

Supporting Online Figures

Supplementary Figure 1. Complete amino acid sequence of CCR5-targeting ZFNs

>CCR5; ZFN-L

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIIHGVPAAEAERPFQCRICMRNFSSDRS
NLSRHIRTHTGEKPFACDICGRKFAISSNLNSHTKIHTGSQKPFQCRICMRNFSRSDNL
ARHIRTHTGEKPFACDICGRKFATSGNLTRHTKIHLRGSQLVKSELEEKSELRHKLKY
VPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDY
GVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVS
GHFKGNYKAQLTRLNHI TNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGE INF

>CCR5; ZFN-R

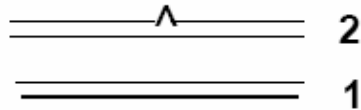
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PHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDY
VIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVSG
HFKGNYKAQLTRLNHI TNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGE INF

Supplementary Figure 1. The complete amino acid sequences of the CCR5-targeting ZFNs. Underlined are the recognition α -helices (from position -1 to position+6).

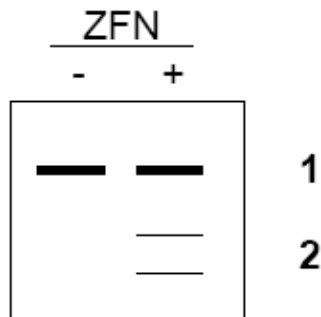
Supplementary Figure 2. The Surveyor Assay: Principle and Validation

2a.

1. Denature and allow PCR products to re-anneal.



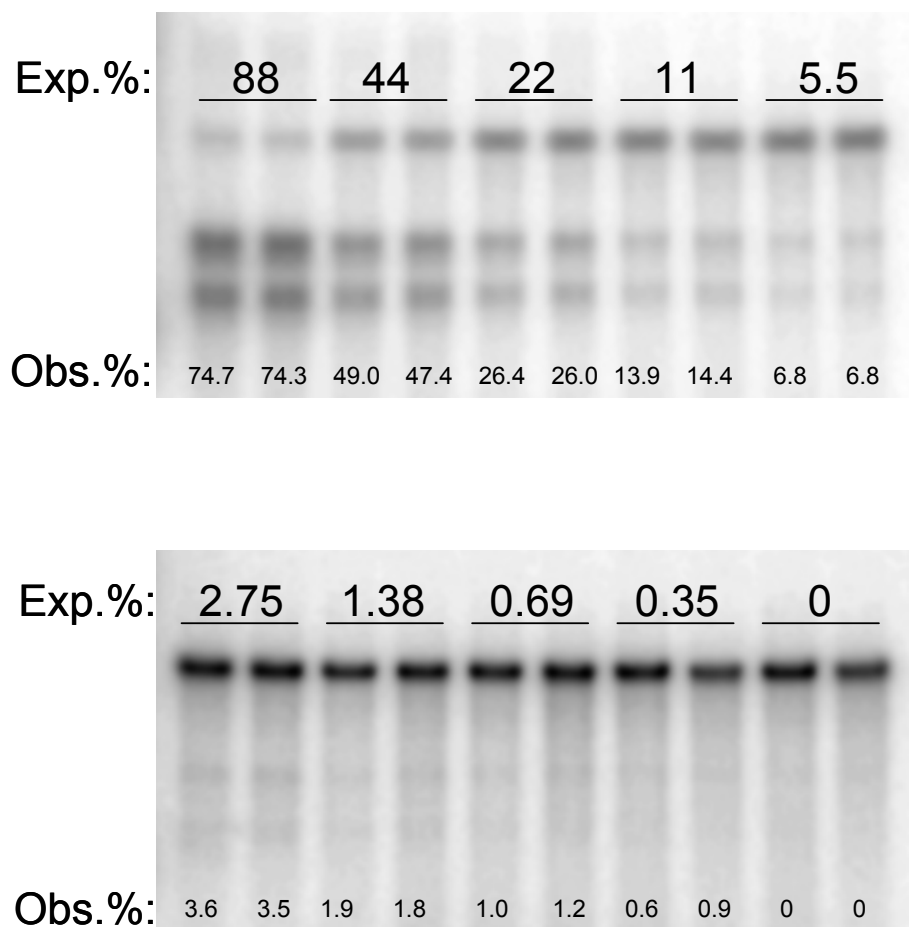
2. Digest re-annealed products and analyze by PAGE.



Supplementary Figure 2a: Mismatch selective endonuclease assay for measuring ZFN-mediated gene disruption. The level of ZFN-induced mutations were quantified by PCR amplification of the ZFN target region from genomic DNA, followed by denaturing and allowing wild type and mutant alleles to re-anneal together to create hetero-duplexes¹. The re-annealed PCR products are then digested with the Surveyor nuclease (Transgenomic) that preferentially cuts DNA at sites of duplex distortions. Briefly, radioactive PCR (50 ul reactions) was performed (AccuPrime PCR kit (Invitrogen)) on 100ng of genomic DNA extracted from modified and control cells using the MasturePureTM DNA purification kit (Epicentre Biotechnologies) and supplemented with 5 uCi α -P³² dATP and 5 uCi α -P³² dCTP. A 292-bp fragment of the CCR5 locus encompassing the CCR5-ZFN

target site was amplified for 30 cycles (95 °C - 30 sec., 60 °C - 30 sec., and 68 °C - 30 sec.) using the primers C5_Cel_160_F1: AAGATGGATTATCAAGTGTCAAGTCC; and C5_Cel_160_R1: CAAAGTCCCCTGGGCG. The PCR product was spun through a G-50 column (GE Healthcare) and 1 ul of the purified product was mixed with 1 ul of 10X annealing buffer (1X annealing buffer - 10 mM Tris, 100 mM NaCl) and water to a final volume of 10ul. The DNA was denatured and re-annealed in a PCR block using a program that allows heteroduplexes to form (95°C - 10 min; 95°C to 85°C at -2°C/s; and 85°C to 25°C at -0.1°C/s). After re-annealing, 1ul of the Surveyor nuclease (Transgenomics), 1ul 10X AccuPrime PCR buffer II, and water were added to a total volume of 20 ul. The reaction was incubated at 42°C for 20 min to digest heteroduplexes, and the cleaved products were resolved on a non-denaturing 10% TBE polyacrylamide gel (Bio-Rad). The gel was dried, exposed, and the level of ZFN-induced target gene disruption monitored by phosphorimager to determine the ratio of the uncleaved parental fragment to the two lower migrating cleaved products. The proportion of ZFN-disrupted CCR5 alleles in the original sample is calculated using the formula: $(1 - \sqrt{\text{Parental fraction}}) * 100$. The assay is sensitive to single nucleotide changes and has a detection limit of ~1% ZFN-modified alleles.

