Peer Review File

Manuscript Title: Rejuvenating aged immune systems by depleting myeloid-biased hematopoietic stem cells

Parts of this Peer Review File have been redacted as indicated to remove third-party material

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):
In this manuscript, Ross, Myers et al attempt to use antibody-mediated depletion of myeloid-biased hematopoietic stem cells (HSCs) as a strategy to promote rejuvenation of the aged immune system. The authors identify four key cell surface proteins, CD150, CD62p, Neo1, and CD41 that exhibit elevated expression in aged and myeloid-biased HSCs. They then develop approaches to optimize depletion of myeloid-biased HSCs using antibodies against those surface proteins in combination with antibodies targeting CD47 and cKit, and they evaluate the effects on the hematopoietic system in vivo. The authors report that putative depletion of myeloid-biased HSCs promotes expansion of lymphoid progenitors and naïve B and T cells in older animals as well as a decline in inflammatory cytokines. Finally, the authors demonstrate that some of these targets are similarly enriched in aged human HSCs, raising the possibility that this strategy could be employed to promote rejuvenation of the human blood/immune system.

Overall, this is an intriguing and innovative study with high potential impact. However, there are two critical limitations of the study that must be thoroughly addressed:

1. This study exclusively depends on expression levels of CD150 to distinguish between myeloid-biased and balanced HSCs. While this marker is well-established under steady state and aging conditions in mice, it is not at all clear whether this is sufficient to distinguish between these subsets of HSCs after antibody treatment, especially with anti-CD150. The authors must validate that antibody treatment functionally depletes myeloid-biased HSCs rather than just altering their cell surface phenotype. Ideally, this would be done with transplantation assays in vivo and could be complemented by gene expression data.

2. Effects of antibody treatment on hematopoietic cell populations are reported only in terms of frequencies. This is extremely problematic as cell frequencies can be significantly influenced by off-target effects of the antibody treatment rather than by changes in HSC output. In all cases, absolute numbers of cells must be reported.

In addition to these overall issues, the following specific points should be addressed:

3. The increase in CLP frequency after just 1-week (Fig. 2F-H) is interpreted by the authors as increased output from HSCs. The kinetics of this rejuvenation suggest that HSCs are rapidly contributing to hematopoiesis following antibody treatment. What are the effects of antibody

depletion on HSC and progenitor proliferation?

4. Other possible explanations for the increased frequency of CLPs is that there is broad depletion of other hematopoietic cells and/or that these progenitors expand in response to a loss of mature lymphoid cells. A much more comprehensive assessment of the effect of acute antibody treatment on B lineage cell numbers (progenitors and mature cells) is warranted.

5. The restoration of naïve T cells in aged, antibody-conditioned mice implies functional restoration of the involuted thymus. Characterization of thymic cellularity should be included.

6. Depletion with anti-CD150 also induces increased frequency of myeloid progenitors (Fig. 2F). What is the effect of antibody depletion on mature myeloid, megakaryocytic and erythroid lineage cells? Taken together, points 4-6 emphasize the need to more fully characterize the effects of acute antibody treatment on hematopoietic cell numbers.

7. There are some striking inconsistencies regarding HSC frequencies and how data are reported. For example, Extended Data Fig. 4F shows HSC frequency as being ~1% of live cells, which is off by two orders of magnitude. Extended Data Fig. 4D and 4G reports myeloid-biased HSCs as a fraction of total HSCs at a value of “2”, which implies that 200% of HSCs are myeloid-biased. Again, it would be much more straight forward if all data include reporting of absolute numbers.

8. It is unclear that inclusion of anti-CD47 and anti-cKit help to optimize myeloid-biased HSCs. Extended data Fig. 4A shows no additive effect of these antibodies on myeloid-biased HSC depletion, although it inexplicably increases the frequency of balanced HSCs. Similarly, combining these antibodies with CD41 also has no effect on myeloid-biased HSCs (Extended data Fig. 4B). The authors justify that inclusion of these additional antibodies also has no effect on total HSC numbers, but the relevant experiment appears statistically underpowered to draw that conclusion (Extended Data Fig. 4F), which shows that 2/4 mice exhibit more than a 50% decline in HSC frequency. Instead of using an anti-cKit antibody that would broadly recognize hematopoietic progenitors, could additive effects be achieved by combining CD150, Neo1 and/or CD62p antibodies?

9. The relevance of human HSC expression data is questionable. CD150 has been shown to not be an effective surface marker for identifying human HSCs (Blood 2011; 117(5):1550–1554.). The data would be much more compelling and impactful if the study included identification and characterization of myeloid-biased human HSCs. Otherwise, the human data may be better suited as a supplementary figure.

10. In addition to a more comprehensive assessment of off-target effects in the hematopoietic system (comments 4-6), the authors should discuss potential effects on non-hematopoietic tissues. To what extent are these markers expressed by other tissues? Are changes in other tissues noted?

11. How do the authors explain the durability of the response to a single course of antibody administration (Fig. 3B)? Have antibody levels been measured in the blood at different timepoints after treatment?

12. Antibody conditioning increases the frequency of balanced HSCs in old mice. Is there any expectation that the HSCs are rejuvenated or are they still functionally compromised as compared to young adult HSCs?

13. There is a minor spelling mistake in the word “clone” in the x-axis labels of Extended Data Fig. B-D and F-H.

Referee #2 (Remarks to the Author):

The manuscript by Ross et al provides evidence for a novel therapeutic strategy to restore lymphopoiesis and adaptive immune function in aged individuals. Their rationale is that selective depletion of myeloid-biased HSCs, which is observed with aging, will allow remaining lineage-balanced HSCs to reconstitute lymphopoiesis. They discover using antibody-mediated depletion of several target molecules selectively expressed on myeloid-biased HSCs that they are able to restore production of lymphoid progenitor cells and mature lymphocytes in vivo in aged mice. Further, they demonstrate that these same antigens are expressed on the surface of human HSCs demonstrating proof-of-principle that a similar antibody-mediated depletion strategy may work in aged humans.

The work is original and significant, and addresses a critical health problem in the aging population. The approach appears valid with good data quality and appropriate use of statistics, as well as appropriate credit to previous work. However, several conclusions are not adequately supported by experimental data and additional experiments and controls are needed to robustly support the conclusions of this paper.

1. While CD150 expression has been demonstrated to enrich for functional myeloid-biased versus lineage-balanced HSCs, this same rigorous definition has not been applied to the new markers of myeloid-biased HSCs that they propose here; Neo1, CD41 and CD62p. To formally demonstrate that these populations are myeloid-biased in function, prospective isolation and transplant of high vs. low (or positive vs. negative) populations should be performed to demonstrate that these markers enrich for functional myeloid-biased HSCs.

2. Assessment of relative specificity of myeloid-biased HSCs markers to this population relative to lineage-balanced HSCs and downstream HPCs (Figure 1) is very strong. However, this flow cytometric analysis should also include mature hematopoietic cell populations to provide insight into potential off-target effects of antibody-based depletion.

3. In Figure 2, the authors show efficacy of their depletion protocol using flow cytometric characterization of stem & progenitor cell populations. This should be supported by quantitation of total numbers of each of these populations, to support the conclusion that lymphoid populations are restored to similar 'levels'. Furthermore, complete blood counts and mature hematopoietic cell frequencies in the peripheral blood are needed to rule out off-target effects and to show restoration of 'youthful' hematopoiesis. Lastly, to formally prove that differences in progenitor output are truly derived from different proportions of HSCs rather than a direct effect on progenitors, lineage tracing

studies, or ex vivo single HSC output studies, are needed to track output of individual HSCs to myeloid vs. lymphoid progenitors.

4. In the experiment shown in Figure 3A, for the same rationale as above, complete blood count data should be included for these mice. To directly support the conclusion of 'rejuvenating the immune system', functional T and B cell assays should be performed on material isolated from these mice.

5. The human data collected so far shown in Figure 4 is promising, however, demonstration that these markers increase at the surface level in CD34+ cells in aged vs. young humans is needed to support the conclusion. Furthermore, as suggested for the murine studies above, prospective isolation of CD62p+ vs. CD62p- followed by transplant into recipient xenografted animals is needed to prove that these markers enrich for myeloid-biased HSCs in the human context, as this has not been demonstrated previously.

6. The section 'Antibody-mediated depletion of myeloid-biased HSCs in vivo' could be much more succinct and streamlined; much of this information is more appropriate for supplemental materials.

Referee #3 (Remarks to the Author):

In this study, Ross et al. present a series of intriguing and interesting experiments predicated on the idea that the age-related myeloid deviation in the HSC compartment could be remedied by selective depletion of myeloid-biased HSC (My-HSC), and by consequently allowing balanced HSC (Bal-HSC) to dominate hematopoiesis and generate adequate numbers of lymphoid cells. The authors begin their study by examining differential gene and protein expression on the two HSC subsets in an attempt to identify potential targets to eliminate/reduce My-HSC. They present a careful and systematic validation of potential targets, along with optimization of dosage, antigenic density and depleting antibody isotypes. They then characterize the impact of My-HSC depletion on CLP / lymphoid (IL-7Ra+) progenitors (presumably in the bone marrow) and on age-sensitive T and B cell subsets and cytokines/chemokines in peripheral blood of treated mice. They finish their work with a preliminary analysis of analogous molecular targets in human bone marrow, and conclude that their conditioning regimens rejuvenate the old immune system.
The work is conceptually original and for the most part logically described. There are, however, critical missing experiments necessary to evaluate the authors' claims, as well as omissions, inaccuracies and errors in statistical approaches.
Major criticisms:
1. Data on immune rejuvenation is inconclusive and incomplete. The authors analyze peripheral blood, which is hardly a relevant compartment for residence and function of naïve T and B cells. Secondary lymphoid organs (SLO) are critical sites of naive T and B cell residence, maintenance and function, and there is accumulating evidence that these organs, particularly lymph nodes, suffer alterations with aging that compromise their structure and functon(Becklund, B., et al., Sci. Rep. 2016; Richner, J.C. et al., Plos Pathogens, 2015; Thompson, H.L. et al., Aging Cell, 2019). There is further evidence from the work of Dorshkind and coworkers that bone marrow stromal environment, in addition to the HSC compartment, also undergoes a series of age-related changes,

and the same seems to be true of thymic (Lancaster, J.N. et al., Aging Cell, 2022) and lymph node (Sonar, S.A. et al., PNAS, 2022) stroma. Most relevant to this work, even when thymic rejuvenation via sex steroid blockade or growth factor administration is achieved, and blood shows an increase in newly produced T cells, or if naïve T cells are injected exogenously, SLO are not necessarily able to host such newly produced cells and this does not result in improved functional immunity (Thompson, H.L. et al., Aging Cell, 2019; Sonar, S.A. et al, PNAS 2022). Therefore, the authors need to document significant improvements in naïve lymphocyte numbers in SLO and to demonstrate whether or not this yields improved survival and immunity against infection. This is a necessary set of experiments.

2. In many experiments, including the critical experiments in Fig. 3, panels b-I, the authors have used unpaired parametric one-tailed t tests to evaluate statistical significance. This is not appropriate when three treatments are compared, and also may not be appropriate if data are not normally distributed, which is often the case with the analysis of older animals. Authors need to justify their use of this test, which is known to be susceptible to both type I and II errors.

3. To interpret results in Figure 3, all subsets need to be analyzed not only by % change but also by absolute numbers. Similarly, the main memory cell subsets should be analyzed as well to be able to interpret whether changes in frequencies are reflective of absolute changes in one or the other cell subset(s). Specifically, is the treatment truly producing more naïve lymphocytes, and is that the reason for the decline of “exhausted” or age-related cell subsets, or are the latter also diminishing in absolute numbers?

4. In Figure 3b, the authors need to explain how and why would the numbers of CLP be further increasing from week 1 post treatment (not significant) to week 8 and 16 (progressively more and more significant).

5. In Fig. 3j, did the authors analyze the most commonly increased cytokines with aging, such as IL-6, CRP and TNFa? These are commonly increased in 24-mo old mice.

6. Human studies are a reasonable, albeit preliminary, proof of principle. However, myelodysplastic syndrome and malignancy data is a clear add-on, that at the present should be deleted and incorporated into a more complete study.

Other comments:

• The manuscript needs thorough editing and tightening of repetitive parts. There are several sections that meander while repeating considerations on antibody depletion therapy (e.g. lines 114-116 and then on lines 127&128; on myeloid progenitor stages on page 9, in two places, and a long section on lines 201-208 that can be condensed).

• Important information is difficult to find in figures and the text. Time points, tissue sources and other critical experimental details need to be transparently listed everywhere.

