Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9

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CCR5 serves as an essential coreceptor for human immunodeficiency virus type 1 (HIV-1) entry, and individuals with a CCR5Δ32 variant appear to be healthy, making CCR5 an attractive target for control of HIV-1 infection. The CRISPR/Cas9, which functions as a naturally existing adaptive immune system in prokaryotes, has been recently harnessed as a novel nuclease system for genome editing in mammalian cells. Although CRISPR/Cas9 can be readily delivered into cell lines, due to the large size of the Cas9 protein, efficient delivery of CCR5-targeting CRISPR/Cas9 components into primary cells, including CD4+ T-cells, the primary target for HIV-1 infection in vivo, remains a challenge. In the current study, following design of a panel of top-ranked single-guided RNAs (sgRNAs) targeting the ORF of CCR5, we demonstrate that CRISPR/Cas9 can efficiently mediate the editing of the CCR5 locus in cell lines, resulting in the knockout of CCR5 expression on the cell surface. Next-generation sequencing revealed that various mutations were introduced around the predicted cleavage site of CCR5. For each of the three most effective sgRNAs that we analysed, no significant off-target effects were detected at the 15 top-scoring potential sites. More importantly, by constructing chimeric Ad5F35 adenoviruses carrying CRISPR/Cas9 components, we efficiently transduced primary CD4+ T-lymphocytes and disrupted CCR5 expression, and the positively transduced cells were conferred with HIV-1 resistance. To our knowledge, this is the first study establishing HIV-1 resistance in primary CD4+ T-cells utilizing adenovirus-delivered CRISPR/Cas9.

INTRODUCTION

More than 33 years have elapsed (CDC, 1981) since the first reported case of AIDS. Although the introduction of effective antiretroviral therapy (ART) has resulted in huge reductions in rates of human immunodeficiency virus type 1 (HIV-1) illness, HIV-1 infection remains incurable. In addition, ART encounters a number of challenges, including a persistent latent viral reservoir, the requirement for lifelong adherence, and the potential development of drug resistance and toxicity (Chun et al., 2008; Corbeau & Reynes, 2011; Kent et al., 2013; Richman et al., 2009; Sendagire et al., 2009). Thus the development of novel therapeutic methods that may enhance current therapeutic options and even lead to curative approaches would significantly expand our portfolio of strategies to counteract HIV-1/AIDS.

Nuclease-mediated genome editing represents one promising strategy for HIV-1 therapy. Nucleases including zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) recognize genome locus based on protein–DNA interactions (Mak et al., 2012; Pavletich & Pabo, 1991). By construction of a tandem array of various artificially engineered modules, ZFN and TALEN can target virtually any DNA sequence (Boch et al., 2009;
Urnov et al., 2005). However, these two technologies to some extent present limitations and remain not only technically complex but also laborious and time-consuming to manipulate. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein 9 (Cas9), derived from a type II CRISPR/Cas system naturally existent in bacteria, has been harnessed as a novel nuclease tool to mediate genome editing in mammalian cells (Cong et al., 2013; Mali et al., 2013b). By simple delivery of two essential components, a human codon-optimized Cas9 protein and a single-guided RNA (sgRNA), the CRISPR/Cas9 complex formed can be programmed to target any genomic locus followed by a 5′-protos spacer adjacent motif (PAM) sequence of NGG, with the specificity determined by the sgRNA containing a 20 nt guide sequence complementary to the genome locus of interest (Cong et al., 2013; Mali et al., 2013b). Upon the guidance of sgRNA, Cas9 protein is programmed to cleave the targeted DNA at each strand, resulting in double-stranded breaks (DSBs) that trigger cellular repair mechanisms. In eukaryotes, the DSBs are more commonly repaired by the mechanism of error-prone non-homologous end joining (NHEJ), therefore generating sequence changes, for instance insertions and deletions (indels), around the DSBs (Kim & Kim, 2014). Owing to the simplicity of manipulation and versatility, the CRISPR/Cas9 system has been utilized as an attractive tool for various applications, such as genome-wide screening (Shalem et al., 2014; Zhou et al., 2014), gene repression and activation (Cheng et al., 2013; Doench et al., 2014; Gilbert et al., 2014), targeted fluorescence imaging (Tanenbaum et al., 2014) and novel approaches against pathogens including hepatitis B virus (Lin et al., 2014a; Seeger & Sohn, 2014), human papillomavirus (Kennedy et al., 2014), Epstein-Barr virus (Wang & Quake, 2014; Yuen et al., 2015), malaria (Wagner et al., 2014) and HIV-1 (Ebina et al., 2013; Hu et al., 2014; Ye et al., 2014).