2b.

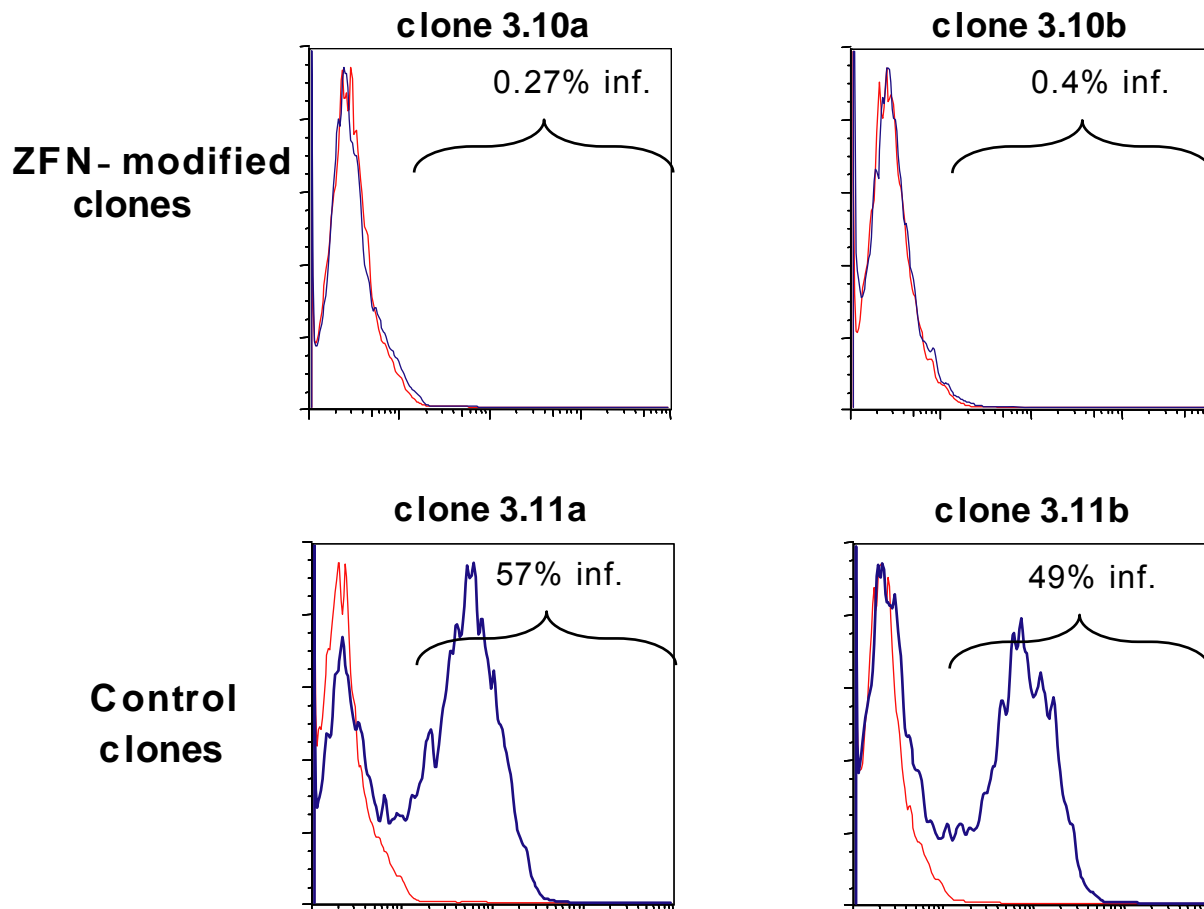


Supplementary Figure 2b: Validation of mismatch sensitive Surveyor nuclease assay. A 292-bp fragment of the CCR5 locus encompassing the ZFN-target site was PCR amplified from control cells and from a pool of cells with a sequence-confirmed known level of ZFN-disrupted CCR5 alleles of 88%. The PCR product from the modified cell population was serially diluted with PCR product from unmodified control cells in 2-fold increments. These mixed DNA pools were denatured, re-annealed, digested with the Surveyor nuclease, and the digested

products resolved and analyzed on a non-denaturing PAGE gel, as described (Supp. Fig. 2a). The observed (experimentally determined) percentage of modified alleles in the population was compared to the expected. Experimentally determined levels of CCR5-disrupted alleles accurately reflected input for mixes containing mutant alleles at between 0.69% and 44% with the square of the correlation coefficient being greater than 0.99.

Supplementary Figure 3. Block in HIV entry by ZFN-induced disruption of CCR5 and rescue by expression of the CCR5 cDNA

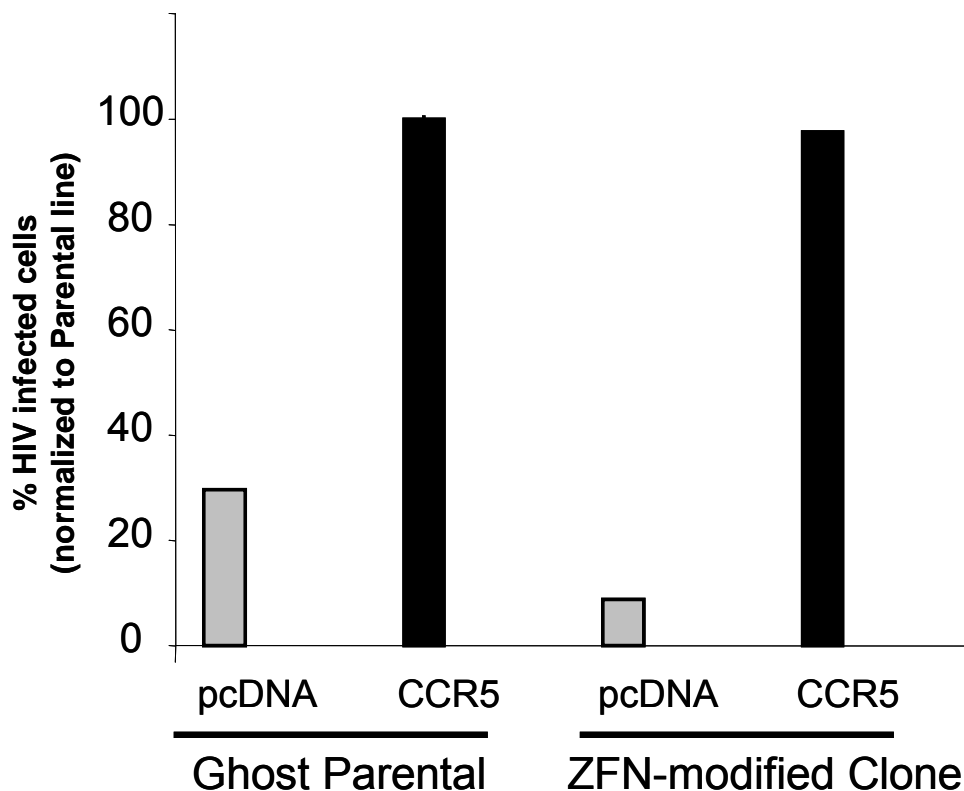
3a.



