nature portfolio

Peer Review File

High frequency CCR5 editing in human hematopoietic stem progenitor cells protects xenograft mice from HIV infection

Corresponding Author: Dr Christian Boutwell

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Claiborne et al present a manuscript entitled “High frequency CCR5 editing in human hematopoietic stem progenitor cells protects xenograft mice from HIV infection” for publication in Nature Communications. The goal of this study was to identify the threshold level of CCR5-inactivated hematopoietic cells that supports full protection against infection with CCR5-tropic HIV-1 in a humanized mouse model.

Four lead human CCR5 gRNA candidates were first identified through a rigorous combination of in silico screening and ex vivo HSPC-based assays. Experiments in primary T-cells further focused on candidates TB48 and TB50, alone or in combination. At 2 days post-editing, CCR5 protein levels in these 3 conditions were each reduced greater than 10-fold. In the TB48/TB50 dual-guide condition, up to 97% HSPC editing was observed ex vivo and cells behaved comparably to controls in a colony forming assay. Although engraftment in NBSGW mice was delayed relative to controls, reconstitution was comparable by 8 weeks post-transplant. Similarly robust findings were observed in an NSG mouse model. CCR5-edited animals were highly resistant to CCR5-tropic (but not dual-tropic) HIV challenge: a mathematical model predicted that >100 HIV challenges would be required to infect an animal with 98% CCR5-edited cells. The authors close by positing that the barrier presented by CCR5-inactivated cells in this setting should translate to a barrier in suppressing viral replication following reactivation from latency.

This is a well-written and thorough analysis that will broadly inform the promise and feasibility of CCR5 editing to cure HIV infection. Additional details on engraftment kinetics of edited HSPCs early after transplantation, and on selection of NSG vs. NBSGW mouse models for specific experiments, would further augment what already is a highly successful study.

Major Points
-Can the authors speculate on the delay in engraftment observed with dual guide-edited HSPCs (Figure 4C)? For example, were chromosomal translocations detected in these cells? If so, one possibility is that the engraftment delay was due to a DNA damage response that eliminated a subset of the dual-edited cells. This is especially important to address since the authors assert that there is “no discernable decrease in engraftment potential” of these cells (lines 279-280).

-The data in Figure 7D suggest that at suboptimal levels of editing, there is not a clear virus-dependent selection for CCR5-inactivated cells. However individual animals’ trends cannot be discerned from this bar graph. Are there other ways to address virus-dependent selection, e.g. the proportion of animals that showed an increase in CCR5 editing between weeks 20 and 24?

Minor Points
-The abstract and introduction mention that this platform is “scalable.” Please expand either with data (e.g. validation that clinical-scale products can be edited with similar efficiency) or provide references to support this statement.

-The correlation data in Figure 3D appears to be strong; if possible, please apply a statistical test.

-The data suggest that NSG and NBSGW models were equally informative in HIV infection assays, but it would be helpful to clearly indicate in the results text which model was used in which experiment, and the rationale. For example, why was NBSGW chosen in Figure 7?

-Lines 198-199: are reference(s) available to clarify what is meant by non-productive or “cryptic” HIV infection?

-Figure 6B: is the difference in CD4+ cells between mock and CCR5-edited animals statistically significant?