• Animal strains and ages should similarly be consistently defined everywhere.

• It would be really useful for the field if this group would use its unique strengths to demonstrate at what point My-HSC become detrimental to lymphopoiesis – it seems that having 65% Bal-HSC is not too shabby, but the data in this manuscript suggest that this is already too little. This is not a must for the present study, but if the authors have a cross-sectional data set, it would be nice to include it.

Author Rebuttals to Initial Comments:

Referees' comments:

Referee #1 (Remarks to the Author):
In this manuscript, Ross, Myers et al attempt to use antibody-mediated depletion of myeloid-biased hematopoietic stem cells (HSCs) as a strategy to promote rejuvenation of the aged immune system. The authors identify four key cell surface proteins, CD150, CD62p, Neo1, and CD41 that exhibit elevated expression in aged and myeloid-biased HSCs. They then develop approaches to optimize depletion of myeloid-biased HSCs using antibodies against those surface proteins in combination with antibodies targeting CD47 and cKit, and they evaluate the effects on the hematopoietic system in vivo. The authors report that putative depletion of myeloid-biased HSCs promotes expansion of lymphoid progenitors and naïve B and T cells in older animals as well as a decline in inflammatory cytokines. Finally, the authors demonstrate that some of these targets are similarly enriched in aged human HSCs, raising the possibility that this strategy could be employed to promote rejuvenation of the human blood/immune system.

Overall, this is an intriguing and innovative study with high potential impact. However, there are two critical limitations of the study that must be thoroughly addressed:

Author's Response
We thank the reviewer for the excellent summary of our findings and for the thorough critique of our work.

1. This study exclusively depends on expression levels of CD150 to distinguish between myeloid-biased and balanced HSCs. While this marker is well-established under steady state and aging conditions in mice, it is not at all clear whether this is sufficient to distinguish between these subsets of HSCs after antibody treatment, especially with anti-CD150. The authors must validate that antibody treatment functionally depletes myeloid-biased HSCs rather than just altering their cell surface phenotype. Ideally, this would be done with transplantation assays in vivo and could be complemented by gene expression data.

Author's Response
1.1: We thank the reviewer for raising several important points related to the use of cell-surface markers to distinguish between myeloid-biased and balanced HSCs, including after antibody treatment. We agree with the reviewer on the importance of these points, as we also carefully considered each of these factors at the outset of our study, prompting us to incorporate a series of controls and independent analyses into our experimental design. As the reviewer discusses, it is well-established that CD150 expression distinguishes between myeloid-biased and balanced HSCs under steady state and aging conditions in mice[1]. We considered $CD150^{Hi}$ cells to be age related and myeloid biased HSCs in our study, but we did not exclusively depend on CD150 expression to define HSC subsets. We applied several orthogonal approaches to verify our examination of my-HSCs at baseline and after antibody-mediated depletion. First, in addition to CD150, we verified the steady state and depletion levels of my-HSCs using the independent cell-surface protein NEO1, which we have reported phenotypically define functional my-HSCs *in vivo*[2-4] (**Ext. Data Fig. 4a–d** and new **Ex. Data Fig. 4p–q**). Second, to address the use of cell-surface markers after antibody treatment, we validated multiple independent 'non-masking' antibodies, which enabled the detection of target cells in the presence of antibody treatment (**Ext. Data Fig. 3a–l** and **Ext. Data Fig. 5l–q**). Third, we developed several antibody-conditioning protocols targeting the independent my-HSC markers CD150, NEO1, and CD62p to deplete my-HSCs (**Fig. 2** and new **Ex. Data Fig. 4p–q**). Fourth, we conducted long-term *in vivo* functional studies after my-HSC depletion demonstrating the reversal of phenotypes associated with my-HSCs (**Fig. 3**), including new *in vivo* experiments on functional immunity (new **Fig. 4** and new **Ex. Data Fig. 9**). Further details are below:

(i) Independent my-HSC markers: To demonstrate that our results do not exclusively depend on a single marker to define my-HSCs, we evaluated the depletion of my-HSCs based on an independent functionally validated marker of my-HSCs: $NEO1^{2}$. We demonstrate that in addition to depleting $CD150^{Hi}$ HSCs (**Ext. Data Fig. 4a**), anti-CD150 depletes $NEO1^{+}$ HSCs by frequency (**Ext. Data Fig. 4c)** and absolute number (new **Ext. Data Fig. 4d**).

(ii) Independent non-masking antibodies: To address the use of cell-surface markers to distinguish between myeloid-biased and balanced HSCs after antibody treatment, we performed experiments to control for antibody 'masking', whereby *in vivo* antibody might prevent the detection of target cells[5]. We identified multiple independent non-masking antibodies to CD150, which enabled the identification of my-HSCs in the presence of saturating concentrations of anti-CD150 *in vivo* (**Ext. Data Fig. 3a–l**). Analogous experiments were conducted for anti-NEO1 (**Ext. Data Fig. 5l–q**). These experiments address using CD150 or NEO1 to distinguish myeloid-biased and balanced HSCs after treatment with anti-CD150 or anti-NEO1 *in vivo*.

(iii): Independent my-HSC targets: We developed three independent antibody protocols targeting three different my-HSC antigens: CD150, NEO1, and CD62p. Each protocol depleted my-HSCs based on the well-established $CD150^{Hi}$ definition (**Fig. 2c–h** and new **Ex. Data Fig. 4p**), despite the absence of anti-CD150 in the anti-NEO1 and anti-CD62p regimens. In addition, all three protocols resulted in depletion of $NEO1^{+}$ myeloid-biased HSCs (new **Ex. Data Fig. 4p**). Importantly, there was a strong statistically significant positive correlation between the frequency of my-HSCs as defined by $CD150^{Hi}$ HSCs or $NEO1^{+}$ HSCs at steady-state, or after antibody treatment with any of the three regimens, whether targeting CD150, NEO1, or CD62p (new **Ex. Data Fig. 4q**).

(iv) Long-term functional studies: To account for the possibility that antibody treatment impacted cell-surface phenotype but not function, we conducted long-term functional time-course experiments 8-16 weeks after treatment. Depletion of my-HSCs resulted in long-term functional restoration of multiple phenotypes that are suppressed by my-HSC abundance and associated with bal-HSCs, including increased CLPs (**Fig. 3d**), increased frequencies and absolute numbers of nascent, or naïve lymphocytes (T and B cells) (**Fig. 3e–f** and new **Ex. Data Fig. 7a, 7d**), and reduced inflammatory cytokines (**Fig. 3i–j**). In the revised manuscript, we further demonstrate that depletion of my-HSCs (2 months prior) enhances functional immunity to infection with a live, pathogenic virus *in vivo* (new **Fig. 4a–c** and new **Ex. Data Fig. 9a–j**). These observations are unlikely to be explained by alterations to cell-surface phenotype induced by these antibodies, since these time-point are after the estimated clearance of these antibodies *in vivo*[6-8], and the expected lifespan of short-term hematopoietic progenitors[9-13]. These results are consistent with clearance of my-HSCs enabling bal-HSCs to repopulate open niches and proliferate through self-renewal. This is further supported by new analysis that my-HSC depletion **increases** the absolute numbers of **bal-HSCs** by approximately 2-fold (new **Ex. Data Fig. 4b**) after approximately one-week, consistent with a model whereby the depletion of my-HSCs clears niches that are then repopulated by bal-HSCs, which then proliferate through self-renewal and increase in total numbers[14].

1.2: We appreciate the reviewer's suggestion to conduct *in vivo* transplantation assays to support functional depletion of myeloid-biased HSCs. Although we agree these experiments could be informative, prospective isolation and *in vivo* transplantation of HSCs would introduce confounding factors due to the impact of conditioning protocols required for engraftment[15,16]. For example, irradiated hosts maintain the phenotypic HSC pool with far fewer quiescent, G0 HSC than unirradiated mice[17]. In contrast, *in vivo* antibody-mediated depletion of my-HSCs more faithfully represents their endogenous function, which is the focus of our study. Furthermore, our experiments serve as a pre-clinical proof-of-principle model to be translated in humans, unlike transplantation, which would not provide insight into the impact of our conditioning protocol to reverse immune decline in aged animals. In addition to applying multiple independent markers and protocols to control for cell-surface changes, our long-term studies demonstrate that my-HSC depletion reverses multiple age-related phenotypes associated with my-HSC abundance and promotes phenotypes associated with bal-HSCs (**Fig. 3**), including new experiments that demonstrate enhanced *in vivo* functional immunity months after my-HSC depletion (new **Fig. 4** and new **Ex. Data Fig. 9**).

2. Effects of antibody treatment on hematopoietic cell populations are reported only in terms of frequencies. This is extremely problematic as cell frequencies can be significantly influenced by off-target effects of the antibody treatment rather than by changes in HSC output. In all cases, absolute numbers of cells must be reported.

Author's Response

2: We appreciate the reviewer's suggestion and agree that quantifying absolute cell numbers could inform our analysis, including revealing potential off-target effects. Following the reviewer's request, we have reported absolute numbers of cells in the revised manuscript for critical experiments. Quantification of absolute numbers supports our conclusions that antibody treatment depletes my-HSCs (new **Ext. Data Fig. 4b** and new **Ext. Data Fig. 9c**) and increases naïve T cells (new **Ext. Data Fig. 7a**) and mature B cells (new **Ext. Data Fig. 7d**). We observed concordance between the analysis of frequencies and absolute numbers, supporting that results are not due to off-target effects. Comparison of cell frequencies and absolute numbers for multiple independent cell types (my/bal-HSCs, myeloid/lymphoid progenitors, etc.) in the BM at steady-state or after antibody treatment demonstrated a strong statistically significant positive correlation ($r=0.9547$, $p<0.0001$) spanning 6 orders of magnitude (new **Ex. Data Fig. 4r**). In our experience, frequencies are more reliable in accounting for experimental variability between mouse strain, age, and tissue processing, and so we selected these analyses for the main figures.

In addition to these overall issues, the following specific points should be addressed:

3. The increase in CLP frequency after just 1-week (Fig. 2F-H) is interpreted by the authors as increased output from HSCs. The kinetics of this rejuvenation suggest that HSCs are rapidly contributing to hematopoiesis following antibody treatment. What are the effects of antibody depletion on HSC and progenitor proliferation?

Author's Response

3: We agree with the reviewer's interesting observation that the kinetics of rejuvenation suggest HSCs are rapidly contributing to hematopoiesis following antibody treatment. To experimentally address the impact of antibody depletion on HSC proliferation, we quantified the absolute number of my-HSCs and bal-HSCs approximately 1-week after antibody conditioning. Approximately one week after treatment, anti-CD150 **decreased** the absolute number of **my-HSCs** over 4-fold (new **Ex. Data Fig. 4b**), but **increased** the absolute numbers of **bal-HSCs** by approximately 2-fold (new **Ex. Data Fig. 4b**) in the total bone-marrow. These results support bal-HSC proliferation via self-renewal after antibody treatment, consistent with a model where depletion of my-HSCs clears niches that can be repopulated by bal-HSCs, which then proliferate through self-renewal and increase in total numbers[14].

4. Other possible explanations for the increased frequency of CLPs is that there is broad depletion of other hematopoietic cells and/or that these progenitors expand in response to a loss of mature lymphoid cells. A much more comprehensive assessment of the effect of acute antibody treatment on B lineage cell numbers (progenitors and mature cells) is warranted.