Using a pair of sgRNAs targeting the LTR of HIV-1, it was shown that HIV-1 provirus can be removed from the genome of infected cell lines (Ebina et al., 2013; Hu et al., 2014). By combining TALEN or CRISPR/Cas9 with PiggyBac technology, researches have generated induced pluripotent stem cells (iPSC) homozygous for the naturally occurring CCR5<sup>Δ32</sup> variant resistant to HIV-1 infection (Ye et al., 2014). However, owing to the large size of the Cas9 coding sequence with a total length of more than 5 kb when combining with sgRNA, promoters and other essential elements for efficient expression, delivery of CRISPR/Cas9 components into primary cells, for instance, CD4<sup>+</sup> T-lymphocytes, the primary target for HIV-1 infection in vivo, remains challenging. In the current study, after optimized design and screening of a panel of sgRNAs in cell lines, we obtained several sgRNA sequences with high potency to target CCR5. By utilizing a chimeric adenoviral vector, we demonstrated the efficacy of CCR5 editing and established HIV-1 resistance in primary CD4<sup>+</sup> T-cells.

RESULTS

Design of sgRNA guide sequences

The ORF of HIV-1 coreceptor CCR5 is located on the fourth or last exon of the CCR5 gene, which maps to the short arm of chromosome 3 at position 21.31 (3p21.31), with base pair range from 46 370 854 to 46 376 206 of the forward strand based on GRCh38 coordinates (Fig. 1). The sgRNA was designed at the online platform http://crispr.mit.edu/ (Hsu et al., 2013). Based on the presence of a PAM with a 5′-NGG sequence, a total of 124 possible sgRNAs can be designed to target the 1059 bp CCR5 ORF or its complementary strand (Table S1, available in the online Supplementary Material). CRISPR/Cas9 was found to tolerate mismatches (MMs) between the sgRNA sequence and recognition site and the properties of MMs, in particular the number and location of MMs, were shown to affect off-target effects (OTEs) (Hsu et al., 2013; Kucscu et al., 2014). Therefore, we further selected sgRNAs among the 20 top-ranked sgRNAs of the 124 candidates by applying the following rules: (1) MMs ≥ 2 bp; (2) for MMs = 2 bp, both locate in the PAM-proximal half; (3) for MMs = 3 bp, at least 1 bp locates in the PAM-proximal half; and (4) for overlapping sgRNAs with only 1 bp difference, choose the sgRNA with the highest score. These rules were set up artificially, mainly on the principle of increasing MMs, particularly MMs in the PAM-proximal half of the guide sequence, and diversity of the targeting region of our designed sgRNAs. As a result, eight top-ranked sgRNAs, designated sgR5-3 to -10, were generated (Fig. 1, Table S2). Two previously reported sgRNAs targeting CCR5 (sgR5-1 and -2) (Cradick et al., 2013) and a non-targeting sgRNA (sgNeg) (Shalem et al., 2014) were employed as positive and negative controls, respectively.

Efficient editing of the CCR5 locus by the RNA-guided Cas9 nuclease

sgRNA-guided Cas9 cleaves target DNA at both strands and generates DSBs which commonly trigger NHEJ and result in indels (Cong et al., 2013; Mali et al., 2013). We generated new sgRNAs using a T7 endonuclease I (T7EI) mutation detection assay. HeLa-derived TZM-bl cells were infected with LentiCRISPR expressing Cas9 and sgRNA, followed by extraction of genomic DNA and amplification of a 1228 bp fragment encompassing the CCR5 ORF (Table S3). T7EI analysis of the amplicons showed that all 10 sgRNAs effectively guided CCR5 editing with various efficacies, while the scrambled sgRNA (sgNeg) guided no detectable editing. The top two effective sgRNAs, sgR5-5 and -8, resulted in 74.1 % and 63.8 % indels in the CCR5 allele, respectively. In contrast, the least effective sgRNAs, sgR5-9 and -7, generated 27.1 % and 30.8 % indels, respectively (Fig. 2a). Of interest, the sgRNAs exhibiting higher editing efficacy mainly locate in the region from nt 254 to 315 of the CCR5 ORF. However, using a
chromatin immunoprecipitation (ChIP) assay, we did not find a significant difference in terms of binding affinity between sgR5-5 and sgR5-7, of which one targets the region between nt 254 and 315 of the CCR5 ORF and the other targets outside this region (Fig. S1).