Supplementary Figure 3a. Resistance of CCR5 ZFN treated GHOST-CCR5 cell clones to HIV infection. Single cell derived clones were isolated from CCR5-ZFN transduced GHOST-CCR5 cells (Fig. 1), expanded over a period of several weeks. The CCR5 transgene was genotyped and clones possessing only ZFN-disrupted CCR5 alleles (clone 3.10a and 3.10b) were tested for resistance to HIV infection by HIV-1_{BAL}. Clones obtained in parallel possessing an intact CCR5

transgene (3.11a and 3.11b) remained HIV-1 infectable. Key: Red, Mock infection; Blue, HIV infection.

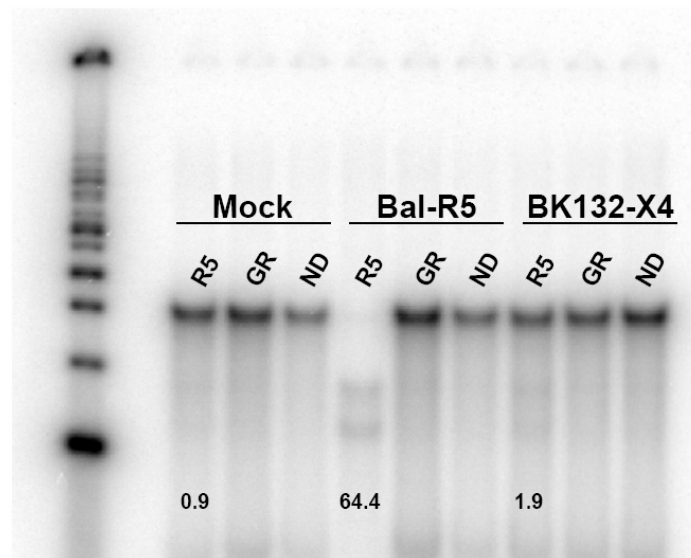
3b.



Supplementary Figure 3b. CCR5 complementation recovers HIV-1 infection in CCR5-ZFN transduced GHOST-CCR5 cell clones. GHOST parental cells or ZFN-disrupted CCR5 null GHOST-CCR5 cells were transfected by Nucleofection (Amaxa) with a CCR5 expression plasmid (CCR5) or control plasmid (pcDNA). GHOST parental cells express CD4 and GFP driven by the HIV LTR. All groups were exposed to HIV-1_{BAL} and infection assessed by GFP fluorescence 48 hours after HIV-1 challenge. For each condition the percentage of infected cells was determined and the results normalized to the parental GHOST cells transfected with CCR5.

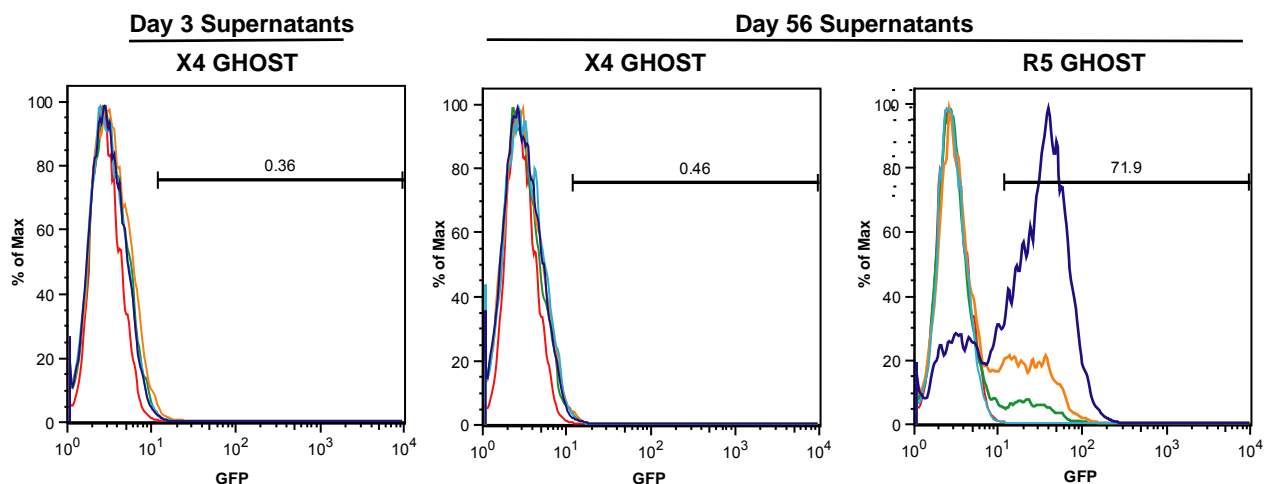
Supplementary Figure 4. Analysis of HIV tropism and viral envelope evolution in CCR5-ZFN treated, HIV challenged cells

4a.