Author's Response

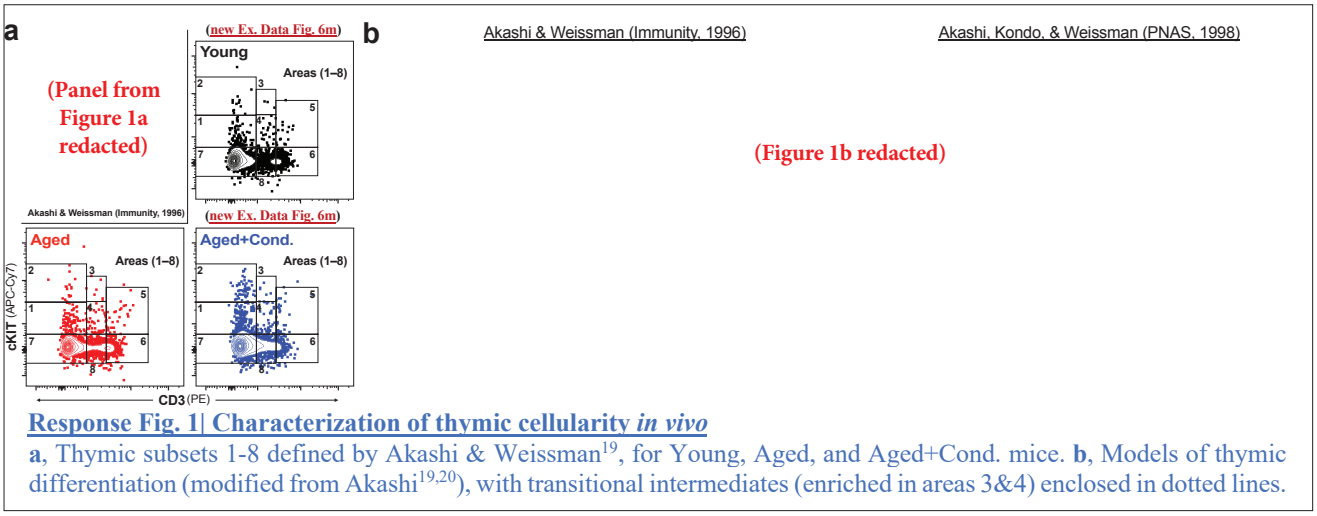
4: The reviewer raises an interesting alternative explanation for the increased frequency of CLPs and requests a more comprehensive assessment of the acute effects of antibody treatment on B lineage cells. We examined B lineage cells (progenitors and mature cells) in the bone-marrow approximately one week after the anti-NEO1 antibody-conditioning protocol and found there was no significant impact on the frequency of mature B cells ($B220^+CD19^+CD43^-CD93^-IgM+IgD^+$) (new **Ex. Data Fig. 6j**) or progenitor B cells ($B220^+CD19^-$) (new **Ex. Data Fig. 6k**). Given these results and the limited self-renewal of CLPs[18] compared to HSCs (which self-renew indefinitely), it is unlikely the increase in CLPs is due to their proliferation.

5. The restoration of naïve T cells in aged, antibody-conditioned mice implies functional restoration of the involuted thymus. Characterization of thymic cellularity should be included.

Author's Response

5: We thank the reviewer for suggesting that we characterize thymic cellularity. We did not observe significant differences in thymus weight between aged mice with or without antibody treatment (new **Ex. Data Fig. 6l**). We further characterized thymus cellularity based on our previous interrogation of thymic subsets during mouse T cell development (Akashi & Weissman[19]) (new **Ex. Data Fig. 6m** and **Response Fig. 1**). Young, aged, and aged mice

receiving the anti-NEO1 antibody-conditioning protocol demonstrated similar frequencies for all of these previously characterized thymic progenitor subsets (new **Ex. Data Fig. 6n**), including the subset (areas 3&4) enriched for transitional intermediate (TI) cells, a population that we previously demonstrated is enriched for thymic precursors committed to the CD4 or CD8 lineages (**Response Fig. 1**)[19-21]. The presence of these progenitors supports the potential capacity of aged mice receiving antibody conditioning to contribute to thymic output. Although these analyses may not be sensitive enough to identify subtle changes, the lack of an effect of the antibodies on thymic precursors could be due to the blood-thymus barrier, which has been reported nearly 60 years ago by several investigators (e.g. Raviola and Karnofsky, reviewed by Ribatti[22]).



Response Fig. 1| Characterization of thymic cellularity *in vivo*

a, Thymic subsets 1-8 defined by Akashi & Weissman[19], for Young, Aged, and Aged+Cond. mice. **b**, Models of thymic differentiation (modified from Akashi[19,20]), with transitional intermediates (enriched in areas 3&4) enclosed in dotted lines.

6. Depletion with anti-CD150 also induces increased frequency of myeloid progenitors (Fig. 2F). What is the effect of antibody depletion on mature myeloid, megakaryocytic and erythroid lineage cells? Taken together, points 4-6 emphasize the need to more fully characterize the effects of acute antibody treatment on hematopoietic cell numbers.

Author's Response

6.1: We thank the reviewer for noting the increased frequency of myeloid progenitors with anti-CD150. This phenotype appeared to be specific for the anti-CD150 protocol. The frequency of myeloid progenitors was not increased with other protocols, including anti-NEO1 (**Ext. Data Fig. 4l–m**), which is one reason we favored anti-NEO1 for our comprehensive time-course, age-impact, and immune function studies (**Fig. 3** and new **Fig. 4**). The reviewer's question prompted us to extend our analysis to megakaryocytic and erythroid lineage progenitors (MEPs). We performed this analysis for all conditioning protocols, which similarly demonstrated that all protocols (including anti-NEO1 but not anti-CD150) either decreased or did not significantly impact MEPs (new **Ex. Data Fig. 4n**).

6.2: The reviewer makes the excellent suggestion to provide information on the impact of antibody depletion on mature myeloid lineage cells. We examined mature myeloid cells after anti-NEO1 conditioning, which was the protocol used in our functional experiments. Acute treatment with anti-NEO1 did not significantly impact the frequency of $CD11b^{+}Ly6G/C^{+}$ (new **Ex. Data Fig. 6h**) or $CD11b^{+}SIRPa^{+}$ (new **Ex. Data Fig. 6i**) myeloid cells.

7. There are some striking inconsistencies regarding HSC frequencies and how data are reported. For example, Extended Data Fig. 4F shows HSC frequency as being ~1% of live cells, which is off by two orders of magnitude. Extended Data Fig. 4D and 4G reports myeloid-biased HSCs as a fraction of total HSCs at a value of "2", which implies that 200% of HSCs are myeloid-biased. Again, it would be much more straight forward if all data include reporting of absolute numbers.

Author's Response

7.1: We greatly appreciate the reviewer's close review of our data and suggestions to improve our presentation. We especially thank the reviewer for noticing the "fraction of total HSCs at a value of 2" – this was a typo (the correct fraction is "0.2"). To enable more straight-forward presentation of these results, we changed "fraction of total" to "percentage of total" in the revised manuscript (e.g., **Fig. 2c–e**).

7.2: We thank the reviewer for highlighting differences in HSC frequencies across different experiments. Two factors that could contribute to these differences when comparing experiments within our study or to others are: (i) mouse age, and (ii) cKIT-enrichment. We performed additional analyses to determine any impact of these factors:

(**i**) Our study involved mice from 10-weeks to >100-weeks of age. Since the frequency and absolute number of HSCs increases with age, differences would be expected when comparing between experiments of mice from different ages. To determine the impact of mouse age on HSC frequency and total number, we quantified the frequencies of HSCs in total bone marrow for a subset of experiments. We verified that the frequency of HSCs in the total bone marrow is consistent with prior reports for young-adult (20-weeks) mice (~0.01% HSCs)[23] and for very old (~100 weeks) mice (~0.1% HSCs)[24] (new **Ex. Data Fig. 2j**). To control for age-related differences, all treatment comparisons were conducted with **age-matched controls**. In the revised manuscript, we have indicated mouse age in all figure legends for clarity.

(**ii**) Our study involved the analysis of stem and progenitor cells, which are extremely rare cells in the bone-marrow. To accurately quantify changes to these rare cells, most experiments included cKIT-enrichment prior to analysis of HSCs and/or HPCs (e.g., my-HSCs, bal-HSCs, CMPs, CLPs, etc.). For HSCs, the increase in frequency between cKIT-enriched and total bone marrow was approximately 10-fold (new **Ex. Data Fig. 2j**). Importantly, the frequency of HSCs in total or cKIT-enriched bone marrow was highly correlated and similar across mouse age, from approximately 20 to 100 weeks old (new **Ex. Data Fig. 2j**). To further evaluate if cKIT-enrichment impacted our results, we examined a depletion experiment that included paired analysis of total bone-marrow and cKIT-enriched bone-marrow. Comparing the frequency of live cells for **11 critical cell types** (e.g., HSCs, my/bal-HSCs, CLPs, etc.) in the total vs. the cKIT-enriched bone marrow revealed a highly statistically significant correlation ($r=0.9467$, $p<0.0001$) over 4 orders of magnitude: (i) at steady-state, and (ii) upon antibody-treatment (new **Ex. Data Fig. 4s**). These experiments support using either method of analysis. Importantly, for all experiments involving a comparison or treatment, we only compared samples that were processed the same way (e.g., cKIT-enriched or total bone marrow). In the revised manuscript, we have indicated whether the samples represented total or cKIT-enriched bone-marrow in the figure legends for clarity.

7.3: We appreciate the reviewer's suggestion to provide absolute numbers to make the data reporting more straight forward. In the revised manuscript, we provide absolute numbers to validate and to confirm the depletion of my-HSCs with anti-CD150 (new **Ext. Data Fig. 4b**) and anti-NEO1 protocols (new **Ext. Data Fig. 9c**). For reporting absolute numbers of cells in the bone marrow, we always examined total bone-marrow (or paired total bone-marrow & cKIT-enriched bone-marrow). We also verified that depletion of my-HSCs increases the absolute numbers of naïve T cells (new **Ext. Data Fig. 7a**) and mature B cells (new **Ext. Data Fig. 7d**). Importantly, when reporting absolute numbers, we do it for the tissues examined; it is not feasible to present total body numbers, as the methods to do so comprehensively and accurately in a reasonable time frame simply aren't currently available. Extrapolations from single tissues or organs or blood are feasible, but could be inaccurate unless the distribution of cell subsets is constant from tissue to tissue, which it isn't.

8. It is unclear that inclusion of anti-CD47 and anti-cKit help to optimize myeloid-biased HSCs. Extended data Fig. 4A shows no additive effect of these antibodies on myeloid-biased HSC depletion, although it inexplicably increases the frequency of balanced HSCs. Similarly, combining these antibodies with CD41 also has no effect on myeloid-biased HSCs (Extended data Fig. 4B). The authors justify that inclusion of these additional antibodies also has no effect on total HSC numbers, but the relevant experiment appears statistically underpowered to draw that conclusion (Extended Data Fig. 4F), which shows that 2/4 mice exhibit more than a 50% decline in HSC

frequency. Instead of using an anti-cKit antibody that would broadly recognize hematopoietic progenitors, could additive effects be achieved by combining CD150, Neo1 and/or CD62p antibodies?

Author's Response

8.1: The reviewer comments that it is unclear if anti-CD47 and anti-cKIT help to optimize the depletion of myeloid-biased HSCs. We added anti-CD47 and anti-cKIT to our protocols since this combination promotes the engraftment of infused HSCs in our prior studies (Chhabra et al.[25] and George et al.[26]). We reasoned that adding anti-cKIT and anti-CD47 to a my-HSC specific antibody (e.g., anti-CD150) would clear at least some my-HSCs from their niches and enable niche-replacement by competing non-targeted bal-HSCs, which could then proliferate and expand post-treatment. Consistent with this model, adding anti-cKIT+anti-CD47 to anti-CD150 **depleted my-HSCs** and **increased bal-HSCs** (new **Ex. Data Fig. 4b**) approximately one-week after treatment. We agree with the reviewer that there could be other ways to potentiate the depletion of my-HSCs or to develop other protocols that omit anti-CD47 and/or anti-cKIT.

8.2: We thank the reviewer for drawing attention to our experiments on the additive effects of these antibodies on my-HSC depletion. We agree with the reviewer that the decreased proportion of my-HSCs to bal-HSCs from adding anti-CD47 and anti-cKIT appears to be driven by an **increase** in bal-HSCs, as opposed to **further depletion** of my-HSCs (**Ex. Data Fig. 4a**). We analyzed absolute cell numbers in addition to cell frequencies, which confirmed these observations: the absolute number of **my-HSCs decreased** over 4-fold (new **Ex. Data Fig. 4b**), while the absolute numbers of **bal-HSCs increased** by approximately 2-fold (new **Ex. Data Fig. 4b**). These results are consistent with a model where anti-CD47 and anti-cKIT results in more efficient depletion of my-HSCs, opening niches for replacement by bal-HSCs, which subsequently expand through self-renewal[14].

