal analysis, B.Y., Y.L., Y.-C.C., I.Z. and F.L.; investigation, B.Y., Y.L., Y.-C.C., I.Z. and F.L.; data curation, B.Y., Y.L., Y.-C.C., I.Z. and F.L.; writing, B.Y., Y.L., Y.-C.C., I.Z. and F.L.; supervision, F.L.; project administration, F.L.; funding acquisition, F.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.
13. Anderson, J.; Akkina, R. CXCR4 and CCR5 shRNA transgenic CD34+ cell derived macrophages are functionally normal and resist HIV-1 infection. *Retrovirology* 2005, 2, 53. [CrossRef]
22. Daffarian, P.M.; Kumar, A.; Kryworuchko, M.; Diaz-Mitoma, F. IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by tumor necrosis factor-alpha. *J. Immunol.* 1996, 157, 12–20. [CrossRef] [PubMed]
27. Scherer, L.J.; Rossi, J.J. Approaches for the sequence-specific knockdown of mRNA. *Nat. Biotechnol.* 2003, 21, 1457–1465. [CrossRef]
29. Wong-Staal, F.; Poeschl, E.M.; Looney, D.J. A controlled, Phase 1 clinical trial to evaluate the safety and effects in HIV-1 infected humans of autologous lymphocytes transduced with a ribozyme that cleaves HIV-1 RNA. *Hum. Gene Ther.* 1998, 9, 2407–2425. [CrossRef]


39. Yeni, P. Update on HAART in HIV. *J. Hepatol.* **2006**, *44*, S100–S103. [CrossRef]


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