To determine the detailed sequence changes resulting from CRISPR/Cas9-mediated gene editing, we amplified the genomic segment encompassing the predicted cleavage site and sequenced the amplicons by deep sequencing. Results showed that diverse changes were generated (Figs 2b and S2), consistent with previous findings (Cong et al., 2013). For sgR5-5, various mutations from a 1 bp insertion to a 51 bp deletion were frequently read out. The mutation with the highest reads number was a 16 bp deletion at the predicted cleavage site (Fig. 2b). Similar results were observed for sgR5-8, while the mutation with the most reads number was an 11 bp deletion at the predicted cleavage site (Fig. S2). The above results collectively

**Fig. 1.** Diagram of the composition and genomic location of CCR5 and the sgRNA sequences designed. The genomic location of the CCR5 gene maps to the short arm of chromosome 3, with a base pair range from 46 370 854 to 46 376 206. The four exons (magenta boxes) and two introns (magenta lines) of the CCR5 gene are shown. The ORF of CCR5 is located in the fourth exon. Blue bars represent sgRNA sequences designed to target specific sites of the CCR5 ORF, as shown by nucleotide sequences. Orange bars, corresponding to the red NGG sequences in the CCR5 ORF, denote the PAM region. The ORF is numbered starting from the start codon.
Fig. 2. Efficacy of on-target editing of the CCR5 locus by sgRNA-guided Cas9 nuclease. (a) Analysis of gene editing efficacy in TZM-bl cells by T7EI cleavage assay. Following transduction of TZM-bl cells with LentiCRISPR expressing Cas9 and various sgRNAs targeting CCR5 (sgR5-1 to -10) or non-targeting sgRNA (sgNeg) (m.o.i. = 1), the genomic DNA of the positively transduced cells was extracted and used as template to amplify a 1228 bp fragment encompassing the CCR5 ORF. The amplicons were purified, denatured and self-annealed, followed by T7EI digestion and agarose gel analysis. Mock, the sample from untreated cells. One representative out of three independent experiments is shown. (b) Alignment of the sgR5-5-edited sequences from deep sequencing. The amplicons derived from the genomic DNA of sgR5-5-edited cells were appended with a barcode and subjected to deep sequencing by using the Illumina MiSeq system. Acquired sequences with high reads number (top 32) were aligned and are shown. The reads numbers of different sequences were ranked and are indicated by the background colour scale, with red and light pink representing the highest and the lowest reads number, respectively.
demonstrated that CRISPR/Cas9 is an efficient tool to edit the CCR5 locus.

To determine the specificity of our designed sgRNAs, we measured OTEs of sgR5-5, -6 and -8, which exhibited the highest efficacy of on-target editing. For each of the three sgRNAs, the 15 top-ranked off-target sites in the whole human genome were analysed (Tables S3 and S4). Approximate 500 bp segments spanning the potential off-target sites were amplified from genomic DNA of TZM-bl cells and subjected to T7EI analysis. No significant cleavage was detected for all of these potential off-target sites (Fig. S3). Furthermore, because CCR2 shares a high extent of sequence homology with CCR5, we tested OTEs of the three sgRNAs in CCR2. For sgR5-6, the off-target sites in CCR2 were the same as OTE-56-15 (Fig. S3, Tables S3, S4 and S5). For sgR5-5 and -8, we observed no significant cleavage at their homologous regions in CCR2 (Fig. S4, Table S5), demonstrating high specificity of the Cas9 nuclease directed by the three sgRNAs.

HIV-1 resistance in susceptible cell lines conferred by disruption of CCR5 expression

Next, we measured CCR5 protein expression on the cell surface following CRISPR/Cas9-mediated gene editing. Consistent with the T7EI cleavage assay, CCR5 expression on TZM-bl cells assayed by flow cytometry (FCM) was found to be correspondingly disrupted by the panel of sgRNAs (Fig. 3a). Maximum disruption was observed on cells edited by sgR5-5 and -8, which exhibited the most potent capability in mediating CCR5 cleavage. To determine the cell-type dependence of these results, we further measured CCR5 disruption on CHO-CCR5, a CHO-K1 derivative with stable CCR5 expression (Hu et al., 2005), and C8166-CCR5 cells, a human T-lymphocyte cell line supporting HIV-1 growth (Soda et al., 1999). Similarly, CCR5 expression on CHO-CCR5 and C8166-CCR5 cells were abrogated by sgR5-5 and 8 to a similar extent as that observed on TZM-bl cells (Fig. 3a).

We then confirmed if disruption of coreceptor CCR5 expression by CRISPR/Cas9 conferred HIV-1 resistance. TZM-bl cells edited by sgR5-5 and 8 were subjected to HIV-1 BaL (R5-tropic) infection, followed by luciferase reporter assay to quantify the infection. Results revealed that BaL infection in these cells was largely decreased compared with that in cells edited by sgNeg or without editing (Fig. 3b), consistent with the indispensable role of CCR5 in R-tropic HIV-1 infection (Alkhatif et al., 1996). As expected, infection with NL4-3, an X4-tropic HIV-1, was not affected whether the cells were CCR5 edited or not (Fig. 3b).