-There are several references to “Supplementary Text” which doesn’t appear to be included in the manuscript.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
Claiborne and colleagues describe here a CRISPR-Cas9/RNP platform capable of producing a human HSPC transplant with high frequency CCR5 editing. They successfully reconstitute the immune system in xenograft mice which are highly refractory to HIV replication, following several challenges with CCR5-HIV strains. Moreover, upon grafting mice with different proportions of CCR5 edited HSPC (0% to 96%) they show that the protective value of HSPC CCR5 editing decreases quickly and becomes negligible between 26% and 54% CCR5 editing. Overall their results suggests that it is potentially possible to recapitulate a curative CCR5 32/ 32 transplant in an autologous HSCT context, although high frequency of CCR5 editing is needed. The manuscript is clear, well written and although similar papers have been published dealing with CCR5 editing in human HSCT prior to in vivo testing in mice, this is the first time that a CCR5 editing reaches values approaching 100% achieving a complete protection in vivo.
However, there are some points that need further clarification.
Major comments
1. The authors do not show any data on cell viability after transfection and CRISPR-Cas9 editing. Another important issue is the % recovery of edited cells related to the input. This data is key to assess the feasibility of applying the technique in a clinical setting.
2. Despite the detailed description of off-target effects at DNA level that is used for selecting those guideRNA without off-targets, there is no reference on putative effects on cell functionality. Authors should demonstrate that immune cell function is preserved upon CRISPR-Cas9 treatment in terms of in vitro activation upon general stimuli and/or the lack of major changes at transcriptomic level.
3. Although data on % of CCR5 expression is pretty clear in mice blood, additional data on biodistribution in tissues is missing, especially in cases where editing is below 90%. Are CCR5 edited cells equally distributed between tissues? How is the situation in lymph nodes? This data would be relevant for latency establishment/reservoir replenishment if CCR5 edited cells are not equally distributed thorough the body.
4. The authors propose 2 potential protective mechanisms of CCR5 editing. Although the “barrier at inoculation” is pretty clear from the data presented, this is not the case for “Barrier at proliferation” mechanism. This latter mechanism, albeit plausible, is not well-supported by the data presented. The most relevant experiment that supports is that shown in Fig 7H, although a clear correlation between CCR5-editing and VL is not observed with the data provided.
Minor comments
5. The number of replicates in some in vivo experiments should be increased or all animals have to be included in the graph, as is the case of Fig 6C.
6. The authors use 2 distinct mice strains. However, the choice of one or the other is not clear. Why a given strain is used and which are the reasons to change from one to the other? Please clarify in Fig 4-5 and results section.
7. Line 131-132. The authors comment that the frequency of CCR5+ cells is maintained for 10 days in culture. As far as I know, CRISPR-Cas9 editing should be stable and irreversible. Please clarify.
8. Fig 4B. If results represent the number of colonies derived from a single donor, what do the error bars represent? Are there replicates? Please clarify.
9. Line 232-234. Statistics is missing in figure, although results section claims for a significant reduction.
10. Supplementary Texts are missing, but mentioned in the text (lines 258-259, 310 and 630-631)
11. Fig 6A. There is a symbol in one CCR5 edited mice at 1 week post challenge and no legend is provided.
12. Fig 6C. It would be nice to have data for all animals tested, those in Fig 6A.
13. Fig 7B. The 0% CCR5 edited group (which is mentioned in line 218) should be added as a control
14. Fig 7E. VL of the animals included in the experiment should be shown. It is relevant to show the quantification of viral replication. This may help to clarify the relation between VL and CCR5 editing that is claimed in the next 3 subfigures (7F-H)
15. Figure 7. Colour code is confusing and makes it difficult to distinguish between the distinct group of mice.

(Remarks on code availability)
Out of my expertise

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have sufficiently addressed my comments and questions.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have successfully adressed all my previous concerns, significantly improving the manuscript and also its impact for the scientific community. At this point, the manuscript is significantly better and in my opinion should be accepted for publication.

(Remarks on code availability)
Out of my expertise

Reviewer #3

(Remarks to the Author)
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(Remarks on code availability)
As of now, the code reports an 404 error "The page you requested could not be found.
It either never existed or it was removed by the owner.".

We sincerely thank the reviewers for their insightful comments, and we believe that in addressing them, we have provided an improved manuscript for your review. The revised manuscript addresses key points that enhance clarity of the concepts put forth in the original text. In addition, each critique raised by the reviewers is addressed in a point-by-point response below, with the response text highlighted in blue.

Reviewer's Comments:

Reviewer #1 (Remarks to the Author)

Claiborne et al present a manuscript entitled "High frequency CCR5 editing in human hematopoietic stem progenitor cells protects xenograft mice from HIV infection" for publication in Nature Communications. The goal of this study was to identify the threshold level of CCR5-inactivated hematopoietic cells that supports full protection against infection with CCR5-tropic HIV-1 in a humanized mouse model.

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This is a well-written and thorough analysis that will broadly inform the promise and feasibility of CCR5 editing to cure HIV infection. Additional details on engraftment kinetics of edited HSPCs early after transplantation, and on selection of NSG vs. NBSGW mouse models for specific experiments, would further augment what already is a highly successful study.

Major Points
-Can the authors speculate on the delay in engraftment observed with dual guide-edited HSPCs (Figure 4C)? For example, were chromosomal translocations detected in these cells? If so, one possibility is that the engraftment delay was due to a DNA damage response that eliminated a subset of the dual-edited cells. This is especially important to address since the authors assert that there is "no discernable decrease in engraftment potential" of these cells (lines 279-280).

While it is possible that the transient engraftment delay observed with dual-guide edited HSPCs could be due to a DNA damage response, this is unlikely to be a cause of long-term engraftment

deficit, as reconstitution levels were identical at 8-weeks post-transplant and following between mock and dual-guide edited HSCs.
Additionally, initial viability 48 hours post electroporation was not dramatically lower in dual-guide edited HSCs compared to mock, and this data is captured below:

HSCs					
Edited					
Donor	**Total live cells Prior to EP**	**Viability prior to EP**	**Total live cells two days post EP**	**Viability two days post EP**	**% Recovery**
1	4.50E+07	95%	4.40E+07	N/A (>90% when thawed for engraftment)	97.78%
2	4.00E+07	90%	3.82E+07	86%	95.50%
3	9.00E+07	94%	9.01E+07	85%	100.11%
Mock Editing (GFP)					
Donor	**Total live cells Prior to EP**	**Viability prior to EP**	**Total live cells two days post EP**	**Viability two days post EP**	**% Recovery**
1	4.50E+07	95%	6.20E+07	N/A	137.78%
2	4.00E+07	90%	4.81E+07	91%	120.25%
3	4.50E+07	94%	6.02E+07	92%	133.78%

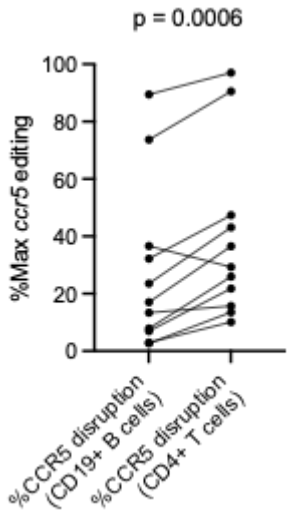
The above table is now included as **Supplementary Table 1**.