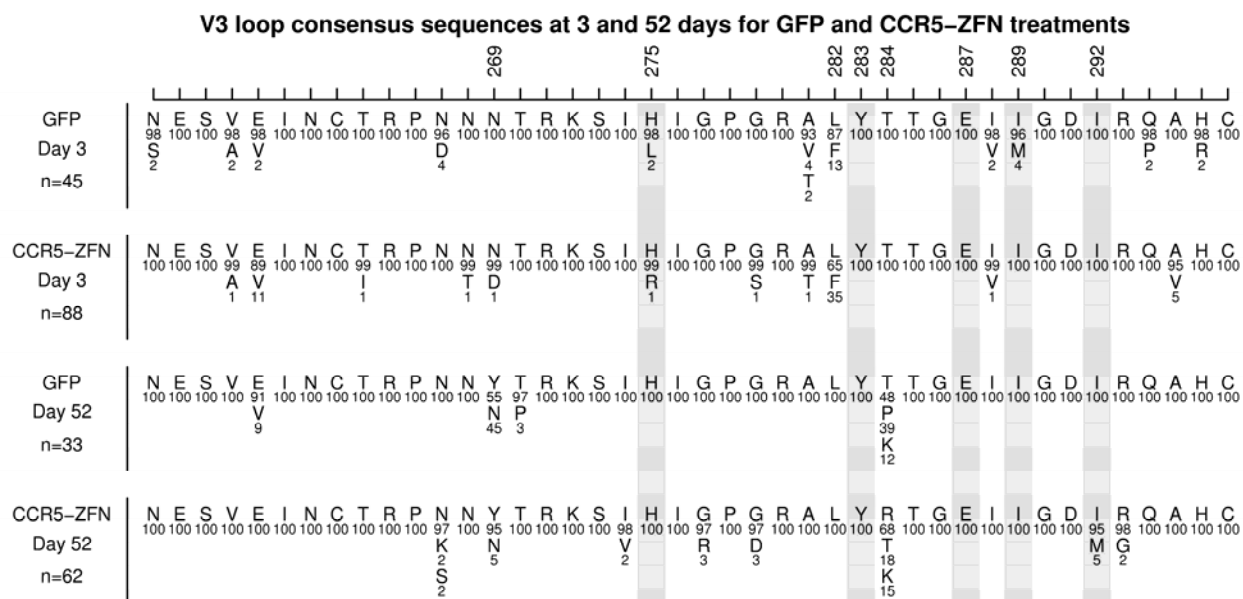
Supplementary Figure 4a. CCR5-ZFNs confer stable, heritable resistance to CCR5-tropic HIV-1 infection. PM1 cells were mock transfected, or transfected with plasmids encoding the CCR5-ZFNs (R5) or a control glucocorticoid receptor (GR) ZFN pair as described in manuscript Fig. 2. These cell populations were challenged with HIV-1_{BAL} and on day 59 post-infection a portion of each sample was mixed with parental, non-transfected PM1 cells and re-infected with either CXCR4-tropic HIV-1_{BK132} or CCR5-tropic HIV-1_{BAL}. These re-infected cultures were followed over time and analyzed for CCR5 gene disruption frequency on day 21 post-reinfection (day 80 post-initial infection).

4b.



Supplementary Figure 4b. Functional co-receptor preference of HIV-1 isolates derived from HIV-1 challenge of CCR5-ZFN modified PM1 cells on days 3 and 56 of culture. GHOST-CXCR4 cells challenged with supernatants (300 μ l) from cultures of HIV-1 infected CCR5-ZFN transfected PM1 cells removed at early (day 3) and late (day 56) time points showed no CXCR4 dependent infection. In contrast, the same supernatants applied to GHOST-CCR5 cells remained infectious with the exception of the CCR5-ZFN transfected sample, suggesting that the >30-fold enrichment for CCR5 null PM1 cells had resulted in greatly reduced viral infectivity by day 56 of the culture. Key to supernatants tested: **dark blue**, non-transfected cells; **light blue**: CCR5 ZFN transfected; **green**, Control (GR) ZFN transfected; **orange**, GFP transfected; **red**, auto-fluorescence background stain control.

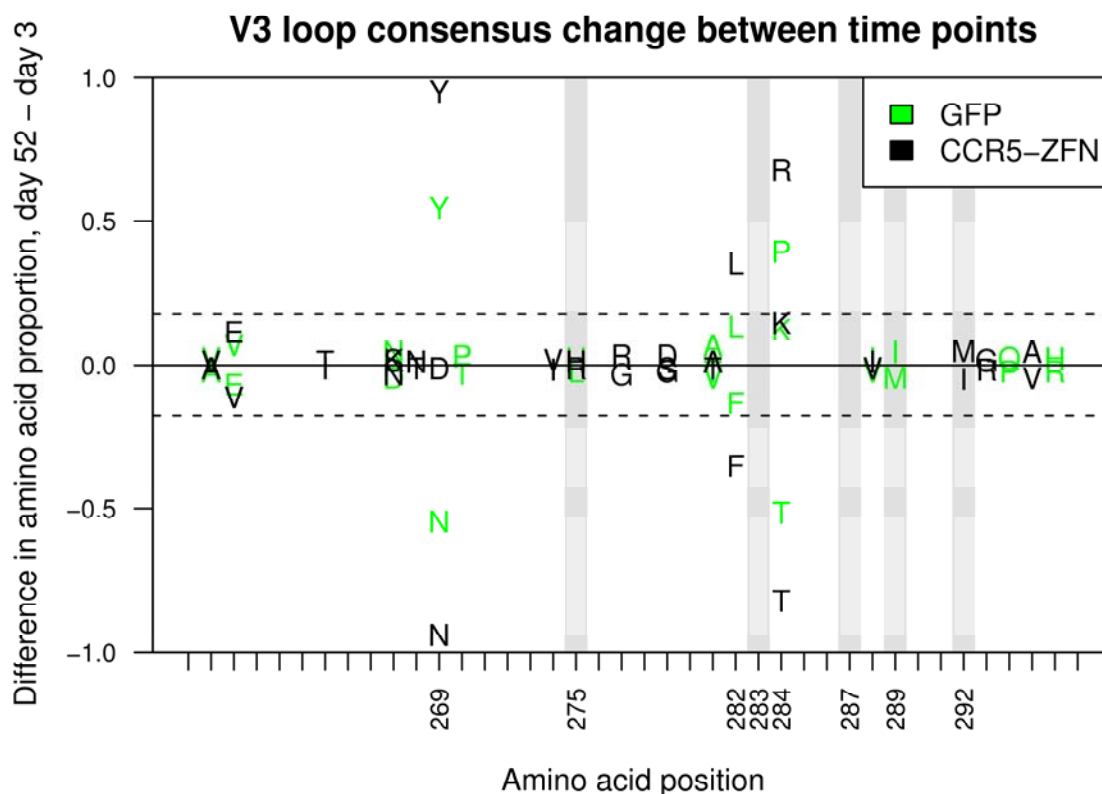
4c.



Supplementary Figure 4c. V3 loop sequences indicate that CCR5-ZFN modified cells do not force rapid evolution of CCR5-tropic HIV toward CXCR4-dependent or dual tropic variants. V3 loop sequences obtained from supernatants of HIV-1 challenged PM-1 cells transfected with plasmids expressing either CCR5-ZFNs or a GFP control were studied to determine the effects ZFN generated CCR5 null cell enrichment exerted on viral tropism over time. 150 proviral HIV DNA sequences were isolated from longitudinal cultures of HIV-1_{BAL} infected CCR5 ZFN-treated PM-1 cells, and of these, 88 were from day 3 and 62 were from day 52 after infection. As a control, 78 HIV DNA sequences were isolated from the HIV infected GFP-treated PM-1 cells, including 45 from day 3 and 33 from day 52. The sequences were evaluated for changes in tropism by matching the R5, R5X4, or X4 consensus V3 loop sequences from Hung et al²; critical residue positions involved in determining virus tropism are shaded. All V3

loop sequences from the GFP and CCR5-ZFN treated cells at both day 3 and day 52 samples most closely matched the CCR5 consensus sequence, suggesting no rapid evolution toward alternative co-receptor usage.