Delivery of CRISPR/Cas9 system by a chimeric Ad5F35 adenoviral vector

HIV-1 mainly targets CD4+ T-cells in vivo. Although the lentiviral vector transduces T-cells with high efficacy, the capacity of lentivirus is relatively small, resulting in a low titre of lentivirus carrying the large CRISPR/Cas9 insert. In addition, the integrative property of the lentiviral vector is of critical concern in therapeutic applications. We thus sought to deliver the CRISPR/Cas9 components into primary CD4+ T-cells using a chimeric Ad5F35 adenoviral vector, which has been shown to transduce CD34+ haematopoietic cells with high efficacy (Gaggar et al., 2003; Shayakhmetov et al., 2000). We constructed the recombinant adenoviral vectors pAd5F35.Cas9.sgR5-5/8/sgNeg.EGFP (designated pAd5F35.gCas9) carrying the 5.9 kb CRISPR/Cas9 and an EGFP reporter (Fig. 4a, Table S6). The 35.2 kb genome size of pAd5F35.gCas9 was smaller than the theoretical packaging capacity of E1E3-deleted adenoviral vectors (Bett et al., 1994). Recombinant Ad5F35.Cas9 virus was rescued by transient transfection of HEK293 cells. Apparent cytopathic effects were observed at 10–16 days post-transfection (Fig. 4b). Purified viral stocks with high titre (up to 1013 infectious viral particles ml⁻¹) were obtained by CsCl banding following several rounds of amplification in HEK293 cells. Following infection of HEK293 cells with the purified Ad5F35.Cas9 viral stocks, Cas9 protein expression was detected (Fig. 4c), confirming that the large CRISPR/Cas9 components can be successfully packaged by Ad5F35 vector. TZM-bl cells transduced with Ad5F35.gCas9 exhibited significant CCR5 editing, while cells transduced with Ad5F35.EGFP (Ad5F35 carrying EGFP alone) or Ad5F35.gCas9 showed no editing (Fig. 4d), validating the functionality of the recombinant viral stocks. Notably, our results showed that the efficacy of editing varied between adenovirus- and lentivirus-delivered CRISPR/Cas9 (Figs 2a and 4d). This most likely resulted from the differences in transduction m.o.i., integration property and antibiotic selection of these two approaches.

Gene editing of CCR5 in primary CD4+ T-cells by Ad5F35-delivered CRISPR/Cas9

We subsequently investigated the editing efficacy of the Ad5F35 viruses produced on CD4+ T-cells. Primary CD4+ T-cells were activated with Phytohaemagglutinin (PHA) for 24 h and then exposed to Ad5F35 viruses. The m.o.i. was set as 30 and 100 according to previous reports (Perez et al., 2008; Shayakhmetov et al., 2000) and under these conditions no significant cytotoxic effect was observed by trypan blue staining. Transduction efficiency was monitored by GFP expression, which appeared as early as 24 h post-transduction (p.t.), peaked at 4 days p.t. on average and remained nearly constant for at least 10 days p.t. At peak level, CD4+ T-cells transduced with Ad5F35.Cas9 at an m.o.i. of 30 or 100 exhibited 24.6% (n=9) or 30.3% (n=3) of GFP expressing cells correspondingly, while 45.3% (n=3) of cells exposed to m.o.i. 30 of Ad5F35.EGFP were GFP-positive (Fig. 5a), comparable with previous reports (Shayakhmetov et al., 2000). Gene editing of CCR5 in the total cells exposed to Ad5F35.gCas9 was determined at various time points p.t. and the maximum level of editing was achieved at approximate 8 days p.t. (Fig. S5). Therefore, we further purified the positively transduced (GFP+) cells...
at least 8 days p.t. by FCM sorting and analysed CCR5 editing. Results showed that for either sgR5-5 or -8, more than 30% indels were generated in the CCR5 allele derived from the GFP+ cells, while no obvious indels were generated by the Ad5F35.g5/8Cas9 (Fig. 5b). Correspondingly, cell surface CCR5 expression was also significantly disrupted by Ad5F35.g5/8Cas9 (Fig. 5c), but not Ad5F35.gNCas9 or Ad5F35.EGFP. We further assayed potential OTEs in CD4+ T-cells. Utilizing the same approach as in TZM-bl cells, we tested the 15 top-ranked potential off-target sites for both Ad5F35.g5Cas9 and Ad5F35.g8Cas9 (m.o.i. 30) and observed no significant OTEs.