However, we cannot rule this out with the possibility that dual-guide editing and the subsequent DNA damage repair mechanisms affected engraftment kinetics. This is now further expounded upon in the discussion (lines 292 – 300), where we propose that though high levels of editing followed by DNA repair may have impeded the engraftment of short-term progenitors, there was no apparent reduction in engraftment of long-term progenitors, human cell numbers 6-months post-transplant were indistinguishable among HSC groups.

-The data in Figure 7D suggest that at suboptimal levels of editing, there is not a clear virus-dependent selection for CCR5-inactivated cells. However individual animals' trends cannot be discerned from this bar graph. Are there other ways to address virus-dependent selection, e.g. the proportion of animals that showed an increase in CCR5 editing between weeks 20 and 24?

The frequency of CCR5+ CD4+ T cells among experimental groups depicted in Figure 7D is meant to demonstrate the intended spread in CCR5 frequencies among HSCP mixtures given the distinct experimental groups and to demonstrate that these levels are relatively stable over time in the T cell compartment (as demonstrated in the myeloid compartment in Figure 7C). These data are all derived from pre-infection time points, thus no virus selection has yet occurred. We have denoted in the figure legend (lines 630 – 631) that this is from pre-infection time points to clarify this point.

However, the reviewer of course brings up a very salient question regarding whether virus infection and continued replication selects for CCR5 edited, and thus protected, cells over time, especially in mice with suboptimal levels of edited HSCPs where infection is more frequent. To address this, we can compare the levels of CCR5 edited B cells vs CCR5 edited CD4+ T cells at necropsy in the bone marrow of HIV-infected mice. We would suggest that B cells represent a reference population (approximating pre-infection CCR5 editing levels), as they cannot be frequently infected with HIV and thus are unlikely to demonstrate significant enrichment for CCR5 null cells, especially as B cells do not naturally express CCR5. However, CD4+ T cells, within the same mouse, should have an advantage if null for CCR5 and would become enriched over time. Comparison to the B cell population thus could control for initial levels of editing, which are variable among mice even in the same HSPC mix engraftment group (Fig. 7B). The figure below demonstrates that there is indeed a significant increase in CCR5 gene disruption in the CD4+ compartment compared to the B cell compartment, suggesting enrichment for protected/edited CD4+ T cells post infection. However, other publications have demonstrated an enrichment in edited cells when editing is suboptimal, and as these studies were not set up to measure this specifically, we did not include these results in the manuscript.



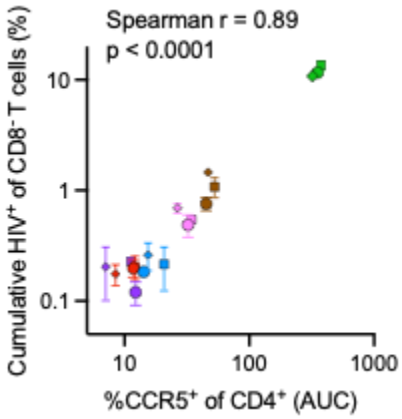
Minor Points

-The abstract and introduction mention that this platform is “scalable.” Please expand either with data (e.g. validation that clinical-scale products can be edited with similar efficiency) or provide references to support this statement.

We have now further expanded on this point by linking this study and process to the two clinical trials (NCT03745287, NCT03655678) which use the same process and methodology (line 88). We have also referenced the manuscript in which these results were reported to more clearly associate the approach used here with the clinical trials initiated by Vertex Pharmaceuticals and CRISPR Therapeutics.

-The correlation data in Figure 3D appears to be strong; if possible, please apply a statistical test.

Thank you for pointing out this omission. We have now added the results of a statistical test to support the visual association depicted in Figure 3D (as shown below), and described the statistical test used in the figure legend.



-The data suggest that NSG and NBSGW models were equally informative in HIV infection assays, but it would be helpful to clearly indicate in the results text which model was used in which experiment, and the rationale. For example, why was NBSGW chosen in Figure 7?

NBSGW mice were chosen for the study in Figure 7 for two reasons:
1) The study required efficient engraftment to recapitulate the HSC mix given, and these mice have been shown to have an advantage, albeit modest, for engraftment of human HSCs [McIntosh BE, Stem Cell Reports, 2015].
2) NBSGW mice do not require higher doses of radiation to create space for human HSC engraftment, which leads to a longer life span and results in an overall more robust model, which we anticipated would be required for the extensive time frame needed to evaluate hematopoiesis and resistance to HIV challenge in the repeat challenge model depicted in Figure 7A.