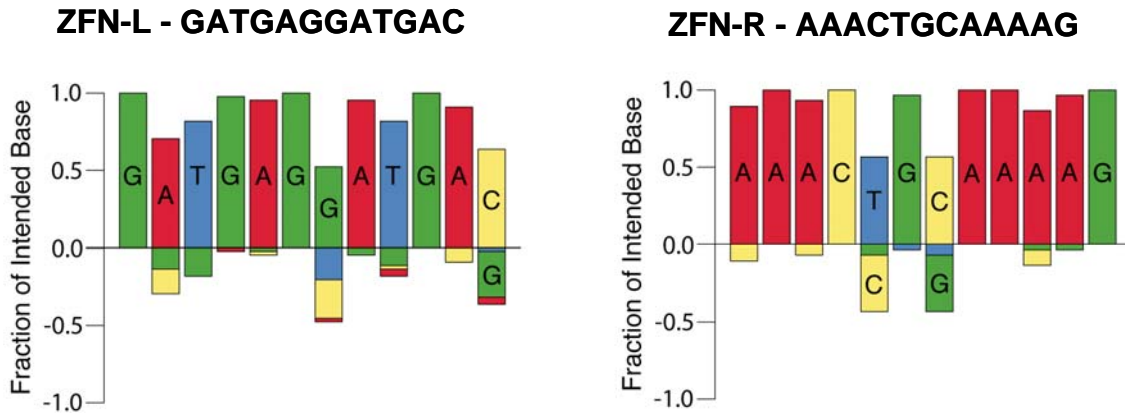
4d.



Supplementary Figure 4d. V3 loop sequence comparison between day 3 and day 52 samples show CCR5-ZFN modified and GFP transfected cells exhibit a similar pattern of change over time. Changes in the V3 loop residues at day 52 for both the GFP-treated (green) and CCR5 ZFN-treated (black) PM-1 cells were plotted along the y-axis, whereby the proportion of an acquired amino acid at day 52 occurs above the x-axis and those lost below the x-axis. The shaded positions mark the V3 loop positions that are critical for determining HIV tropism². No significant differences were observed in the residue changes between the GFP and CCR5-ZFN samples.

Supplementary Figure 5. Specificity testing of CCR5-ZFN reagents

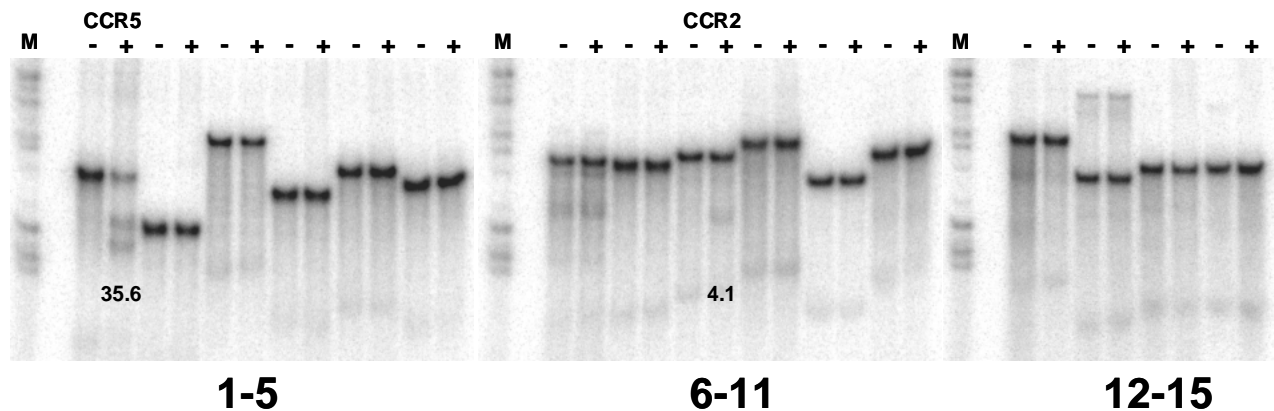
5a.



5b.

Rank	Score	Chrom.	ZFN-L Target	Mismatch	ZFN-R Target	Mismatch	Gene	Intron/Exon
Intended	0.0045156	3	GATGAGGATGAC	0	AAAGTCAAAAAG	0	CCR5	Exon
1	0.0004681	4	GATGAGGATGAC	0	GATGAGGATGcC	1	ABLIM2	Intron
2	0.0001714	14	AAAGTCAAAAAC	1	AAAGTCAAAAAG	0	None	N/A
3	4.92E-05	17	cAAcAGCAAAAAG	2	AAAGTCAAAAAG	0	ABCA6	Intron
4	1.66E-05	16	GATGAGGAgGcC	2	GgTGAGGATGAC	1	None	N/A
5	1.07E-05	1	GcTGAGGATGAC	1	GATGAacATGAC	2	CACHD1	Exon
6	9.52E-06	21	AAcAGCAAAAAC	2	AAAGTCAAAAAG	0	None	N/A
7	9.52E-06	8	AAcAGCAAAAAC	2	AAAGTCAAAAAG	0	None	N/A
8	7.87E-06	3	GATGAGGAcGAC	1	AAAGTCAAAAa	1	CCR2	Exon
9	4.37E-06	5	gAACTGGA AAAAG	1	AAAGTCAAAAAG	1	None	N/A
10	3.94E-06	18	AAAGTGA AAAa	1	AAtCTGCAAAAAG	1	None	N/A
11	3.69E-06	8	AAAGTGCAtAAG	1	AAAGTGA AAAa	1	None	N/A
12	2.88E-06	X	GcTGAGGATGAt	2	AAcAGGA AAAAG	1	None	N/A
13	2.48E-06	7	AAAGTGAAcAG	1	GcTGAGGATaAC	2	WBSCR17	Intron
14	2.27E-06	X	GATGAGGATGAg	1	AcAaTGCAAAAAG	2	DGKK	Intron
15	2.02E-06	2	AAcAGcAgAAG	2	GcTGAGGATGAC	1	GALNT13	Intron

5c.



5d.