Fig. 3. Conferred HIV-1 resistance by disruption of CCR5 expression in susceptible cell lines. (a) Followed by transduction with lentivirus expressing Cas9 and various sgRNAs targeting CCR5 or sgNeg, TZM-bl, CHO-CCR5 and C8166-CCR5 cells were stained with anti-CCR5-PE (clone 2D7) and assayed for CCR5 expression by FCM. (b) HIV-1 infection of TZM-bl cells following disruption of CCR5 expression. TZM-bl cells edited by sgR5-5-, sgR5-8- or sgNeg-guided Cas9, or without editing were infected with 2 ng or 10 ng p24 of HIV-1 BaL (R5-tropic, left) or NL4-3 (X4-tropic, right). Virus infection was determined by luciferase activity assay and is presented as percentage of infection. HIV-1 infection of TZM-bl cells without CCR5 editing was set as 100%. One representative out of three independent experiments is shown.
Inhibition of HIV-1 infection of primary CD4⁺ T-cells by Ad5F35-delivered CRISPR/Cas9

To test if CD4⁺ T-cells with CCR5 edited by CRISPR/Cas9 gained HIV-1 resistance, the positively transduced CD4⁺ T-cells at least 8 days post-transduction were sorted by FCM as described above and exposed to HIV-1 infection. Virus infection was determined by measurement of p24 in the culture supernatants. Results showed that at 2 ng p24 inoculum of HIV-1 BaL but not X4-tropic NL4-3, infection of CD4⁺ T-cells transduced with Ad5F35.g⁵/⁸Cas9 was markedly decreased compared with that of cells transduced with Ad5F35.g⁸Cas9 or Ad5F35.EGFP (Figs 6a and S6). When virus inoculum was increased to 10 ng of p24, comparable inhibition was detected at 7 days post-infection (Fig. 6b). Transmitted/founder (T/F) HIV-1 strains isolated very early after transmission are thought to be more physiologically relevant than laboratory-adapted strains (Keele et al., 2008). We examined if CRISPR/Cas9-mediated disruption

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of CCR5 also affected CH042, a T/F strain infection. Similar results were obtained (Fig. 6c), confirming the indispensable role of CCR5 for R5 HIV-1 infection.

DISCUSSION

Although ART is one of the most prominent achievements in HIV-1/AIDS research, AIDS remains an incurable disease (Lewin, 2013; Richman et al., 2009). Gene therapy represents one alternative approach for treatment of HIV-1 infection (DiGiusto et al., 2013; Herrera-Carrillo & Berkhout, 2015; Tebas et al., 2014). Here, we demonstrated that CRISPR/Cas9 could efficiently cut the CCR5 locus, resulting in various sequence changes and disrupted CCR5 expression. At the same time, the high on-target efficacy was not observed to be associated with obvious OTEs. Importantly, we efficiently delivered the CRISPR/Cas9 components into primary CD4+ T-cells using recombinant adenoviral vectors, which inhibited HIV-1 infection in the positively transduced cells following CCR5 disruption.

Owing to the indispensable role of CCR5 for HIV-1 entry and the natural presence of the CCR5Δ32 variant, a number of gene editing studies have targeted CCR5 by various approaches (Badia et al., 2014; Holt et al., 2010; Huang et al., 1996; Mussolino et al., 2014; Perez et al., 2008; Saito et al., 2014; Ye et al., 2014). In a recently finished Phase I clinical trial, autologous CD4+ T-cells with CCR5 modified by ZFN showed promise in alleviating the disease condition of some HIV-1 patients (Tebas et al., 2014). However, genome editing utilizing early developed nucleases, including ZFN and TALEN, remains technically challenging, and the broad application of these technologies is consequently somewhat limited. The prokaryote-derived CRISPR/Cas9 system is a robust genome editing tool with versatile applications (Mali et al., 2013a).