8.3: The reviewer comments on our justifications for adding anti-CD47 and anti-cKIT to our antibody protocols. To clarify, we speculated that anti-CD47 +/- anti-cKIT would improve the depletion of my-HSCs when combined with a my-HSC-specific antibody (e.g., anti-CD150). Indeed, adding anti-CD47+/-anti-cKIT to anti-CD150 improved the depletion of my-HSCs relative to bal-HSCs (**Ex. Data Fig. 4a**), decreasing the absolute numbers of my-HSCs and increasing the absolute numbers of bal-HSCs (new **Ex. Data Fig. 4b**). Our control experiments with anti-CD47 +/- anti-cKIT were conducted to determine the impact of these antibodies in the absence of a my-HSC-specific antibody. Since my-HSCs and bal-HSCs expressed CD47 and cKIT at similar levels (**Ex. Data Fig. 2k**), we reasoned that low-doses of this combination might lower the threshold for cell clearance without substantially depleting **total HSCs** (e.g., my-HSCs + bal-HSCs). As the reviewer points out, there does appear to be some impact on **total HSCs** (e.g., my-HSCs + bal-HSCs) with anti-cKIT+anti-CD47, and we agree that the relevant experiment (**Ex. Data Fig. 4g**) appears statistically underpowered to draw a definitive conclusion. However, although **total HSCs** may be impacted by treatment with anti-CD47 +/- anti-cKIT, these antibodies did not alter the **relative proportion of my-HSCs to bal-HSCs** unless they were combined with anti-CD150 (**Ex. Data Fig. 4a, 4f, 4h**). These results are consistent with the requirement of a my-HSC-specific antibody to preferentially clear my-HSCs. In the revised manuscript, we have modified the text for this section to make it clearer and more succinct.

8.4: We agree with the reviewer's excellent suggestion that additive effects could be achieved by combining CD150, Neo1 and/or CD62p antibodies instead of adding anti-cKIT antibody and this will be the focus of future studies.

9. The relevance of human HSC expression data is questionable. CD150 has been shown to not be an effective surface marker for identifying human HSCs (Blood 2011; 117(5):1550–1554.). The data would be much more compelling and impactful if the study included identification and characterization of myeloid-biased human HSCs. Otherwise, the human data may be better suited as a supplementary figure.

Author's Response

9.1: We thank the reviewer for highlighting the important work of Morrison and Dunbar (Blood 2011; 117(5):1550–1554.)[27], demonstrating that CD150 has been shown not to be an effective surface marker to identify human HSCs, which we have added as a reference in the revised manuscript. We agree with the reviewer and the work by Morrison

and Dunbar that CD150 is not a specific marker of human HSCs. In the revised manuscript, we conducted additional analysis confirming that CD150 is not as specific for HSCs as for downstream progenitors (new **Ex. Data Fig. 11j**), as compared to CD90, CD62p, or other markers we examined (i.e., ESAM, CD166) (new **Ex. Data Fig. 11h–m**).

9.2: To clarify the purpose of our human HSC experiments, our interest in CD150 (and other markers) was not as a specific marker to **isolate** human HSCs, but to determine if a subset of human HSCs **differentially express** these markers, **identifying them as potential candidate human my-HSC markers and clinical targets for antibody-treatment in patients**. We found that a subset of human HSCs (as defined by $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$)[28] are $CD150^{Hi}$ (**Fig. 5h–i** and **Ex. Data Fig. 11g**). These results are consistent with the original study by Morrison and Dunbar[27], which also demonstrated that CD150 is expressed by a subset of human HSCs (as defined by $CD34^{+}$). Both studies support future work to determine the relative myeloid/lymphoid potential of $CD150^{Hi}$ vs. $CD150^{Lo}$ human HSCs.

9.3: We appreciate that our study would be more compelling and impactful if we identified and characterized myeloid-biased human HSCs. This is an ongoing project of our laboratory that will be the focus of additional manuscripts. These experiments require transplantation into recipient xenografted SCID-hu immunodeficient mice[29,30], which is currently limited by recent politics resulting in the closing of nonprofit providers of the required tissues. To further expand upon our findings, we performed additional analysis of candidate human my-HSC markers by evaluating their relative expression levels on HSCs and multipotent progenitors (new **Ex. Data Fig. 11h–m**).

10. In addition to a more comprehensive assessment of off-target effects in the hematopoietic system (comments 4-6), the authors should discuss potential effects on non-hematopoietic tissues. To what extent are these markers expressed by other tissues? Are changes in other tissues noted?

Author's Response

10.1: We thank the reviewer for prompting us to examine the potential effects on non-hematopoietic tissues and to determine the expression of these markers by other tissues. To determine the expression of these markers in other tissues, we examined gene expression data from two independent mouse tissue compendium studies[31,32], including the *Tabula Muris* Consortium[31]. Examination of >10 distinct mouse tissues revealed the relative specificity of these markers to the hematopoietic system (new **Ex. Data Fig. 1n–o**), suggesting a limited impact from our treatment.

10.2: We thank the reviewer for asking if we noted changes in other tissues after antibody treatment. We did not observe any obvious signs of toxicity in animals receiving antibody-conditioning, consistent with the relative restriction of the gene expression of these markers to the hematopoietic system (new **Ex. Data Fig. 1n–o**). Importantly, treatment of aged mice up to >20 months old (which can be frail), did not result in any preferential signs of disease or toxicity up to 2-4 months after treatment. We cannot exclude subclinical toxicities of treatment.

10.3: Information on side-effects and potential off-target effects is available for many of the antibodies used in our study. We previously reported 500ug (higher than the 100ug in our study) anti-cKIT (ACK2) is tolerated in mice and does not result in toxicity based on histologic examination and hematologic parameters[6]. The anti-CD47 antibody used in this study has been extensively characterized for off-target toxicity[33,34], with or without anti-cKIT[33,34]. Several independent groups have administered anti-NEO1 to mice without reporting any significant side effects[35-37]. We did not observe any obvious side effects from anti-CD62p administration. Collectively, the limited tissue expression, prior toxicity studies, and time-course data supports our treatments being well-tolerated.

11. How do the authors explain the durability of the response to a single course of antibody administration (Fig. 3B)? Have antibody levels been measured in the blood at different timepoints after treatment?

Author's Response

11.1: We appreciate the reviewer's request to explain the durability of the response to a single course of antibody administration. Our findings are consistent with our hypothesis for the antibody-mediated depletion of my-HSCs resulting in a clonal shift of the total HSC population towards bal-HSCs, as opposed to the long-acting presence of antibody *in vivo*. The long-term differences (≥8 weeks) we observed in my-HSC/bal-HSC abundance is not consistent with a continued impact of antibody treatment, since this is after the estimated time for elimination of the antibody isotypes in our study. The half-lives of antibody isotypes used in this study have been reported: mouse IgG2a (6-8 days) and IgG2b (4-6 days), rat IgG2a (9-10 days) and IgG2b (2-3 days)[6-8,38]. The half-life of goat IgG in mice would not be expected to be greater than these mouse/rat antibodies. The referenced studies likely reflect upper limits of clearance, since they were conducted with non-specific antibodies, and the antibodies used in our study would be expected to be further reduced by antigen 'sink' resulting from binding to antigen-positive cells. Since we estimate clearance of all antibodies by <8 weeks (i.e., minimum >5.5 half-lives equaling >97% elimination), the durability of response to a single administration is unlikely due to the persistent *in vivo* antibody.

11.2: The long-term durability of response is consistent with our original hypothesis for shifting the clonal composition of the HSC compartment by depleting my-HSCs and enabling niche-repopulation by bal-HSCs. We previously demonstrated that total HSC numbers are restricted by niche availability[14]. Thus, we speculated that depletion of my-HSCs would open niches that could be occupied by bal-HSCs to enable their proliferation by self-renewal[14]. This is supported by data demonstrating the expansion of bal-HSCs shortly after antibody-conditioning (new **Ex. Data Fig. 4b**) and by our long-term experiments, where we detect fewer my-HSCs **several months** after antibody conditioning (new **Ex. Data Fig. 9c**). Since even a single HSC can repopulate and provide input to the entire hematopoietic compartment, even subtle shifts in HSC clonal composition could extensively impact the overall composition of mature hematopoietic progeny, including downstream progenitors and differentiated cells.

12. Antibody conditioning increases the frequency of balanced HSCs in old mice. Is there any expectation that the HSCs are rejuvenated or are they still functionally compromised as compared to young adult HSCs?

Author's Response

12: The reviewer raises the important question if balanced HSCs in old mice after antibody conditioning are rejuvenated compared to young adult HSCs. Several studies by our group and others demonstrate that bal-HSCs in aged animals retain their phenotype[1,24]. Thus, we expect that phenotypic bal-HSC in aged mice act as bal-HSCs. This is consistent with our long-term studies and new data demonstrating improvement in multiple features associated with bal-HSC function upon depletion of my-HSCs, including increased CLPs (**Fig. 3d**), increased nascent, or naïve lymphocytes (T and B cells) (**Fig. 3e–f**), reduced inflammatory cytokines (**Fig. 3i–j**), and new experiments demonstrating enhanced functional immunity to infection (new **Fig. 4** and new **Ex. Data Fig. 9**).

13. There is a minor spelling mistake in the word "clone" in the x-axis labels of Extended Data Fig. B-D and F-H.

Author's Response

13: We thank the reviewer for pointing out this spelling mistake, which we have corrected in the revised manuscript.

Referee #2 (Remarks to the Author):

The manuscript by Ross et al provides evidence for a novel therapeutic strategy to restore lymphopoiesis and adaptive immune function in aged individuals. Their rationale is that selective depletion of myeloid-biased HSCs, which is observed with aging, will allow remaining lineage-balanced HSCs to reconstitute lymphopoiesis. They discover using antibody-mediated depletion of several target molecules selectively expressed on myeloid-biased HSCs that they are able to restore production of lymphoid progenitor cells and mature lymphocytes in vivo in aged mice. Further, they demonstrate that these same antigens are expressed on the surface of human HSCs demonstrating proof-of-principle that a similar antibody-mediated depletion strategy may work in aged humans.

The work is original and significant, and addresses a critical health problem in the aging population. The approach appears valid with good data quality and appropriate use of statistics, as well as appropriate credit to previous work. However, several conclusions are not adequately supported by experimental data and additional experiments and controls are needed to robustly support the conclusions of this paper.

Author's Response

We thank the reviewer for the critical review of our work and suggestions to improve our study.

1. While CD150 expression has been demonstrated to enrich for functional myeloid-biased versus lineage-balanced HSCs, this same rigorous definition has not been applied to the new markers of myeloid-biased HSCs that they propose here; Neo1, CD41 and CD62p. To formally demonstrate that these populations are myeloid-biased in function, prospective isolation and transplant of high vs. low (or positive vs. negative) populations should be performed to demonstrate that these markers enrich for functional myeloid-biased HSCs.

Author's Response

1: We agree with the reviewer that CD150 expression enriches for functional myeloid-biased versus lineage-balanced HSCs, as we and others have previously demonstrated[1,24]. For consistency across this study, we defined my-HSCs and bal-HSCs based on this definition: my-HSCs are $CD150^{Hi}$, and bal-HSCs are $CD150^{Lo}$. The reviewer recommends that we prospectively isolate and transplant high vs. low populations for Neo1, CD41, and CD62 HSCs to formally demonstrate these markers enrich for functional myeloid-biased HSCs. Functional studies have already been conducted by our group and others to formally demonstrate the myeloid-biased function *in vivo* for Neo1 (our group[2]), CD41 (Nakauchi[39] and Graf[8]), and CD62p (Bystrykh[4]), and so we believe repeating these experiments would not be further informative.

To clarify, our study focused on my-HSC markers that could be targets for antibody-mediated depletion of my-HSCs. We identify CD150, NEO1, and CD62p as candidate targets to deplete my-HSCs, and we demonstrate that antibodies to each of these targets results in the depletion of my-HSCs ($CD150^{Hi}$ HSCs) *in vivo* (**Fig. 2c–h**). We further validate that independent targeting of CD150, NEO1, or CD62p all result in depletion of my-HSCs defined by either the $CD150^{Hi}$ or $NEO1^{+}$ phenotype (new **Ex. Data Fig. 4p–q**). The depletion of functional my-HSCs with antibody-conditioning is further supported by our long-term *in vivo* studies demonstrating the restoration of numerous phenotypes associated with their depletion and shift to bal-HSCs (**Fig. 3**), including new experiments demonstrating improved functional immunity to live, pathogenic viral infection *in vivo* (new **Fig. 4** and new **Ex. Data Fig. 9**).

2. Assessment of relative specificity of myeloid-biased HSCs markers to this population relative to lineage-balanced HSCs and downstream HPCs (Figure 1) is very strong. However, this flow cytometric analysis should also include mature hematopoietic cell populations to provide insight into potential off-target effects of antibody-based depletion.