However, we did not perform controlled engraftment experiments in NSG vs NBSGW mice using the same HSC donor, and thus did not want to comment on the superiority of the model system, as the studies presented in this manuscript were not designed to formally evaluate the differences in immunodeficient transplant recipients.

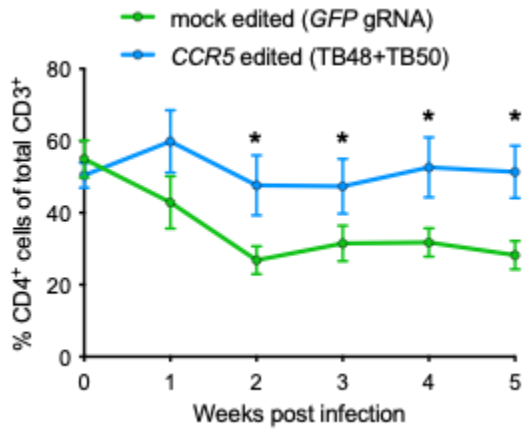
There are now publications formally comparing these mouse backgrounds and for an application relevant to this manuscript [Choo S. et al., Bood Advances, 2024]. Thus, we have now cited these comparisons in text (lines 217 – 291) as justification for the use of the NBSGW model.

-Lines 198-199: are reference(s) available to clarify what is meant by non-productive or "cryptic" HIV infection?

The term "cryptic" in HIV infection and persistence is typically used to refer to very low-level variants present near the time of HIV transmission that subsequently become the dominant species many months later (PMID: 28759651). This was meant to reference the potential presence of low-level infection that could have been undetectable by plasma viremia, but could have grown out eventually if the humanized mouse studies would have been carried forward longer. However, this is not the best terminology to describe this potential scenario, thus we have removed the word to reduce confusion and added additional text to clarify (line 201).

-Figure 6B: is the difference in CD4+ cells between mock and CCR5-edited animals statistically significant?

Yes, the frequency of CD4+ T cells within the total T cell compartment is significantly lower in HIV-infected humanized mice with mock edited HSCs, except at the pre-infection (week 0) and 1-week post infection time points. Asterisks have been added to Figure 6B to denote p values < 0.05 and the statistical test has been added to the figure legend (example of update figure below).



-There are several references to "Supplementary Text" which doesn't appear to be included in the manuscript.

We sincerely apologize for this mistake. Though available at the time, the Supplementary Text detailing specifics of the mathematical modeling was accidentally omitted from the original submission. It has now been included in its entirety in the resubmitted document under "**Supplementary Text 1. Mathematical Modeling**" (lines 769 – 1111).

Reviewer #2 (Remarks to the Author)

Claiborne and colleagues describe here a CRISPR-Cas9/RNP platform capable of producing a human HSPC transplant with high frequency CCR5 editing. They successfully reconstitute the immune system in xenograft mice which are highly refractory to HIV replication, following several challenges with CCR5-HIV strains. Moreover, upon grafting mice with different proportions of CCR5 edited HSPC (0% to 96%) they show that the protective value of HSPC CCR5 editing decreases quickly and becomes negligible between 26% and 54% CCR5 editing. Overall their results suggests that it is potentially possible to recapitulate a curative CCR5D32/ D32 transplant in an autologous HSCT context, although high frequency of CCR5 editing is needed. The manuscript is clear, well written and although similar papers have been published dealing with CCR5 editing in human HSCT prior to in vivo testing in mice, this is the first time that a CCR5 editing reaches values approaching 100% achieving a complete protection in vivo. However, there are some points that need further clarification.

Major comments

1. The authors do not show any data on cell viability after transfection and CRISPR-Cas9 editing. Another important issue is the % recovery of edited cells related to the input. This data is key to assess the feasibility of applying the technique in a clinical setting.

The reviewer raises an important point regarding the feasibility of translating this approach to a clinical setting. We found that viability was >85% in all 3 HSCP donors post electroporation and CRISPR/Cas9 editing. Most importantly, the percent recovery was >95% for all 3 donors tested. We have now included this data as a supplemental table **(Supplementary Table 1)** in the resubmitted manuscript (referenced in line 163). Additionally, the approaches and methodology used are the same as those used for two current clinical trials (NCT03745287, NCT03655678), and this has now been referenced in text (line 88), suggesting that the approaches utilized in this manuscript are sufficient to translate this approach to the clinic.

2. Despite the detailed description of off-target effects at DNA level that is used for selecting those guideRNA without off-targets, there is no reference on putative effects on cell functionality. Authors should demonstrate that immune cell function is preserved upon CRISPR-Cas9 treatment in terms of in vitro activation upon general stimuli and/or the lack of major changes at transcriptomic level.