Fig. 5. Transduction and gene editing efficacy of Ad5F35 adenoviruses in primary CD4+ T-cells. (a) Transduction efficacy of Ad5F35 in primary CD4+ T-cells. CD4+ T-cells were stimulated with PHA and mock transduced or transduced with Ad5F35.EGFP (m.o.i. 30) or Ad5F35.gCas9 (m.o.i. 30 or 100). The dot plots shown are EGFP expression assayed by FCM at 8 days p.t. (b) Gene editing efficacy of the CCR5 locus in the positively transduced (GFP+) CD4+ T-cells. The GFP+ cells, transduced with various Ad5F35 (m.o.i. 30), were obtained by FCM sorting followed by T7EI cleavage assay as described in the legend of Fig. 2(a). (c) CCR5 expression on GFP+ CD4+ T-cells. At 8 days p.t., CD4+ T-cells transduced with various Ad5F35 (m.o.i. 30) were stained with anti-CCR5-PE and subjected to FCM assay. Data shown are one representative out of three independent experiments using CD4+ T-cells derived from different donors.
CCR5 editing by adenovirus-delivered CRISPR/Cas9

CCR5. However, the efficacy varied markedly for various sgRNAs, consistent with previous findings that the efficacy of editing is mainly determined by the guide sequence (Cong et al., 2013). One interesting finding is that, compared with other regions of the CCR5 ORF, there was a trend for sgRNAs targeting the region from nt 254 to 315 to exhibit higher efficacies of gene editing. Of interest, our ChIP results suggest that, at least for the sgRNAs tested, such higher efficacies are unlikely due to the difference of binding affinity between sgRNAs targeting this region and those targeting other regions. The underlying mechanisms and whether other genes also possess such hotspots for CRISPR/Cas9 targeting remains to be explored in future studies.

The sgRNA/Cas9 complex recognizes target sites based on the rule of Watson–Crick base pairing, conferring overall high specificity of the CRISPR/Cas9 system (Hsu et al., 2013; Smith et al., 2014; Veres et al., 2014). However, CRISPR/Cas9 was found to tolerate MMs between its sgRNA and target sites (Duan et al., 2014; Kuscu et al., 2014; Lin et al., 2014b). We selected the sgRNAs according to the guidelines of increasing the number and position of

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**Fig. 6.** HIV-1 infection of primary CD4+ T-cells following CRISPR/Cas9-mediated disruption of CCR5. (a) HIV-1 BaL infection of Ad5F35-positively transduced CD4+ T-cells. PHA-stimulated CD4+ T-cells were mock transduced or transduced with various Ad5F35 adenoviruses (m.o.i. 30). The positively transduced GFP+ cells were separated by FCM sorting and infected with HIV-1 BaL (2 ng p24 of virus). Half of the culture medium was collected at the indicated number of days post-infection (p.i.), followed by measurement of p24 content. (b, c) HIV-1 BaL (b) and CH042 (c) infection of Ad5F35-positively transduced CD4+ T-cells. The experiment was carried out as in (a), except that two inocula of 2 ng and 10 ng p24 of HIV-1 were conducted and p24 concentration at 7 days p.i. is shown. Each treatment was conducted in sextuplicate. Data shown (mean ± SD) are representative of three independent experiments using CD4+ T-cells derived from different donors.
MMs, particularly MMs locating at the PAM-proximal half of the sgRNAs which were shown to be critical for determining OTEs (Hsu *et al.*, 2013). Among the total 45 top-ranked off-target sites (15 for each of the three most potent sgRNAs) tested, we observed no significant OTEs, confirming the specificity of the CRISPR/Cas9 system. However, due to the limited sites we analysed and the restricted sensitivity of the T7EI cleavage assay, lower incidences of OTEs for these sgRNAs at a genome-wide scale remain to be determined by more powerful approaches, such as ultra-deep next-generation sequencing of the whole genome (Hu *et al.*, 2014; Smith *et al.*, 2014; Veres *et al.*, 2014). Studies aimed to develop modified CRISPR/Cas9 systems are also promising to further improve the on-target fidelity of CRISPR/Cas9 (Guillinger *et al.*, 2014; Qi *et al.*, 2013; Ran *et al.*, 2013).