Author's Response

2: We appreciate the reviewer's suggestion to provide more insight into potential off-target-effects of antibody-based depletion. To address the reviewer's request, we conducted additional flow cytometric and gene expression

analysis on mature hematopoietic cell populations. First, we evaluated the flow-cytometry expression of CD150, NEO1, CD41, and CD62p on mature cells positive for lineage ($CD3^+$ or $Ly\text{-}6G^+/Ly\text{-}6C^+$ or $CD11b^+$ or $B220^+$ or $Ter\text{-}119^+$) markers (new **Ex. Data Fig. 2g–h**). Second, we determined the impact of acute antibody-depletion on mature myeloid cells (new **Ex. Data Fig. 6h–i**) and B cells (new **Ex. Data Fig. 6j–k**). Third, we expanded our gene expression analysis of the hematopoietic cell tree to include additional mature and progenitor populations (new **Ex. Data Fig. 1m**). These data are consistent with limited off-target effects of antibody-based depletion on mature cells.

3. In Figure 2, the authors show efficacy of their depletion protocol using flow cytometric characterization of stem & progenitor cell populations. This should be supported by quantitation of total numbers of each of these populations, to support the conclusion that lymphoid populations are restored to similar 'levels'. Furthermore, complete blood counts and mature hematopoietic cell frequencies in the peripheral blood are needed to rule out off-target effects and to show restoration of 'youthful' hematopoiesis. Lastly, to formally prove that differences in progenitor output are truly derived from different proportions of HSCs rather than a direct effect on progenitors, lineage tracing studies, or ex vivo single HSC output studies, are needed to track output of individual HSCs to myeloid vs. lymphoid progenitors.

Author's Response

3.1: The reviewer raises several important points related to quantification of total cell numbers and we appreciate the reviewer's recommendation to report absolute numbers of cells in addition to cell frequencies. In the revised manuscript, we provide both absolute numbers of cells and cell frequencies for critical experiments. Importantly, we observed that antibody-conditioning resulted in the depletion of my-HSCs by both frequency and absolute numbers (new **Ext. Data Fig. 4b** and new **Ext. Data Fig. 9c**), verifying the efficacy of my-HSC depletion. We also confirmed increases in the absolute numbers of naïve T cells (new **Ext. Data Fig. 7a**) and mature B cells (new **Ext. Data Fig. 7d**). There was a strong statistical correlation ($r=0.9547$, $p<0.0001$) between absolute numbers and frequencies for several independent cell types (including my/bal-HSCs, myeloid and lymphoid progenitors, etc.) in the bone-marrow across 6 orders of magnitude (new **Ex. Data Fig. 4r**), supporting either method of analysis.

3.2: The reviewer requests complete blood counts and mature hematopoietic cell frequencies in the peripheral blood to rule out off-target effects. In the revised manuscript, we provide absolute numbers of total $CD45^+$ leukocytes in the blood (new **Ext. Data Fig. 7e**), which demonstrated no significant of impact of antibody treatment in aged mice.

3.3: The reviewer suggests that we conduct lineage tracing studies, or *ex vivo* single HSC output studies. Since transplantation introduces the confounding impact of protocols required for HSC engraftment[15-17], we believe that antibody depletion is a more suitable system to test the endogenous potential of HSCs. Further, our experiments reflect the consequences of my-HSC depletion on the hematopoietic system, which is the focus of our study.

4. In the experiment shown in Figure 3A, for the same rationale as above, complete blood count data should be included for these mice. To directly support the conclusion of 'rejuvenating the immune system', functional T and B cell assays should be performed on material isolated from these mice.

Author's Response

4.1: We appreciate the reviewer's suggestion to provide functional T and B cell assays to directly support our conclusion of 'rejuvenating the immune system'. In the revised manuscript, we provide new comprehensive experiments that evaluate functional immunity in aged mice after antibody-conditioning *in vivo*, which is incorporated into a new Main Figure (new **Fig. 4**). We demonstrate that antibody-conditioning in aged mice enhances functional immunity to a viral infection that requires T cells (CD4+ and CD8+), B cells, and innate cytokines[40,41]. These experiments demonstrate that my-HSCs depletion enhances functional immunity in aged mice to live, pathogenic infection *in vivo* (new **Fig. 4a–c** and new **Ext. Data Fig. 9a–j**). We thank the reviewer for prompting inclusion of these experiments, which have strengthened our study.

4.2: To address mature cell blood counts, absolute numbers of total $CD45^+$ leukocytes cells in the blood are provided in the revised manuscript (new **Ext. Data Fig. 7e**). As noted above, my-HSC depletion in aged mice increased naïve T cells and mature B cells both by frequency (**Fig. 3e**, **3f**) and absolute number (new **Ext. Data Fig. 7a, 7d**).

5. The human data collected so far shown in Figure 4 is promising, however, demonstration that these markers increase at the surface level in CD34+ cells in aged vs. young humans is needed to support the conclusion. Furthermore, as suggested for the murine studies above, prospective isolation of CD62p+ vs. CD62p- followed by transplant into recipient xenografted animals is needed to prove that these markers enrich for myeloid-biased HSCs in the human context, as this has not been demonstrated previously.

Author's Response

5.1: The reviewer comments that increased surface levels of these markers in aged vs. young human $CD34^+$ cells are needed to support our conclusions. It is important to note that CD34+ cells are not themselves HSC; only the subset that is $CD45^+CD90^+CD38^{lo}Lin^-$ are HSC[28,42]; while these true HSC represent up to 30% of recently mobilized HSC and progenitors in mobilized blood, only about 7% of CD34+ cells resident in bone marrow are HSC[30]. To clarify the data in our manuscript, we only intended to provide preliminary data to help facilitate translation of our antibody conditioning protocol to humans. We agree that we can only make limited conclusions based on the expression of these markers on human HSCs. Specifically, we agree that we cannot conclude that the markers we propose – CD150, NEO1, CD62p – are also markers of human my-HSCs, without conducting prospective isolation of positive and negative populations followed by transplant into recipient xenografted SCID-hu immunodeficient mice[29,30]. Such SCID-hu mice were engrafted with human fetal bones with marrow, fetal liver at stages when it is hematopoietic, human fetal thymus to follow T cell development, and human fetal spleen and lymph nodes to receive the injected antigens for measuring human immune responses[29]. Current politics has resulted in the loss of nonprofit providers of such tissues following extensive ethical and legal work. Thus, these experiments require a significant undertaking and are an ongoing project of our laboratory that will be the focus of additional manuscripts, if we are still allowed to do them. In the revised manuscript, we more clearly discuss the limitations of our conclusions, stating that these data are intended to support further interrogation of these markers.

5.2: We appreciate the reviewer's comment that our human data are promising. To help facilitate translation of our pre-clinical therapy to patients, we extended our human analysis by evaluating the relative expression levels of these cell-surface markers on HSCs and downstream multipotent progenitors (new **Ex. Data Fig. 11h–m**).

6. The section 'Antibody-mediated depletion of myeloid-biased HSCs in vivo' could be much more succinct and streamlined; much of this information is more appropriate for supplemental materials.

Author's Response

6: We appreciate and have followed the reviewer's suggestion to make this section more succinct and supplemental.

Referee #3 (Remarks to the Author):
In this study, Ross et al. present a series of intriguing and interesting experiments predicated on the idea that the age-related myeloid deviation in the HSC compartment could be remedied by selective depletion of myeloid-biased HSC (My-HSC), and by consequently allowing balanced HSC (Bal-HSC) to dominate hematopoiesis and generate adequate numbers of lymphoid cells. The authors begin their study by examining differential gene and protein expression on the two HSC subsets in an attempt to identify potential targets to eliminate/reduce My-HSC. They present a careful and systematic validation of potential targets, along with optimization of dosage, antigenic density and depleting antibody isotypes. They then characterize the impact of My-HSC depletion on CLP / lymphoid (IL-7Ra+) progenitors (presumably in the bone marrow) and on age-sensitive T and B cell subsets and cytokines/chemokines in peripheral blood of treated mice. They finish their work with a preliminary analysis of analogous molecular targets in human bone marrow, and conclude that their conditioning regimens rejuvenate the old immune system.

The work is conceptually original and for the most part logically described. There are, however, critical missing experiments necessary to evaluate the authors' claims, as well as omissions, inaccuracies and errors in statistical approaches.

Author's Response
We appreciate the reviewer's critical feedback and for suggesting additional experiments to support our study.

Major criticisms:
1. Data on immune rejuvenation is inconclusive and incomplete. The authors analyze peripheral blood, which is hardly a relevant compartment for residence and function of naïve T and B cells. Secondary lymphoid organs (SLO) are critical sites of naive T and B cell residence, maintenance and function, and there is accumulating evidence that these organs, particularly lymph nodes, suffer alterations with aging that compromise their structure and functon(Becklund, B., et al., Sci. Rep. 2016; Richner, J.C. et al., Plos Pathogens, 2015; Thompson, H.L. et al., Aging Cell, 2019). There is further evidence from the work of Dorshkind and coworkers that bone marrow stromal environment, in addition to the HSC compartment, also undergoes a series of age-related changes, and the same seems to be true of thymic (Lancaster, J.N. et al., Aging Cell, 2022) and lymph node (Sonar, S.A. et al., PNAS, 2022) stroma. Most relevant to this work, even when thymic rejuvenation via sex steroid blockade or growth factor administration is achieved, and blood shows an increase in newly produced T cells, or if naïve T cells are injected exogenously, SLO are not necessarily able to host such newly produced cells and this does not result in improved functional immunity (Thompson, H.L. et al., Aging Cell, 2019; Sonar, S.A. et al, PNAS 2022). Therefore, the authors need to document significant improvements in naïve lymphocyte numbers in SLO and to demonstrate whether or not this yields improved survival and immunity against infection. This is a necessary set of experiments.

Author's Response
1.1: We thank the reviewer for drawing our attention to the importance of secondary lymphoid organs (SLO) in the regulation of naïve T and B cell function during aging. We have cited the important work by Surh[43], Diamond[44], Nikolich-Žugich[45,46], Ehrlich[47], Dorshkind[48], and coworkers in the revised manuscript. The development, architecture, and cell collaborations in immune response functions dominated our own discoveries from 1960-1988. The reviewer makes the important point that previous studies demonstrate increased production of T cells is not sufficient to result in improved functional immunity. To support our study, the reviewer asks us to evaluate immunity against infection. We had excluded our studies on immune responses to viral infections in SLOs in an attempt to allow our co-authors to co-publish their own work. This review has caused us and our generous collaborators to include vaccination, infection testing, and the effects of depletion of my-HSC in this paper.

1.2: We appreciate the reviewer's comments to provide additional experiments to support immune rejuvenation by demonstrating improved immunity against infection. We have provided a new set of experiments that demonstrate improved immunity against infection of aged animals receiving antibody-conditioning, which is incorporated into **a new Main Figure** (new **Fig. 4)**. In the revised manuscript, **we provide a comprehensive new set of experiments**

to determine if antibody depletion of my-HSCs resulted in improvements in functional immunity. We applied our antibody-conditioning protocol to a mouse model of retroviral infection[41] – murine Friend Virus (FV) – an experimental system that requires complex immune responses for virus control[40]. Although aged mice have severely limited functional immunity to FV infection – even after immunization with a live-attenuated vaccine (new **Fig. 4**) – aged animals receiving antibody-conditioning demonstrated enhanced immunity to viral infection upon vaccination. **These experiments demonstrate that antibody conditioning improves immunity against infection in aged mice and have been incorporated into a new figure (**new **Fig. 4a–c** and new **Ex. Data Fig. 9a–j**).

2. In many experiments, including the critical experiments in Fig. 3, panels b-I, the authors have used unpaired parametric one-tailed t tests to evaluate statistical significance. This is not appropriate when three treatments are compared, and also may not be appropriate if data are not normally distributed, which is often the case with the analysis of older animals. Authors need to justify their use of this test, which is known to be susceptible to both type I and II errors.

Author's Response

2: We appreciate the reviewer's feedback on statistical analyses. In the revised manuscript, we applied statistical tests that adjust for multiple comparisons for experiments with three or more treatments. To account for data not normally distributed or with unequal variance, we applied tests that do not make these assumptions (e.g., Mann-Whitney or Brown-Forsythe/Welch ANOVA), or we applied a data transformation (e.g., log-transformation). One-tailed tests were applied only when we had a pre-test directionality hypothesis (e.g., aged vs. young, and/or aged vs. aged+conditioning). Details on statistical tests for all experiments are provided in the figure legends.

3. To interpret results in Figure 3, all subsets need to be analyzed not only by % change but also by absolute numbers. Similarly, the main memory cell subsets should be analyzed as well to be able to interpret whether changes in frequencies are reflective of absolute changes in one or the other cell subset(s). Specifically, is the treatment truly producing more naïve lymphocytes, and is that the reason for the decline of "exhausted" or age-related cell subsets, or are the latter also diminishing in absolute numbers?