Though the reviewer brings up an important point regarding immune function of T cells derived from edited HSCs, there exists a significant body of literature supporting that CCR5 edited T cells have no apparent functional defects:

1) Tebas et al. infused CCR5-edited (generated through zinc-finger nuclease methodology) autologous CD4+ T cells in aviremic HIV+ individuals. Gene modified CD4+ T cells persisted in the blood and trafficked to the rectal mucosa. These data suggest that the persistence and homing of CD4+, CCR5 edited T cells is intact.

Reference: Tebas et al., Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV, NEJM, 2014. PMID: 24597865

2) A follow up trial by Tebas et al. infused a similar zinc-finger mediated CCR5 edited autologous CD4+ T cell product and with updated cell manufacturing protocols. In this study, infusion of the CCR5-edited CD4+ T cell product was associated with enhanced CD8+ T cells responses in individuals exhibiting post rebound control after a structured treatment interruption. This data suggests that CCR5-edited T cells retain helper function enabling antigen-specific CD8+ T cell responses.
Reference: Tebas et al., CCR5-edited CD4+ T cells augment HIV-specific immunity to enable post-rebound control of HIV replication, J Clin Invest, 2021. PMID: 33571163

3) Knipping et al. disrupt both the CCR5 and CXCR4 co-receptors in primary T cells and hematopoietic stem cells using a base editing methodology. Dual-edited primary T cells demonstrated no deficit in effector cytokine production when stimulated with PMA/ionomycin. Moreover, dual-editing of HSCs resulted in no deficit compared to control edited HSCs with respect to colony formation in methylcellulose assay, much the same as our results in **Figure 4B**.
Reference: Knipping et al., Disruption of HIV-1 co-receptors CCR5 and CXCR4 in primary human T cells and hematopoietic stem and progenitor cells using base editing, Mol Ther, 2022. PMID: 34737067

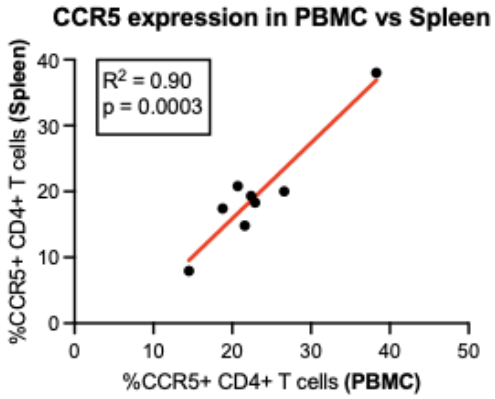
4) Hale et al. generated CD19-targeted CAR T cells which were also edited to ablate CCR5 expression for the treatment of HIV-associated B cell malignancies. CCR5 null CART19 were functionally indistinct from CCR5 intact CART19 in in vitro killing and cytokine production assays and in in vivo tumor suppression experiments using xenograft mice. This further suggests that disruption of the CCR5 locus does not significantly impair T cell function.
Reference: Hale et al., Homology-Directed Recombination for Enhanced Engineering of Chimeric Antigen Receptor T Cells, Mol Ther Methods Clin Dev, 2017. PMID: 28345004

Thus, given the lack of evidence suggesting functional deficits in CCR5-edited T cells in the literature, and our own results demonstrating no defects in T cell differentiation or reconstitution, we would elect to forgo experiments confirming T cell function to pan stimuli in order to streamline the narrative as these experiments are outside the scope of the initially intended study designs.

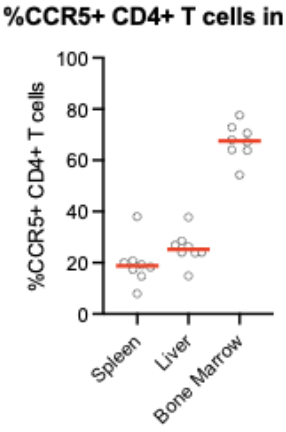
3. Although data on % of CCR5 expression is pretty clear in mice blood, additional data on biodistribution in tissues is missing, especially in cases where editing is below 90%. Are CCR5 edited cells equally distributed between tissues? How is the situation in lymph nodes? This data would be relevant for latency establishment/reservoir replenishment if CCR5 edited cells are not equally distributed thorough the body.

The reviewer is correct in that CCR5 expression levels as measured in the blood can only be a surrogate for tissue expression levels, and would likely be an underestimate of CCR5 expression based on our past unpublished and current metadata associated with this study.