Generally, delivery of CRISPR/Cas9 components into cell lines can be readily achieved. During the process of this manuscript submission, by delivery of CRISPR/Cas9 utilizing an integrative lentiviral vector, Wang *et al.* (2014) reported that targeting of CCR5 rendered CEMss-CCR5, a CD4 \(^+\) T-cell line, resistant to HIV-1 infection. However, efficient delivery of the large CRISPR/Cas9 components into primary cells remains challenging. Another recent report utilized the approach of nucleofection to deliver sgRNA and Cas9 into haematopoietic stem cell and primary CD4 \(^+\) T-cells (Mandal *et al.*, 2014). Although nucleofection is a direct delivery approach, its efficacy and toxicity effects on primary cells restrict its application. We therefore sought to utilize relatively safe viral vectors, which are widely adopted by gene therapy studies (Kootstra & Verma, 2003; Tebas *et al.*, 2014). Considering that (1) only short-term expression is desired for the nuclease to play its role (Cong *et al.*, 2013; Mali *et al.*, 2013b); (2) high capacity is required to carry the large CRISPR/Cas9 components; (3) side effects such as oncogenic potential should be avoided; and (4) tropism for haematopoietic cell lineage is needed to efficiently transduce HIV-1 target cells, we utilized the chimeric adenoviral vector Ad5F35 as the vehicle to deliver the CRISPR/Cas9 components. Our results demonstrated that CRISPR/Cas9 components reaching a length of 5.9 kb when combining with a GFP reporter gene can be successfully packaged into the recombinant Ad5F35 adenovirus, confirming the large capacity of adenoviral vectors (Bett *et al.*, 1994; Cheng *et al.*, 2014; Maggio *et al.*, 2014). Ad5F35 carrying CRISPR/Cas9 efficiently transduced primary CD4 \(^+\) T-cells and disrupted CCR5 expression. However, for the positively transduced GFP \(^+\) cells, we observed that the level of CCR5 editing appeared to be relatively lower in CD4 \(^+\) T-cells compared to that in TZM-bl cells. This probably resulted from various efficacies of expression and activity of the CRISPR/Cas9 components between primary cells and cell lines, or other yet-to-be-defined mechanisms. Nevertheless, CRISPR/Cas9-mediated disruption of CCR5 expression markedly reduced susceptibility of CD4 \(^+\) T-cells to HIV-1 infection, including BaL and a transmitted/founder strain. Whether engraftment of humanized mouse or/and primate animals with the modified CD4 \(^+\) T-cells could confer HIV-1 resistance warrants further investigations.

Here, we targeted CCR5 to confer R5-tropic HIV-1 resistance in primary lymphocytes. Although CCR5 is essential for the entry of R5-tropic viruses and partial ablation of CCR5 may provide clinical benefit for HIV-1 patients, it is likely that targeting CCR5 may drive an HIV-1 coreceptor switch (Poveda, 2015). While the driving force and molecular mechanism of coreceptor switch remain elusive, antiretroviral treatments with CCR5 antagonists tend to select for R5X4 or X4-tropic variants (Fätkenheuer *et al.*, 2008; Moncunill *et al.*, 2008; Mosier, 2009). A recent study has reported that a shift from R5-tropic to X4-tropic HIV-1 variants occurred in a patient following transplantation of stem cells with the CCR5 \(^{X22}\) mutation (Kordelas *et al.*, 2014), further highlighting the importance of coreceptor switch or adaptation in gene therapy targeting CCR5. Nevertheless, by delivery of CRISPR/Cas9 with two sgRNAs simultaneously targeting CCR5 and CXCR4, or CCR5 and viral genes essential for HIV-1 infection, it is theoretically applicable in preventing coreceptor adaptation and virus infection more effectively.

CD4 \(^+\) T-cells or haematopoietic stem cells (HSC) are two branches for delivery of HIV-1 gene therapy (Savkovic *et al.*, 2014). The current study established HIV-1 resistance in primary CD4 \(^+\) T-cells mainly because of their availability in terms of collection and expansion and safety when testing in clinical trials (Stan & Zaia, 2014). In contrast, gene therapy utilizing Ad5F35-transduced HSC is more challenging. Besides the relative complexity for handling HSC, one of the most critical challenges in clinical application is that allogeneic HSC transplantation is usually accompanied by severe immune rejection, which therefore requires prolonged immune-suppressive treatments (Stan & Zaia, 2014). However, Ad5F35-modified HSC have an advantage over CD4 \(^+\) T-cells in some aspects. Like in the Berlin patient, if the HSC transplantation is conducted successfully, one time of therapy may enable long-term control and even cure of HIV-1 infection (DiGiusto *et al.*, 2013; Holt *et al.*, 2010; Hütter *et al.*, 2009).

In summary, here we conducted CCR5 gene editing studies to inhibit HIV-1 infection by utilizing the recently harnessed CRISPR/Cas9 system. Following optimized sgRNA design and selection, Cas9 nuclease can cleave the CCR5 genomic locus around the predicted cleavage site with high efficacy and specificity. Importantly, by utilizing a chimeric Ad5F35 adenoviral vector, we successfully packaged recombinant Ad5F35 adenovirus carrying CRISPR/Cas9 components and transduced primary CD4 \(^+\) T-cells. The positively transduced cells with disrupted CCR5 expression were resolutely conferred with HIV-1 resistance. Considering the properties of versatility and easy manipulation, our results highlight the potential of utilizing CRISPR/Cas9 in HIV-1 gene therapy.
**METHODS**

**Plasmids, antibodies and cell lines.** pLentiCRISPRv2, pAdeasy-1, pShuttle-CMV and pLentiLox3.7 (pL3.7) were from Addgene. pNL4-3.Bal- (+R5-tropic, containing the backbone of NL4-3 and the Env of Bal, referred to as Bal, hereafter) and pNL4-3 (X-tropic), as well as T2M-bl and C8166-CCR5 cell lines were from NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIH.