Author's Response

3.1: The reviewer recommends reporting absolute numbers in addition to frequencies to help interpret our results. In the revised manuscript, we provided absolute numbers of cells for critical experiments. Quantifying absolute numbers supports our conclusions that antibody treatment depletes my-HSCs (new **Ext. Data Fig. 4b** and new **Ext. Data Fig. 9c**), increases naïve T cells (new **Ext. Data Fig. 7a**), and increases mature B cells (new **Ext. Data Fig. 7d**). Thus, these results are not due to absolute changes in one or the other cell subset(s).

3.2: We appreciate the reviewer's suggestion to provide data on the main memory T cell subsets. In the revised manuscript, we examined the main memory T cell subsets, including central memory (CM) and effector memory (EM) cells defined by canonical markers (new **Ex. Data Fig. 7b–c, 7f–g**) and through high-dimensional cluster-based flow-cytometry analysis (new **Ex. Data Fig. 7h–j**). Although we observed changes in these memory populations upon antibody-conditioning, changes in these subsets do not explain the increased frequency of naïve T cells after treatment, since naïve T cells were also increased by absolute numbers (new **Ex. Data Fig. 7a**).

4. In Figure 3b, the authors need to explain how and why would the numbers of CLP be further increasing from week 1 post treatment (not significant) to week 8 and 16 (progressively more and more significant).

Author's Response

4.1: The reviewer asks us to explain the mechanism though which the numbers of CLPs would further increase after one-week post-treatment to become greater at weeks 8-16 and more significant. These experiments may not have been powered enough to make definitive conclusions when comparing between these different time-points. However, these results are consistent with our previous studies on the phenotype and function of CLP, my-HSC, and bal-HSC. CLP, first discovered by us, are not self-renewing cells[18]. Since they are replaced by bal-HSC, but

barely by my-HSC, their continuous rise in the anti-my-HSC conditioned mice is from expanding bal-HSC. After acute antibody-mediated depletion, there could be a delay in the generation of CLP from bal-HSC in very old mice, but this would be expected to peak after several weeks, once there has been a new equilibrium established between bal-HSCs and my-HSCs. Overall, these results are consistent with our original hypothesis for shifting the clonal composition of the HSC compartment by depleting my-HSCs and enabling niche-repopulation by bal-HSCs.

5. In Fig. 3j, did the authors analyze the most commonly increased cytokines with aging, such as IL-6, CRP and TNFa? These are commonly increased in 24-mo old mice.

Author's Response

5: The reviewer asks if we analyzed the most commonly increased cytokines associated with aging, including IL-6, CRP, and TNFa. The cytokines IL-6 and TNFa were included in our original analysis (**Ex. Data. Fig. 6b–d**), but they were not statistically significantly changed between young and aged mice in our cohort. We conducted additional experiments to evaluate CRP, since this required an independent protein assay platform. We did not detect any differences between young and aged animals for CRP or 5 additional proteins included in this independent protein platform (new **Ex. Data Fig. 6g**).

6. Human studies are a reasonable, albeit preliminary, proof of principle. However, myelodysplastic syndrome and malignancy data is a clear add-on, that at the present should be deleted and incorporated into a more complete study.

Author's Response

6.1: We appreciate the reviewer's feedback on our preliminary and proof of principle human data. These analyses were intended to facilitate translation of our antibody-protocol, which we believe will be valuable to the community.

6.2: The reviewer recommends we delete the myelodysplastic syndrome and malignancy data and incorporate it into a more complete study. If acceptable to the reviewer, we favor moving the myelodysplastic syndrome and malignancy data from the main figures to the supplemental (new **Ex. Data Fig. 10f**) rather than deleting entirely, since providing these data to the scientific and relevant clinical community could help to facilitate a more complete study by other groups.

Other comments:

• The manuscript needs thorough editing and tightening of repetitive parts. There are several sections that meander while repeating considerations on antibody depletion therapy (e.g. lines 114-116 and then on lines 127&128; on myeloid progenitor stages on page 9, in two places, and a long section on lines 201-208 that can be condensed).

Author's Response

• We appreciate the reviewer's feedback to tighten repetitive parts, including the sections highlighted above. We have revised these sections and made additional changes to reduce repetitiveness and to increase clarity.

• Important information is difficult to find in figures and the text. Time points, tissue sources and other critical experimental details need to be transparently listed everywhere.

Author's Response

• We appreciate the reviewer's feedback and have made changes to the revised manuscript so that all critical experimental details are transparently listed in the figure legends and/or methods section.

• Animal strains and ages should similarly be consistently defined everywhere.

Author's Response

• For all experiments, we have provided mouse strain and age in the main text, figure legends, and/or methods.

• It would be really useful for the field if this group would use its unique strengths to demonstrate at what point My-HSC become detrimental to lymphopoiesis – it seems that having 65% Bal-HSC is not too shabby, but the data in this manuscript suggest that this is already too little. This is not a must for the present study, but if the authors have a cross-sectional data set, it would be nice to include it.

Author's Response

• We appreciate the reviewer's insight into the range of Bal-HSC required to have adequate lymphopoiesis. We agree that this is an important question for our field that should be pursued in future studies.

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have significantly improved the manuscript and provided key new controls as well as new data on immune function after my-HSC depletion. However, there are still concerns that effects may be driven by off-target effects or changes in progenitor activity. As I previously requested, it is necessary to demonstrate changes in HSC composition/potential are altered after antibody treatment by performing transplant experiments with purified HSCs.

Referee #2 (Remarks to the Author):

The authors have thoroughly addressed my previous comments, recommendations and suggestions in their revised manuscript. The new experiments and data supporting restored T cell immunity in aged mice following myeloid-biased HSC depletion are very exciting and support the overall themes of the paper. I have no further edits to suggest.

Referee #3 (Remarks to the Author):

Ross et al have revised the manuscript to address the reviewers criticisms. While some aspects of the manuscript have been improved, critical experiments have not been performed to address the comments I have raised, and the evidence presented falls short of the authors' claims.

Major criticisms:

1. Overall, I remain unconvinced with the degree of rejuvenation achieved by "rebalancing" of HSC, and the authors did not provide any data to answer my question on what happens in secondary lymphoid organs of old mice upon Ab-mediated "rebalancing" of HSC. In particular, Neo1 depletions produce very modest rebalancing, with Fig. 2e showing a 6.2% reduction in My-HSC (from 13% to 5.8%); this is the most selective treatment used in many experiments, including the ones that were supposed to show more "youthful" immune system after treatment. Is this difference truly biologically significant? Its downstream impact is even more modest, with naive T cell percentage and number increasing somewhat to meet significance in some (Fig. 3e, EFig. 7a) but not other (EFig 7h), but remaining far short of the adult levels. Finally, all this analysis was performed (as far as I can tell) only on peripheral blood. Because the authors stated no improvement in thymic cellularity or other measures of thymic function, it remains questionable how was the stated improvement in naive T cells achieved, and is it actually seen in peripheral lymphoid organs. Similarly, the reduction in "exhausted" cells (and PD1 is by no means enough to call a cell exhausted) is reported by authors, without any change in the effector memory T cell compartment. Because these cells would be expected to be of effector memory phenotype, this result is also surprising and difficult to explain. Authors should perform a comprehensive evaluation of treatment efficacy in different lymphoid

organs (including spleen and lymph nodes) to address these issues, preferably in a model that can track recent thymic emigrant cells in a genetic manner.

2. The most important point I have brought up was the one regarding an improvement in functional immunity in mice treated with anti-My HSC antibodies. In response, the authors present experiments in new Figure 4 with Theiler's murine leukemia virus, to show lower viral loads in vaccinated and antibody-treated ("HSC rebalanced") old mice relative to old mice receiving vaccine only. This result is supposed to support the improvement in naive immune cell function and in immune defense. The experimental design and scope of work is largely insufficient to back up either of these two claims. First, the experiment as set is actually testing immune memory, and not the function of naive, primary immune responses, because it uses vaccination followed by challenge. Moreover, the only measure shown in support of improved immune function is the reduction of splenic viral loads. Is this improvement really due to improved T or B cell responses? There is no data on any measure of adaptive immunity, tetramers or cytokine secretion or function of either of the two main lymphocyte subsets. For the data presented, maybe NK cells are improved in function - we simply do not know that from the data presented. Although the fact that the virus control is improved following vaccination implies some component of immune memory, this needs to be demonstrated. Are the responses to vaccine measurably better?

To evaluate whether the response of naive T and B cells is improved the authors need to use an infectious challenge of unvaccinated mice, and to document T and B cell responses as well as animal survival.

3. Authors need to discuss their approach and results in light of the recent elegant study from Urbanus et al. (Nature Communications volume 14, Article number: 2184 (2023)). In that study, using an unirradiated model of natural aging within the bone marrow niches, the authors document that myelopoiesis occurs continuously due to My-biased HSC. The key difference with aging, then, is not an increased My-HSC bias, but rather an increased number of HSC cells contributes to myelopoiesis. What in that context would Ab treatment be expected to do?

4. In the absence of changes in key signature proinflammatory cytokines (IL-6, CRP, TNFa, sTNFRI and II), this study truly cannot claim reduction in inflammation as a consequence of Ab treatment.

Other points:
- I agree with moving the human data into extended figures.
- Statistical treatment of data has been improved.
- Authors really did not improve the transparency of the manuscript. Tissues analyzed are rarely identified in the legends, experimental design details are lacking and require one to repeatedly visit the Methods section (often in vain). This group is too good to have such poorly written figure legends.

Author Rebuttals to First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have significantly improved the manuscript and provided key new controls as well as new data on immune function after my-HSC depletion.

Authors' Response

We appreciate the Referee's comments that our manuscript has been significantly improved with key new controls and new data on immune function after my-HSC depletion.

However, there are still concerns that effects may be driven by off-target effects

Authors' Response

As we described in our initial response, we provided data on the *in vivo* depletion of my-HSCs using (1) an independent marker of my-HSCs (e.g., NEO1), (2) 'non-masking' antibodies, and (3) several antibody-conditioning protocols, each targeting an independent my-HSC marker (e.g., CD150, NEO1, or CD62p). To further address the use of a single marker to identify and define my-HSCs, we demonstrated that three independent antibody protocols targeting three different my-HSC antigens – CD150, NEO1, or CD62p – all depleted my-HSCs based on the well-established $CD150^{Hi}$ definition, despite the absence of anti-CD150 in the anti-NEO1 and anti-CD62p regimens (**Extended Data Fig. 4p–q**). Further, all three protocols resulted in depletion of $NEO1^{+}$ HSCs (**Extended Data Fig. 4p–q**). These results demonstrate that the effects are not due to downregulation of a single marker, but rather, loss of cells expressing a family of markers that we and others have demonstrated are associated with myeloid-biased HSCs.

or changes in progenitor activity.

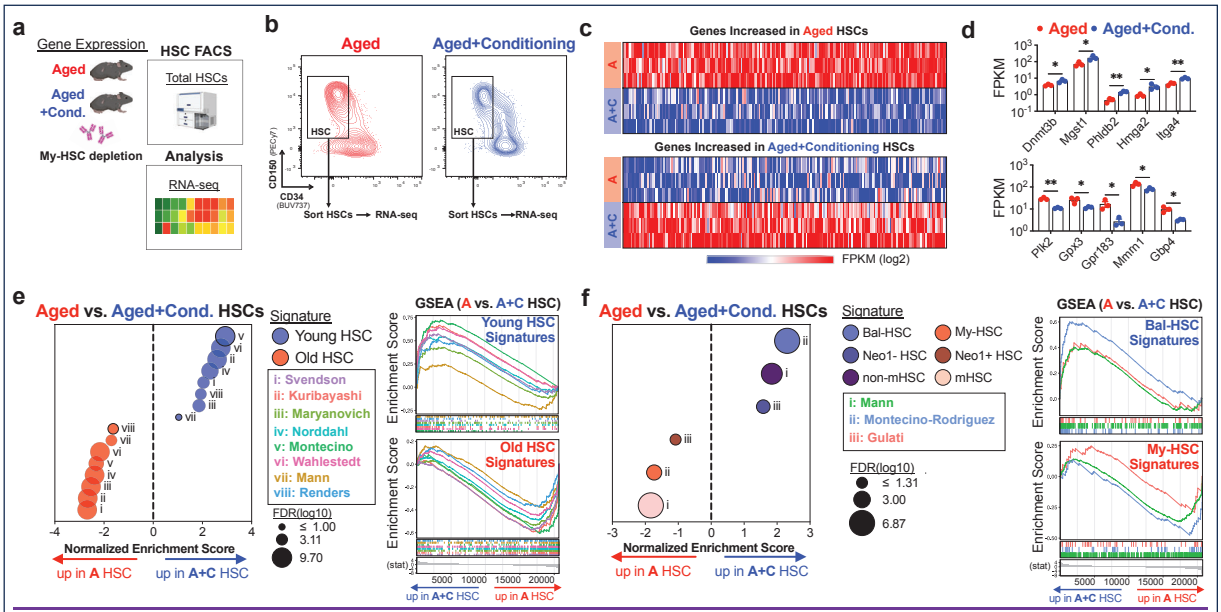
Authors' Response

As we point out in our original response, we have previously demonstrated that CLPs have limited self-renewal[1]. Thus, the increase in CLPs after my-HSC depletion months after treatment cannot be due to their proliferation. Most importantly, our long-term *in vivo* studies demonstrating that my-HSC depletion results in **persistent, long-term changes** to the HSC compartment, long after (>10 weeks) the clearance of these antibodies *in vivo*[2-4] and the lifespan of short-term hematopoietic progenitors[5-9], which do not self-renew.