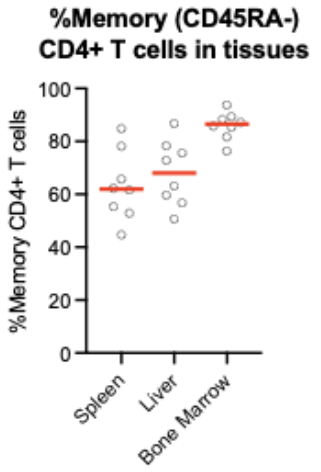
In a re-analysis of unpublished CCR5 expression data in tissues derived from Hu-BLT mice that are HIV-naive, we observe that CCR5 expression in CD4+ T cells in the blood is most closely related to the spleen:



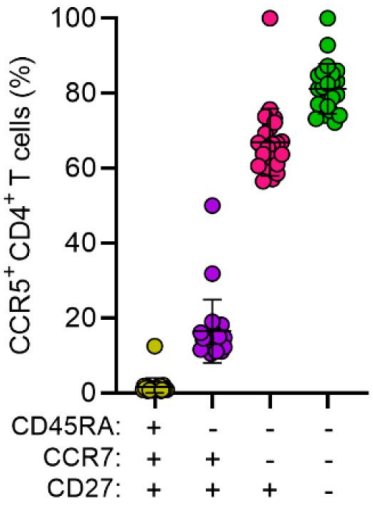
However, other tissues such as the liver, and especially the bone marrow, harbor CD4+ T cells with greater CCR5 expression:



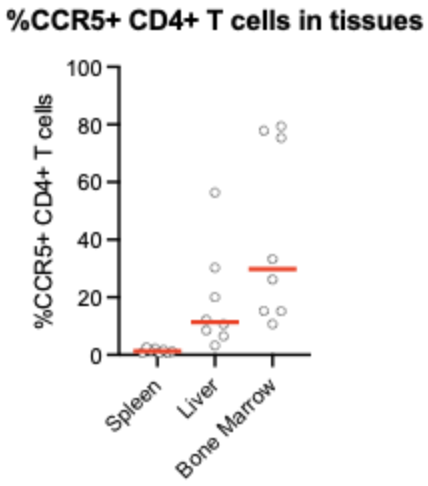
This is likely driven by differences in memory T cell distribution between tissues, and we have seen that the bone marrow harbors a greater frequency of memory CD4+ T cells:



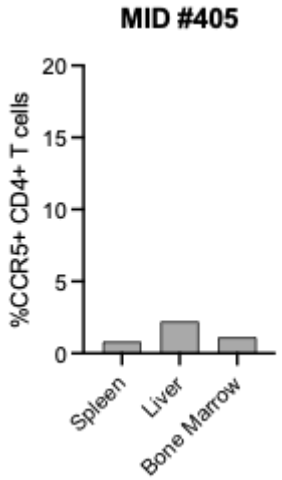
Additionally, we have previously published that effector memory CD4+ T cells that are defined as CD45RA-, CCR7-, and CD27- have the highest frequency of CCR5 expression (data from Maldini & Claiborne et al., *Nat Med*, 2020; Supplementary Figure 3b) which could support that the greater frequency of memory CD4+ T cells in the bone marrow is related to the higher expression of CCR5 on this particular CD4+ T cell compartment:



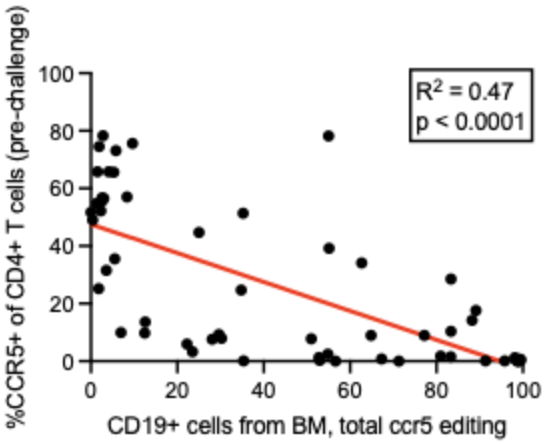
In applying this paradigm to the current study, we observe a similar trend. We have limited flow cytometry data from a subset of animals necropsied after the completion of the study depicted in Figure 7. In an analysis of 8 animals that received mock-edited HSCs only (no CCR5 editing) and were challenged and productively infected, we again observe that there are more CCR5 expressing CD4+ T cells in the bone marrow and liver as compared to the spleen:



However, when editing is robust, even bone marrow resident CD4+ T cells will have low CCR5 expression. For the animal below (MID 405) which belonged to the 50% CCR5-KO HSC experimental group, editing was estimated at a maximum of 73.7% in CD19+ B cells sorted from the bone marrow at necropsy, and CD4s derived from the spleen, liver, and bone marrow all have low CCR5 expression levels:



Finally, the level of editing measured in sorted B cells from the bone marrow at the time of necropsy for the entire cohort was reasonably well correlated with the percentage of CCR5 expressing CD4+ T cells measured in the blood directly prior to study enrollment and HIV challenges:



This correlation suggests that even though we are comparing different cell types (CD19+ B cells vs CD4+ T cells), different time points (post study vs pre study), and different tissue compartments (bone marrow vs peripheral blood), editing frequencies predict CCR5 expression levels. We would extrapolate this data to suggest that though there may be significant differences in the frequency of CCR5 expressing cells in different tissue compartments, there is no evidence for differences in distribution of CCR5-edited cells specifically, as all compartments tested (spleen, liver, BM) were similarly reduced in CCR5 expression when editing was robust. Of note, lymph nodes are very small in Hu-HSC mice, especially humanized mice made with adult mobilized HSCs, and thus it was not technically feasible to sort cells and measure editing in this compartment.