Gene/site	Location	Homo-dimer	Sequences Analyzed	Probable NEHJ Events
CCR2	Chr3 (CCR2)	No	37,028	1,995
ABLIM2	Chr4	Yes	38,023	2
Chr14-873	Chr14-873	Yes	39,285	0
ABCA6	Chr17	Yes	33,699	0
Chr16-463	Chr16	Yes	35,747	0
CACHD1	Chr1	Yes	54,401	0
Chr21-323	Chr21	Yes	24,122	0
Chr8a-783	Chr8a-783	Yes	52,973	0
Chr5-495	Chr5-495	Yes	29,969	0
Chr18-494	Chr18-494	Yes	21,729	0
Chr8-1205	Chr8-1205	Yes	21,142	0
ChrX-136	ChrX-136	No	30,039	0
WBSCR17	Chr7	No	17,869	0
DGKK	ChrX-501	No	24,873	0
GALNT13	Chr2-154	No	42,595	0

5a. Determination of the consensus binding site for CCR5-ZFN (ZFN-224)

SELEX was used to identify the binding site preference for the left and right zinc finger proteins comprising ZFN-224. The experiments were performed as follows: (1) first, an HA-tagged version of the ZFP of interest was expressed via the TnT quick coupled transcription-translation system (Promega), and incubated with a pool of partially randomized DNA sequences in the presence of biotinylated anti-HA Fab fragments (Roche) and poly dIdC competitor DNA (Sigma); (2) the protein – along with any productively bound DNA sequences – was captured on streptavidin coated magnetic beads (Dynal); (3) the magnetic beads were placed in Roche PCR master mix containing the appropriate primers and the bound DNA was then released and PCR amplified. This amplified pool of DNA was then used as the starting DNA pool for subsequent rounds of ZFP binding, enrichment and amplification. Cycles comprising steps (1)–(3) were repeated for a total of four rounds of selection. Finally, DNA fragments amplified after the final round were cloned and sequenced. The randomized region of each DNA sequence was aligned to determine the consensus binding site sequence for the zinc finger DNA binding domain. The nucleotide preferences at each position in the 12-bp binding site for each ZFN comprising ZFN-224 are shown as the fraction of each base, where the intended target base is oriented along the positive Y-axis and the fraction of all other bases are shown along the negative Y-axis.

5b. Bioinformatic determination of the 15 highest similarity putative off-target sites in the human genome for the CCR5 ZFN-224 consensus. Base preferences for the two CCR5 ZFNs as determined by SELEX (5a) were used to guide a genome wide bioinformatic prediction of the top 15 potential off-target sites in the human genome. This bioinformatic analysis searched for and ranked the potential 224 off-target sites as follows:

1. All potential DNA binding sites for ZFN-L and ZFN-R were identified in the human genome allowing for up to and including two (2) base pair mismatches from the consensus determined in the SELEX experiments;
2. All possible cleavage locations were identified using the complete list of binding sites (found in 1.) that allowed any two ZFNs (including homodimerization and heterodimerization events) to bind in the appropriate configuration for nuclease activity (i.e. ZFNs binding on opposite sides of the DNA with either a 5 or 6 bp spacing between them).

The resulting list of potential cleavage sites was then ranked to give priority to those sites with the highest similarity to the consensus for each ZFN as defined by SELEX (5a above). Briefly, the SELEX data was used to create a probability for the recognition of all four nucleotides (A, C, G or T) in each of the 12 positions of the ZFN binding site. Each putative ZFN binding site was scored as the product of these twelve (12) probabilities; note that to eliminate a score or probability of zero every position had a single count added for each nucleotide (A, C, G or T) prior to normalization to ensure no entry in the probability table was zero. Similarly, the score for a given off-target cleavage site (requiring two such ZFN sites to be

occupied) was calculated as the product of the two scores given to each of the two ZFN binding sites comprising the putative cleavage site.

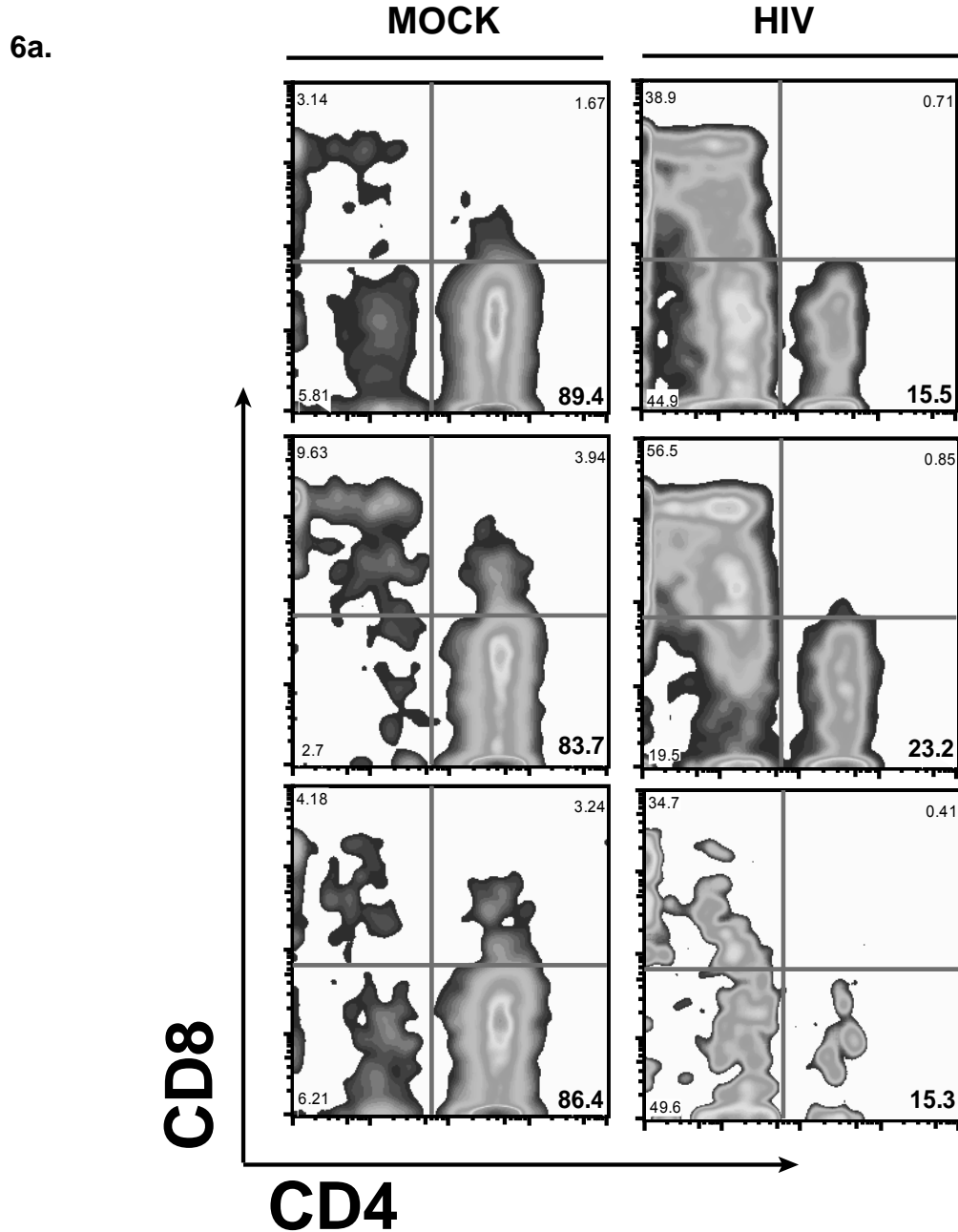
Note: of these 15 sites, 7 fall within annotated genes and 2 fall within exonic sequence. These seven genes share the following characteristics; (i) their mutation or disruption has not been connected with any known pathology; and (ii) with the exception of CCR2, they have no described function in CD4 T-cells.