As I previously requested, it is necessary to demonstrate changes in HSC composition/potential are altered after antibody treatment by performing transplant experiments with purified HSCs.

Authors' Response

In the first review, the Referee requested that we validate changes in HSC composition/potential and suggested we perform transplant experiments or conduct gene expression profiling with purified HSCs. As we discussed in our initial response, we do not believe transplant experiments are an appropriate context to test this hypothesis (see discussion below). We agree that changes to HSC composition can be evaluated by gene expression analysis of purified HSCs after treatment. To verify changes in HSC composition after my-HSC depletion, we have now conducted gene expression profiling with purified HSCs in aged mice with or without antibody-conditioning (**Response Fig. 1 and new Fig. 2i–k in the Revised Manuscript**). We applied Gene Set Enrichment Analysis (GSEA) using (i) 16 independent gene-signatures of purified aged vs. young mouse HSCs obtained from 8 publications[10-17], and (ii) 6 independent gene-signatures of myeloid-biased vs. balanced HSCs from 3 independent publications[14,18,19]. This revealed that purified HSCs obtained from mice receiving antibody-conditioning are enriched in gene expression signatures obtained from young HSCs and balanced/lymphoid biased HSCs. Furthermore, the treated old mice had purified HSCs depleted of gene expression signatures obtained from old HSCs and myeloid-biased HSCs (**Response Fig. 1e–f and new Fig. 2i–k in the Revised Manuscript**). These data support that our treatment alters the composition and molecular phenotype of the HSC compartment and have been added to the revised manuscript.



Response Fig. 1 | HSCs from aged mice with my-HSC depletion are enriched in youthful HSC signatures

a, Schematic for experiment to conduct RNA-sequencing on purified HSCs from aged mice with or without antibody-conditioning. **b**, Representative FACS plots of sorted total HSC from Control mice (Aged) and mice with my-HSC depletion (Aged+Conditioning). **c**, FPKM of top 200 differentially expressed genes between Aged vs. Aged+Conditioning HSCs in both directions based on *p*-value. **d**, Subset of top statistically significant genes increased or decreased in Aged vs. Aged+Conditioning HSCs. Genes enriched in Aged+Conditioning HSCs (top); Genes enriched in Aged HSCs (bottom). **e–f**, GSEA ranked by test statistic applied to HSCs purified from aged mice receiving antibody-conditioning are enriched in young (**e**) and balanced (**f**) HSC signatures and depleted of aged (**e**) and myeloid-biased (**f**) HSCs signatures; *n*=3 mice (Aged), *n*=3 mice (Aged+Conditioning). A, aged mice; A+C, aged mice receiving antibody-conditioning (anti-NEO1+anti-CD62p+anti-cKIT/CD47).

Authors' Response

As we discussed in the initial review, *in vivo* transplantation of HSCs would introduce confounding factors due to the impact of conditioning protocols required for engraftment, as has been described by our group[20] and others[21]. Importantly, it has been demonstrated that HSCs in cell cycle engraft less efficiently upon transplantation[22]. Since we demonstrate that the depletion of my-HSCs results in an increase in the number of bal-HSCs early after treatment – the proliferation of bal-HSCs may introduce a confounding factor that could decrease their efficiency upon transplantation. Further, the Referee raises concerns that effects could be driven by off-target effects or changes in one or another downstream subset of cells. However, unlike the data we present upon antibody-conditioning – that our treatment has limited acute impact on mature hematopoietic cells – it is known that the conditioning required to transplant HSCs has dramatic effects on all hematopoietic cell lineages (and non-hematopoietic) cells. These conditioning protocols – whether they be irradiation or chemotherapy – are known to impact the engraftment of HSCs, chimerism produced, and lineages of cells generated. Thus, in our view, this experiment is not suited to test the hypothesis. For these reasons, we designed our long-term longitudinal studies to track the hematopoiesis resulting after my-HSC depletion after the clearance of the antibodies used[2-4] and after the previously documented lifetime and output of intermediate progenitors[5-9]. To summarize, we validated changes to HSC composition upon antibody therapy in this paper by demonstrating (i) loss of my-HSCs marked by independent myeloid-biased cell-surface markers not targeted by these antibodies, (ii) changes to non-self-renewing hematopoietic populations for up to 16 weeks, and (iii) changes to the transcriptional signature of HSCs isolated and FACS purified from mice receiving antibody-conditioning.

Referee #2 (Remarks to the Author):
The authors have thoroughly addressed my previous comments, recommendations and suggestions in their revised manuscript. The new experiments and data supporting restored T cell immunity in aged mice following myeloid-biased HSC depletion are very exciting and support the overall themes of the paper. I have no further edits to suggest.

Authors' Response
We appreciate the Referee's statement that we have thoroughly addressed their previous comments in the revised manuscript, and that our new experiments and data are very exciting and support the overall themes of our paper.

Referee #3 (Remarks to the Author):
Ross et al have revised the manuscript to address the reviewers criticisms. While some aspects of the manuscript have been improved, critical experiments have not been performed to address the comments I have raised, and the evidence presented falls short of the authors' claims.

Authors' Response
We were very careful with the conclusions in the manuscript, and all claims in the manuscript are backed by experimental verification. Importantly, in this revised manuscript we responded to the major question raised by Referee 3, by including data on the functional immunity after my-HSC depletion. Now, we provide additional data demonstrating that my-HSC depletion results in improved antigen-specific CD8+ T cell response in the spleen of aged animals during vaccination (primary response) and during vaccination followed by infection (secondary response) (**Response Fig. 2 & 3 and new Fig. 4e, 4b in the Revised Manuscript**). This will be added to the manuscript.

Major criticisms:
1. Overall, I remain unconvinced with the degree of rejuvenation achieved by "rebalancing" of HSC,

Authors' Response
1.1: The degree of rejuvenation achieved in this study is based on objective data after depletion of my-HSCs, as measured by the increase in lymphocyte progenitors and naïve cells, decrease in markers of immune dysfunction and inflammation, and most importantly, the functional enhancement of vaccine-induced control of viral infection.

and the authors did not provide any data to answer my question on what happens in secondary lymphoid organs of old mice upon Ab-mediated "rebalancing" of HSC.

Authors' Response
1.2: In the revised manuscript, we demonstrate functional improvement in a secondary lymphoid organ by demonstrating that my-HSC depletion enables robust control of live pathogenic virus in the spleen, using an infectious model with stringent requirements for adaptive immune function. This may have been overlooked by the Referee because they mis-stated that we used "Theiler's" virus, which infects the nervous system, when we in fact used Friend virus, which following i.v. injection infects the spleen, a secondary lymphoid organ. We restored immune elimination of i.v. delivered Friend virus infection in old, vaccinated mice by depleting myeloid HSC with antibodies to their specific cell surface markers. Importantly, we now provide new data demonstrating improved antigen-specific (dextramer-positive) CD8+ T cell responses to vaccination alone (**Response Fig. 1 and new Fig. 4b in the Revised Manuscript**) and to vaccination followed by live viral infection (**Response Fig. 2 and new Fig. 4e in the Revised Manuscript**), which has been previously documented to be required for vaccine-induced FV protection (see **Appendix Figures**).

In particular, Neo1 depletions produce very modest rebalancing, with Fig. 2e showing a 6.2% reduction in My-HSC (from 13% to 5.8%); this is the most selective treatment used in many experiments, including the ones that were supposed to show more "youthful" immune system after treatment.

Authors' Response

1.3: In our view, the reviewer misrepresents the degree of reduction. The 6.2% reduction noted is greater than 2-fold, or a 200%-fold, relative decrease. In the context of HSCs, relative fold-change is the relevant parameter to examine, as HSC are extremely rare, and a single cell can repopulate the entire hematopoietic system. As another example, if the reduction was from 1% to 0.1% – a 1% absolute decrease – this would amount to a 10-fold relative decrease. Regardless, the greater than 2-fold decrease we demonstrate results in numerous physiological changes including increased lymphocyte progenitors and naïve cells, decreased markers of immune dysfunction and inflammation, and most importantly, multi-fold functional enhancement of vaccine-induced control of viral infection, which is all evidence of biological significance.

Is this difference truly biologically significant? Its downstream impact is even more modest, with naive T cell percentage and number increasing somewhat to meet significance in some (Fig. 3e, EFig. 7a) but not other (EFig 7h), but remaining far short of the adult levels.

Authors' Response

1.4: The changes in lymphocyte progenitors, naïve T cells, and markers of inflammation – all of which are downstream of the altered frequencies of My-HSC induced by our therapy – demonstrate biological significance. Furthermore, the enhanced vaccine protection demonstrated in the rejuvenated aged mice is undeniable proof of physiological relevance and biological significance. Nowhere do we claim complete return of the aged immune response to equivalence with youth. We claim significant improvement in aged animals backed by functional experiments. This is, to our knowledge, the first evidence that the adaptive immune deficiency in aging can by altered by depleting my-HSC in aged animals by individuals. Of course, it likely can be improved even more, and perhaps in a preventative mode rather than in already aged mice, which will be the focus of future studies.

Finally, all this analysis was performed (as far as I can tell) only on peripheral blood.

Authors' Response

1.5: Peripheral blood is the appropriate compartment to evaluate the increase in naïve T cells upon my-HSC depletion, since this is the first route of migration of newly formed T cells. As supported by vast literature, naïve lymphocytes belong to the recirculating pool discovered by Gowans that recently antigen activated lymphocytes are temporally depleted from the recirculating [blood and thoracic duct lymph], and these by changes in their homing receptors; upon elimination of antigen the 'stem memory' progeny immunocompetent lymphocytes lose receptors directing them to tissue sites of antigen deposition and regain at least integrin-$\alpha 4\beta 7$ mucosal lymphoid homing receptors and/or CD62L lymph node homing receptors that we discovered previously; that would be expected based on our hypothesis.

Because the authors stated no improvement in thymic cellularity or other measures of thymic function, it remains questionable how was the stated improvement in naive T cells achieved, and is it actually seen in peripheral lymphoid organs.

Authors' Response

1.6: Our data indicate that the improvement in naïve T cells was achieved in response to the provided treatment, which was depletion of myeloid biased HSCs. As described in our initial response, we demonstrate that the cells necessary for the maturation of T cells in the thymus are already present. While

it may be a widely held assumption that improved thymic cellularity is required for the effects we observed, this may, or may not be the case in our model.

Similarly, the reduction in "exhausted" cells (and PD1 is by no means enough to call a cell exhausted) is reported by authors, without any change in the effector memory T cell compartment. Because these cells would be expected to be of effector memory phenotype, this result is also surprising and difficult to explain.

Authors' Response

1.7: We examined $PD1^{+}CD62L^{-}$ T cells based upon previous work, including the recent study by Elyahu (PMID: 31457092), which demonstrated that this population increases with mouse age. We agree that a single marker is not sufficient to state a cell is 'exhausted' and we do not make this claim. In our manuscript, we clearly stated that we examined T cells with "markers" of exhaustion and/or inflammation, in reference to the $PD1^{+}CD62L^{-}$ population defined by Elyahu. Proving these cells are 'truly' exhausted is beyond the scope of this study and would not change our conclusions.

Authors should perform a comprehensive evaluation of treatment efficacy in different lymphoid organs (including spleen and lymph nodes) to address these issues, preferably in a model that can track recent thymic emigrant cells in a genetic manner.

Authors' Response

1.8: As described above, we already present comprehensive treatment efficacy of the spleen – a secondary lymphoid organ – by demonstrating improved functional immunity of a lethal viral challenge that infects this organ and requires multiple lymphocyte subsets for its eradication. The new request to "perform a comprehensive evaluation of treatment efficacy in different lymphoid organs (including spleen and lymph nodes)" is beyond the scope of this study and not necessary to demonstrate functional immunity.