**pCH042, a transmitted/founder HIV-1 molecular clone were kindly provided by Professor Beatrice H. Hahn (Parrish et al., 2013). Anti-CCR5-PE (clone 2D7) and matched isotype control antibodies were purchased from BD Pharmingen. Rabbit anti-Cas9 polyclonal antibody was purchased from Santa Cruz. CHO-CCR5, a CHO-K1 cell line with stable CCR5 expression was described previously (Hu et al., 2005).

**Lentivirus preparation.** The designed sgRNAs (Table S2) were constructed into pLentiCRISPRv2 as described by Shalem et al. (2014). Lentiviruses were produced by co-transfection of HEK293T cells with sgRNA-inserted pLentiCRISPRv2, psPAX2 and pMGG2, followed by concentration of the virus stocks by ultracentrifugation. Virus stocks were titrated by determining the cell number resistant to puromycin selection following infection of HEK293T cells (Shalem et al., 2014; Tiscornia et al., 2006).

Cells were transduced with lentiviruses (m.o.i. 1) in the presence of polybrene for 24 h. Forty-eight hours p.t., puromycin was presented at a concentration to kill untransduced cells within 2–3 days. After removal of puromycin, transduced cells were further cultured until downstream analysis.

**T7El cleavage assay.** Fragments of interest were amplified from genomic DNA by flanking primers (Tables S3, S4 and S5). The amplicons were purified, annealed and subjected to T7 endonuclease I digestion to determine the levels of gene editing as described by Ramakrishna et al. (2014). Indels (%)=100 × [1 − 1/2n] (amount of cut DNA/total DNA) (Cong et al., 2013).

**Production of recombinant adenoviruses.** The shuttle plasmid pShuttle.CRISPR.EGFP was constructed by inserting CRISPR/Cas9 components with an EGFP reporter into the CMV-removed pShuttle-CMV. Ad5F35 adenoviral backbone was constructed by exchanging the fibre shaft and knob region of pAdeasy-1 with that from Ad35 as reported by Shayakhmetov et al. (2000). Recombinant Ad5F35 adenoviral vectors carrying CRISPR/Cas9 (i.e. pAd5F35.Cas9,sgR5-5/8B or pAd5F35.Cas9,sgR5-5/8N) were generated in BSC1 cells using homologous recombination (Luo et al., 2007). Recombinant adenoviral vectors were produced by transfection of HEK293 cells, followed by scale-up propagation and purification via CsCl banding (Luo et al., 2007). Detailed cloning strategies, primers used and virus production methods are available in the online Supplementary Material.

**Adenovirus transduction.** Adenoviral stocks were thawed in a 37 °C water bath and taken out immediately once the ice was dissolved. For cell lines, the cells were seeded in six-well plates overnight to be 30–50% confluent at the time of virus inoculation. An m.o.i. 30 of Ad5F35 viruses was added to each well and incubated for 2–3 h followed by exchange with fresh medium. For primary CD4+ T-cells, the indicated amount of viruses (m.o.i. 30 or 100) was used to transduce 5 × 10^5 cells, which were plated in 24-well plates at the time of transduction in a total volume of 200 μl medium per well. Two hours p.t., an additional 300 μl medium was added and the cells were incubated for another 4 h. Thereafter, the culture medium was removed and the cells were washed once with FBS-free medium to further remove the unbound viruses. The transduced cells were cultured at 37 °C to allow transgene expression and/or gene editing until downstream analysis.

**HIV-1 production and infection.** HIV-1 was produced by transfecting plasmid of HIV-1 infectious clone into 293T cells using Lipofectamine 2000 according to the manufacturer’s instructions. All viral stocks were titrated by p24 ELISA (Beckman Coulter). Virus infection of CD4+ T-cells was conducted in 96-well plates. In each well, 1 × 10^5 cells were infected with the indicated amounts of viruses for 3 h at 37 °C, followed by extensive washes to remove unbound viruses. Cells were resuspended in 200 μl of medium and cultured at 37 °C. The cultured supernatants were collected at the indicated number of days post-infection, lysed with 1% (v/v) Triton X-100 and subjected to p24 quantification.

For virus infection of T2M-bl cells, the cells, pre-seeded into a 96-well plate, were infected with HIV-1 for 48 h, followed by determination of luciferase activity in the cell lysates using a Modulus II Microplate Multimode Reader (Promega) (Jin et al., 2014).

**REFERENCES**


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