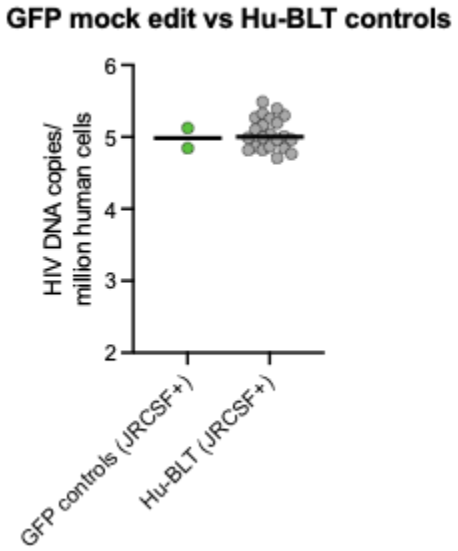
4. The authors propose 2 potential protective mechanisms of CCR5 editing. Although the "barrier at inoculation" is pretty clear from the data presented, this is not the case for "Barrier at proliferation" mechanism. This latter mechanism, albeit plausible, is not well-supported by the data presented. The most relevant experiment that supports is that shown in Fig 7H, although a clear correlation between CCR5-editing and VL is not observed with the data provided.

We agree with the reviewer that the data of this study does not confirm the proposed "Barrier at Proliferation" mechanism. Our intention with proposing this mechanism was to highlight another means by which CCR5 editing may protect from transmission and/or viral rebound post ART interruption and that future studies examining the relevance of the "barrier at proliferation" mechanisms would be enlightening. We apologize for the confusion and have sought to clarify the language used in the last paragraph of the Results (lines 258 – 268), the commentary on viral rebound during ART interruption in the Discussion (lines 318 – 328), and the caption of Figure 7 so as to clearly indicate that the "barrier at inoculation" is supported by the data and the "barrier at proliferation" is an hypothesized mechanisms, which is not evident from this data. Additionally, to more accurately describe the hypothesized mechanism, we have shifted terminology in the manuscript from "barrier at proliferation" to "barrier at dissemination."

Minor comments

5. The number of replicates in some in vivo experiments should be increased or all animals have to be included in the graph, as is the case of Fig 6C.

We included all animals for which tissues were available. The unfortunately limited numbers of animals in Figure 6C is due to the difficulty in locating lymph nodes in Hu-HSC mice generated with adult mobilized HSCs, and the paucity of cells contained within whenever they can be located. Humanized mice generally have visible lymph nodes in the neck (axillary and cervicals) or the mesenteric lymph nodes. The purpose of Figure 6C was to demonstrate that we could not detect any HIV DNA in relevant lymphoid tissues for mice that were refractory to very high dose HIV challenges. Thankfully, we were able to run all possible CCR5-KO mice for HIV DNA measurements by ddPCR, as 1 animal died early from GvHD (denoted by double dagger in Figure 6A) and 1 animal was set aside to demonstrate that mice remained susceptible to CXCR4-tropic strains of HIV (Figure 6D). Unfortunately, only 2 control mice had lymph nodes with enough cells suitable for HIV DNA measurements, but we maintain that these mice still serve as a useful measurement for the typical levels of HIV DNA that we observe in HIV-infected humanized mouse lymphoid tissues. The below data shows the HIV DNA burden, as measured by the same ddPCR method and primers described in this manuscript, for CD4 T cells sorted from viremic HIV-JRCSF infected Hu-BLT mice, where lymph nodes are generally more frequently useable for HIV viral burden measurements:



Thus, though we admit the numbers are suboptimal, we believe that the data clearly demonstrates that we could not find the presence of HIV DNA in CCR5-KO mice that maintained negative plasma viral loads.

6. The authors use 2 distinct mice strains. However, the choice of one or the other is not clear. Why a given strain is used and which are the reasons to change from one to the other? Please clarify in Fig 4-5 and results section.

NBSGW mice were chosen for the study in Figure 7 for two reasons:
1) The study required efficient engraftment to recapitulate the HSC mix given, and these mice have been shown to have an advantage, albeit modest, for engraftment of human HSCs [McIntosh BE, Stem Cell Reports, 2015].
2) NBSGW mice do not require higher doses of radiation to create space for human HSC engraftment, which leads to a longer life span and results in an overall more robust model, which we anticipated would be required for the extensive time frame needed to evaluate hematopoiesis and resistance to HIV challenge in the repeat challenge model depicted in Figure 7A.

However, we did not perform controlled engraftment experiments in NSG vs NBSGW mice using the same HSC donor, and thus did not want to comment on the superiority of the model system, as the studies presented in this manuscript were not designed to formally evaluate the differences in immunodeficient transplant recipients.