5c. Experimental determination of ZFN cleavage at the top 15 off-target sites

in CD4 T cells. Primers specific for each potential off-target cleavage site were used to amplify DNA from CD4 T cells transduced with an Ad5/35 vector expressing CCR5-ZFN-224. Surveyor nuclease assay results for each target are shown. No detectable off-target activity was observed at these 15 sites with the exception of CCR2 (4.1% modification of the alleles). Simultaneous analysis of ZFN cleavage activity at the intended target in CCR5 was measured at 35.6% of the alleles (detection limit of the assay is ~1%).

5d. Ultra deep pyrosequencing of top 15 ZFN sites. Massive deep sequencing of the 15 sites identified by SELEX and shown above in 4b and c was done by 454 pyrosequencing. CD4 T cells were treated with ZFN 224, and the recovered DNA subjected to multiplex PCR and 454 sequencing as described in Methods. The resulting sequences (~17,000 to ~54,000) at each potential site were analyzed for potential NEHJ essentially as described⁵³.

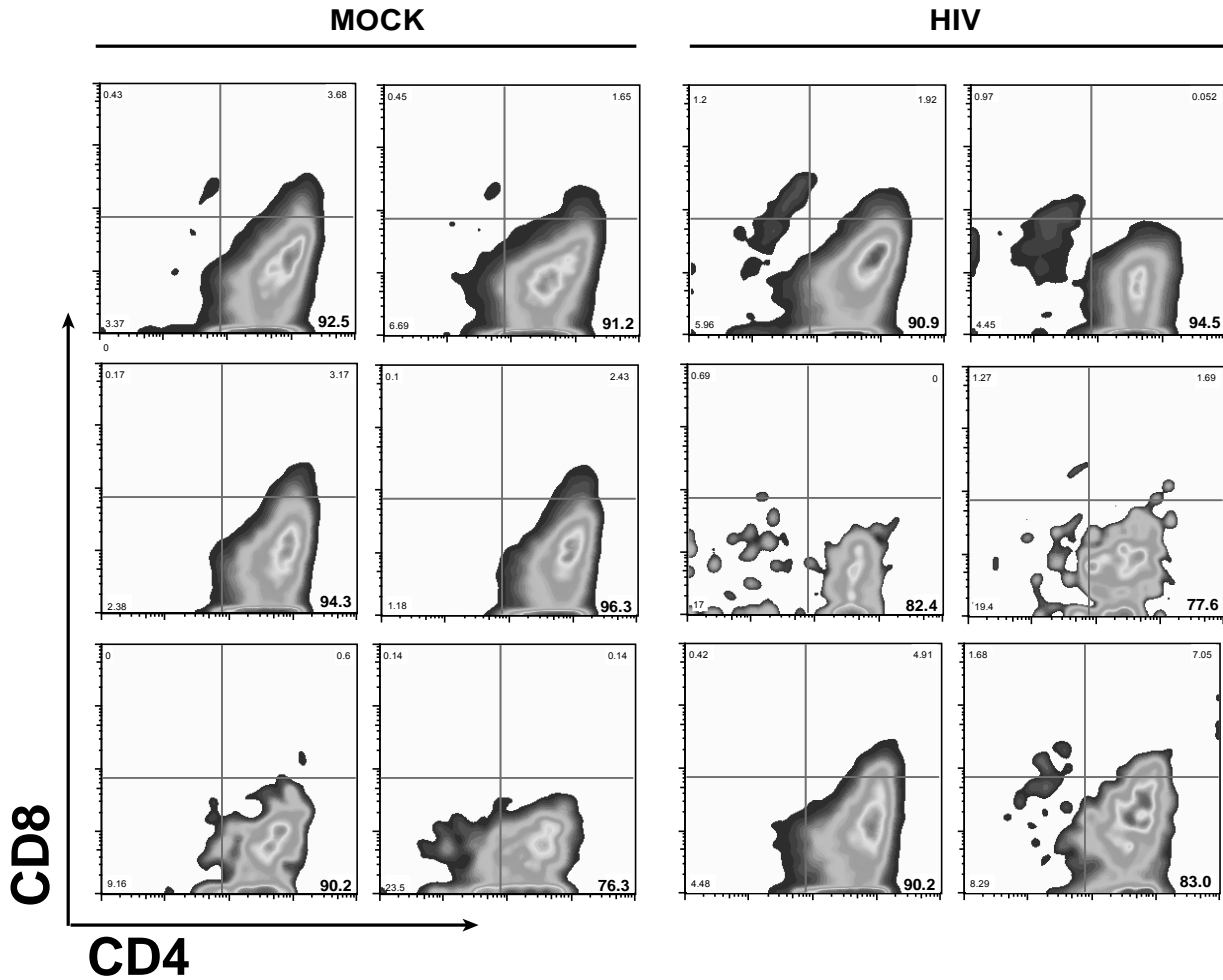
Supplementary Figure 6. CCR5-ZFN treated primary CD4+ T cell engraftment and recovery in NOG mice.



6a. In vivo engraftment of CCR5 disrupted CD4 cells in NOG/SCID mice. Analysis of engraftment of human CD4+ and CD8+ T cells in the peripheral blood

of HIV-infected (n=3) and non-infected mice (n=3) by flow cytometry on day 27 of experiment.

6b.



6b. Analysis of CD4 T cell purity by flow cytometry after column purification of human CD4+ T cells from the spleens of HIV infected and non-infected mice on day 40 of experiment. These samples are from the mice shown in manuscript Figure 4; genomic DNA from these cells was used for CCR5 disruption analysis in Fig. 4. Representative samples from individual Mock (n=6) and HIV infected (n=6) mice are shown.

Supplementary Online Information Perez, et al.

Supplementary Figure 7. Predicted protein sequences generated by CCR5 disruptions.

DELETIONS:

F V G N M L V I L I L I N C K R L K S M T D I Y L L
 TTTTGTGGGCAACATGCTGGTCATCCTCATCTGATAAACTGCAAAAGGCTGAAGAGCATGACTGACATCTACCTGCTC w. t.

F V G N M L V I L I . . T A K G . R A . L T S T C
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F V G N M L V I L I L K L Q K A E E H D . H L P A
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F V G N M L V I L I L K L Q K A E E H D . H L P A
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F V G N M L V I L I D K L Q K A E E H D . H L P A
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F V G N M L V I L T D K L Q K A E E H D . H L P A
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F V G N M L V I L I I N C K R L K S M T D I Y L L
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F V G N M L V I L I . T A K G . R A . L T S T C
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F V G N M L V I L I Q T A K G . R A . L T S T C
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F V G N M L V I L I K L Q K A E E H D . H L P A
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F V G N M L V I R . T A K G . R A . L T S T C
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F V G N M L V I D K L Q K A E E H D . H L P A
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F V G N M L V I L I L I K A E E H D . H L P A
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F V G N M L V I L N C K R L K S M T D I Y L L
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F V G N M L V . . T A K G . R A . L T S T C
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F V G N M L V I L I Q K A E E H D . H L P A
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 F V G N M R K L Q K A E E H D . H L P A
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 F V G N M L T A K G . R A . L T S T C
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 F V G N M Q K A E E H D . H L P A
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 F V G N M K S M T D I Y L L
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 F V G N I . L T S T C
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INSERTIONS:

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F V G N M L V I L I L I K T A K G . R A . L T S T C
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F V G N M L V I L I L M I N C K R L K S M T D I Y L
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F V G N M L V I L I L T D K L Q K A E E H D . H L P
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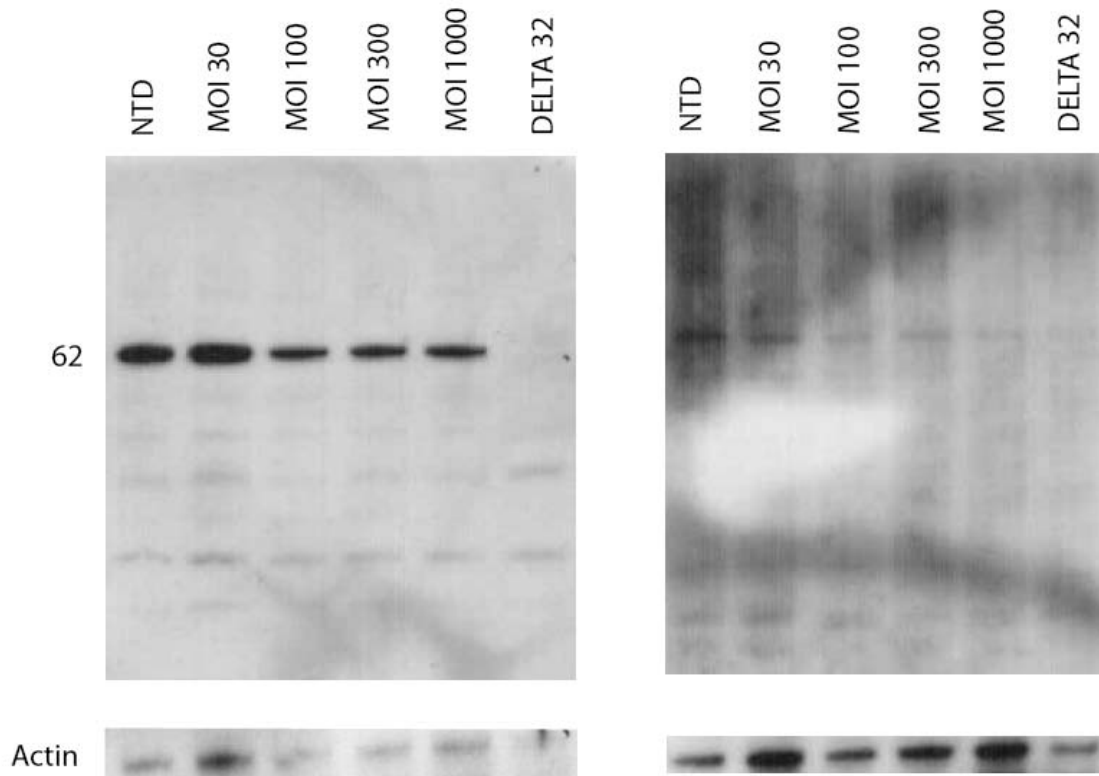
F V G N M L V I L I L I D K L Q K A E E H D . H L P
 TTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATtgatAAACTGCAAAGGCTGAAGAGCATGACTGACATCTACCT +4

F V G N M L V I L I L I . . T A K G . R A . L T S T
 TTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATctgatAAACTGCAAAGGCTGAAGAGCATGACTGACATCTACC +5

F V G N M L V I L I L I . . T A K G . R A . L T S T
 TTTTGTGGGCAACATGCTGGTCATCCTCATCcttaatttaATAAACTGCAAAGGCTGAAGAGCATGACTGACATCTACC +8

Supplementary Figure 7. Predicted protein sequences generated by CCR5 disruptions. The sequence information from the data shown in Fig. 2b was used to translate the predicted protein sequence up to the first stop codon.

Supplementary Figure 8. Western Blot analysis of CCR5-ZFN treated primary CD4⁺ T cells.



Supplementary Figure 8. Western blot analysis of CCR5 expression in CCR5-modified or CCR5 Δ 32 cells. Primary CD4 T cells were transduced with CCR5-ZFN at MOI of 30, 100, 300, and 1000. Nontransduced CD4 T cells from the same donor were used as a positive control, and CD4T cells from a donor with the delta 32 mutation were used as a negative control. Lysates from 1x10⁶ cells/condition were collected on day 5 after transduction. Western blot for CCR5 was performed using commercially available antibodies against C-terminus and N-terminus epitopes of CCR5. (**Left panel**) (CKR5/CCR5 (C-term) Rabbit Monoclonal

Antibody Clone ID E164, Epitomics ; 45523, R&D Systems. **(Right panel)** Anti-C-C Chemokine Receptor Supplementary Online Information Perez, et al. 22 5, N-terminus, Millipore). Secondary antibodies were species-specific hrp-conjugates. Blots were developed using ECL detection kit (GE).

Supplementary References

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