There are now publications formally comparing these mouse backgrounds and for an application relevant to this manuscript [Choo S. et al., Bood Advances, 2024]. Thus, we have now cited these comparisons in text (lines 217 – 219) as justification for the use of the NBSGW model, which was specifically chose for the experiment detailed in Figure 7, where long-term follow up and robust health of the mice was required.

7. Line 131-132. The authors comment that the frequency of CCR5+ cells is maintained for 10 days in culture. As far as I know, CRISPR-Cas9 editing should be stable and irreversible. Please clarify.

The reviewer is correct, and we have made edits to correct our misleading terminology. Our intention was to state that initial editing frequencies observed at 48 hours post EP (Figure 2A-D) resulted in a stable phenotype. As editing in these experiments was not >90% but closer to a median of 60%, there was likely a large population of heterozygous CCR5-KO or CCR5 wt cells, which are not reflected in the total %CCR5+ pool. For example, total CCR5+ cells could have been as great as 50% of the culture, and activation status over time could have changed the initial CCR5 expression frequency over time, and thus our intention was to comment on that only. We have made text edits in an attempt to succinctly clarify this point (lines 133 – 134).

8. Fig 4B. If results represent the number of colonies derived from a single donor, what do the error bars represent? Are there replicates? Please clarify.

Thank you for pointing this out, and we apologize that the initial wording in the figure legend was misleading. The figure legend been updated to clearly state 3 donors were used to generate the data in Figure 4B, and to clarify what the error bars represent: the standard deviation among all 3 donors (lines 580 – 581).

9. Line 232-234. Statistics is missing in figure, although results section claims for a significant reduction.

We have added p-values to Figure 7E for comparisons of survival curves for the various transplant groups.

10. Supplementary Texts are missing, but mentioned in the text (lines 258-259, 310 and 630-631)

We sincerely apologize for this mistake. Though available at the time, the Supplementary Text detailing specifics of the mathematical modeling was accidentally omitted from the original submission. It has now been included in its entirety in the resubmitted document under "**Supplementary Text 1. Mathematical Modeling**" (lines 769 – 1111).

11. Fig 6A. There is a symbol in one CCR5 edited mice at 1 week post challenge and no legend is provided.

Thank you for catching this unintended omission. The double dagger ‡ denotes an animal that required euthanasia before the predetermined experimental endpoint due to health concerns. The figure legend has now been updated with this information (line 612).

12. Fig 6C. It would be nice to have data for all animals tested, those in Fig 6A.

We appreciate the reviewer's concern and can assure that all available data has been included. All possible CCR5-KO mice were included, as there was n=6 CCR5-KO mice in the initial study depicted in Figure 6A, but one mouse was euthanized early (denoted by double dagger), and one mouse was set aside for CXCR4-tropic virus challenge, leaving 4 remaining CCR5-KO mice which are included in Figure 6C. Unfortunately, lymph nodes of sufficient size and cellularity were not able to be obtained from the majority of control mice, but the two where appreciable lymph nodes were recovered are included.

13. Fig 7B. The 0% CCR5 edited group (which is mentioned in line 218) should be added as a control

Thank you for pointing out this mistake. The text refers to a theoretical 0%, not a measured 0%, and thus should not have been referenced with respect to Figure 7B. Though TIDE and ddPCR measurements do not, of course, have 100% sensitivity, the control animals run showed editing percentages <10% (mean of 3.8%, median of 3.2%). These measurements, though helpful for giving a range of sensitivity for the assay, do not reflect real editing percentages. Thus, we would ask to keep the figure as it stands to maintain accuracy, while removing the confusing mention of "mean of 0% editing" in the text (line 223).

14. Fig 7E. VL of the animals included in the experiment should be shown. It is relevant to show

the quantification of viral replication. This may help to clarify the relation between VL and CCR5 editing that is claimed in the next 3 subfigures (7F-H)

To clarify, panels 7F and 7G are intended to demonstrate the relationship between actual CCR5 editing (Figure 7B) rather than experimentally intended (Figure 7A) and the susceptibility to repeated viral challenges, and are not meant to reflect the influence of any viral load data. The raw viral load data for each mouse is less informative than would otherwise be the case due to the repeat challenge model. Here, viral load follow up is not equivalent among mice as it depends on when they were infected in the challenge cycle, which is of course also influenced by CCR5 editing frequency. Thus, unfortunately, comparing longitudinal viral loads is uninformative in this experiment as the study design was not intended to specifically test this. The data in Figure 7H is simply meant to demonstrate that we observed only a trend between editing frequency and the peak viremia among mice, as peak viremia was the only metric that could be reliably obtained for each mouse challenged.

15. Figure 7. Colour code is confusing and makes it difficult to distinguish between the distinct group of mice.

We apologize for any confusion, but would like to keep the color scheme for consistency, where unedited/low editing groups are green in color and higher edited groups are blue in color. This roughly matches with the binary color scheme in Figure 6.

Reviewer #3 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.