Authors' Response

1.9: The Referee requests that we use "a model that can track recent thymic emigrant cells in a genetic manner". Not only is this a new experiment not requested in the first review, such experiments would also unfortunately take years, since any genetic strain would have to be backcrossed to the genetic background used in our study, and then aged 1.5-2 years. These proposed experiments are new and would be of interest if we agree that starting to breed and interbreed and age a new mouse strain susceptible to Friend virus, which we have spent years characterizing; but these new experiments this round of review would not change the conclusions in this manuscript but might further characterize the cellular mechanisms involved. But of course, by that time, new cellular and molecular details will have again changed the playing field and lead to another set of requests from reviewers. We hope the reviewers agree that opening this field doesn't require us to completely explain all of the mechanisms that might satisfy the questions that could be answered to close the field.

2. The most important point I have brought up was the one regarding an improvement in functional immunity in mice treated with anti-My HSC antibodies. In response, the authors present experiments in new Figure 4 with Theiler's murine leukemia virus, to show lower viral loads in vaccinated and antibody-treated ("HSC rebalanced") old mice relative to old mice receiving vaccine only.

Authors' Response

2.1: The Referee implies that we failed to address their "most important point" on "improvement in functional immunity in mice treated with anti-My HSC antibodies". However, in our view, the next comment by the Referee that we "show lower viral loads in vaccinated and antibody-treated ("HSC rebalanced") old mice relative to old mice receiving vaccine only", is clear evidence of "improvement in functional immunity". We conducted these experiments in response to the first round of reviews with Friend

virus, which infects and expands in the spleen following i.v. injection (and not with "Theiler's" virus, which infects and demyelinates the CNS, as mis-stated by the Referee).

Authors' Response

2.2: The Referee incorrectly states that we used "Theiler's" virus instead of Friend virus (FV). This distinction is critical since Theiler's virus infects the nervous system and not the spleen (a secondary lymphoid organ) and has less complex immunological requirements for virus control. FV was selected because of its stringent requirements for vaccine-induced protection, which requires functional T cells (CD4 and CD8), and B cells – with each subset providing unique and non-overlapping functions (see **Appendix Figures**).

This result is supposed to support the improvement in naive immune cell function and in immune defense. The experimental design and scope of work is largely insufficient to back up either of these two claims.

Authors' Response

2.3: We did not claim our experiments on vaccine-induced immune protection are "supposed to support the improvement in naive immune cell function and in immune defense". As stated in our manuscript, our experiments were designed to test immune function after vaccination, a clinically relevant experiment in a well-documented model of viral infection. For example, older subjects require higher doses of vaccines such as influenza in the hope that can make a protective immune response not seen with doses given to younger subjects.

First, the experiment as set is actually testing immune memory, and not the function of naive, primary immune responses, because it uses vaccination followed by challenge.

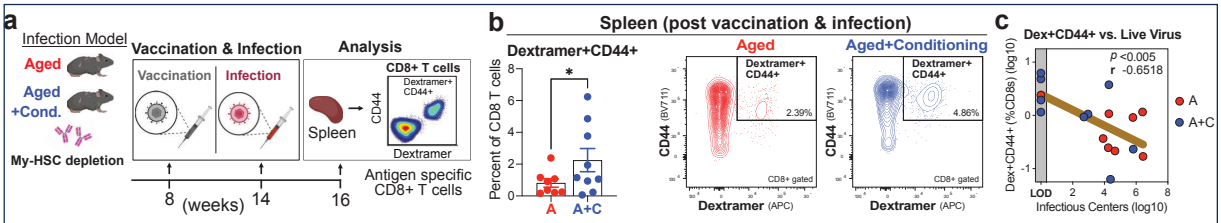
Authors' Response

2.4: We make no claim in the paper that our experiments test primary immune responses. That said, an immune response to a live-attenuated viral vaccine such as that used in our study requires a primary response to generate memory cells. As the Referee states, vaccine protection is a test of immune memory, which in the Friend retrovirus model used in our study, requires functional T cell and B cell responses (Dittmer, *J. Virology* 1999[23] and **Appendix Figures**).

Moreover, the only measure shown in support of improved immune function is the reduction of splenic viral loads. Is this improvement really due to improved T or B cell responses? There is no data on any measure of adaptive immunity, tetramers or cytokine secretion or function of either of the two main lymphocyte subsets. For the data presented, maybe NK cells are improved in function - we simply do not know that from the data presented.

Authors' Response

2.5: In the context of Friend virus infection, increased control of spleen viral loads in vaccinated rejuvenated old mice compared to un-rejuvenated controls is evidence of improved immunity and biological relevance. Measurements of tetramers and cytokines simply further characterize the details of the response, which have been previously reported. Importantly, functional experiments with lymphocyte depletions and adoptive transfer experiments have already been done in the FV model, demonstrating that vaccine-induced viral control requires CD4+ & CD8+ T cells and B cells (see **Appendix Figures**). Nevertheless, to satisfy Referee 3, we have added new data demonstrating that vaccinated rejuvenated aged mice have increased virus-specific CD8+ T cell responses (dextramer+) in the spleen compared to controls (**Response Fig. 2a and new Fig. 4e in the Revised Manuscript**), which is associated with control of live virus (**Response Fig. 2b**). These new data have been added to the manuscript.



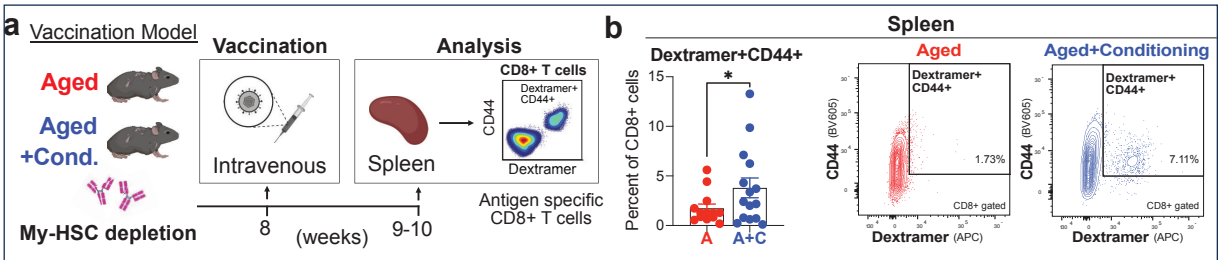
Response Fig. 2 | Aged mice with my-HSC depletion have improved splenic antigen-specific CD8+ response

a, Schematic to evaluate antigen-specific CD8+ T cell response in the spleen of vaccinated and infected aged mice with or without my-HSC depletion. **b–c**, Percent of CD8+ T cells in the spleen that are FV antigen-specific (Dextramer^{+}CD44^{+}) in vaccinated aged mice without (A), or with antibody conditioning (A+C), 14 days after infection with FV (**b**), with correlation to virus control (**c**). For Aged+Conditioning mice, my-HSC depletion was conducted 2 months prior to vaccination. Representative FACS plots of spleens from vaccinated mice (right), gated on CD8^{+} T cells; n=8 mice (Aged), n=9 mice (Aged+Conditioning), pooled from two independent experiments. p-values were obtained by one-tailed unpaired parametric t-test with Welch's correction (**b**) or by one-tailed non-parametric (spearman) correlation (**c**). A, aged mice; A+C, aged mice with antibody-conditioning.

Although the fact that the virus control is improved following vaccination implies some component of immune memory, this needs to be demonstrated. Are the responses to vaccine measurably better? To evaluate whether the response of naive T and B cells is improved the authors need to use an infectious challenge of unvaccinated mice, and to document T and B cell responses as well as animal survival.

Authors' Response

2.6: The Referee asks "Are the responses to vaccine measurably better?". Although we believe we have already satisfied the Referee's requests on improvements in immune function, we provide new data showing improved virus-specific CD8+ T cell responses (dextramer+) following vaccination of rejuvenated old mice compared to old mice (**Response Fig. 3 and new Fig. 4b in the Revised Manuscript)**, which is a primary immune response, rather than a secondary response. Importantly, our prior work has demonstrated that the generation of antigen specific antiviral CD8+ T cells is critical for vaccine-induced immune protection from FV (Dittmer & Hasenkrug, *Virology* 2000[24]). These new data have been added to the revised manuscript. The new request to show improved responses in unvaccinated mice is not required to show improved functional immunity as originally requested, is outside the scope of this study, and would substantially delay publication, negatively impacting the scientific community.



Response Fig. 3|My-HSC depletion improves antigen-specific CD8+ T cell response in the spleen of aged mice

a, Schematic of evaluation of antigen-specific CD8+ T cell response in the spleen to intravenous (i.v.) vaccination in aged mice with or without my-HSC antibody depletion. **b**, Percentage of CD8+ T cells in the spleen that are antigen-specific (Dextramer^{+}CD44^{+}) in aged-matched (20-26 months) mice without (A), or with antibody conditioning (A+C), 10-14 days after i.v. vaccination with live-attenuated virus (left). For Aged+Conditioning mice, my-HSC depletion was conducted 2 months prior to vaccination with anti-NEO1 protocol. Representative FACS plots of spleens from vaccinated mice (right), gated on CD8^{+} T cells; n=13 (Aged), n=15 (Aged+Conditioning), pooled from two independent experiments. p-value was obtained by one-tailed unpaired parametric t-test. A, aged mice; A+C, aged mice with antibody-conditioning.

3. Authors need to discuss their approach and results in light of the recent elegant study from Urbanus et al. (Nature Communications volume 14, Article number: 2184 (2023)). In that study, using an unirradiated model of natural aging within the bone marrow niches, the authors document that myelopoiesis occurs continuously due to My-biased HSC. The key difference with aging, then, is not an increased My-HSC bias, but rather an increased number of HSC cells contributes to myelopoiesis. What in that context would Ab treatment be expected to do?

Authors' Response

3.1: The Urbanus paper confirms the original studies by our group and others over ten years ago, that aging results in "an increased number of HSC cells contributes to myelopoiesis". In the context of the Urbanus study, our treatment would be expected to deplete the increased number of HSCs contributing to myelopoiesis, as demonstrated in this manuscript. And of course, we submitted our paper in 2022 before Urbanus published. Nevertheless, as requested, we have added discussion of the Urbanus paper to our revised manuscript.

4. In the absence of changes in key signature proinflammatory cytokines (IL-6, CRP, TNFa, sTNFRI and II), this study truly cannot claim reduction in inflammation as a consequence of Ab treatment.

Authors' Response

4.1: We agree that IL-6, CRP, TNFa, sTNFRI/II are considered key signature proinflammatory cytokines by many in the field, and as the Referee stated in their initial review, are "commonly increased cytokines with aging". However, in our experiments, IL-6, CRP, and TNFa were not significantly increased in aged versus young animals – therefore, they are not appropriate age-related control cytokines in our system. Importantly, we never claim that we completely reduce all "inflammation". We found that IL-1α (a marker of inflammation), was increased in aged versus young animals, and reduced upon my-HSC depletion. Thus, we stated that we observed a reduction in "markers of inflammation". That we did not observe a decline in some other pro-inflammatory cytokines (e.g., IL-6, CRP, TNFa, sTNFRI and II) does not impact these findings, it just means we did not detect measurable differences in these cytokines. Further, independent investigators have identified IL-1α as a marker associated with age-related HSC decline, which supports the specificity of our treatment to HSC rejuvenation. As IL-1α has been validated to increase in aged mice and humans and is a *bona fide* and well-established target of anti-aging therapeutics and HSC aging[25,26], the decrease in this cytokine after my-HSC depletion is significant and supports our overall conclusions.

Other points:

- I agree with moving the human data into extended figures.

Authors' Response

We thank the Referee for agreeing and for this suggestion.

- Statistical treatment of data has been improved.

Authors' Response

We thank the Referee for their input on statistics and for commenting that this has been improved.

- Authors really did not improve the transparency of the manuscript. Tissues analyzed are rarely identified in the legends, experimental design details are lacking and require one to repeatedly visit the Methods section (often in vain). This group is too good to have such poorly written figure legends.

Authors' Response

As requested in the initial review, we took several steps to improve transparency of the manuscript, including describing all tissues, mouse ages, and time-points in the figure legends. To further satisfy this request to improve transparency, we will carefully review our changes and add additional information to the Figure Legends.

Appendix Figures

Vaccine-induced protection of FV infection requires CD4+ & CD8+ T cells and B cells.

[Appendix figure/panels redacted]

Viremia of FV-challenged mice receiving adoptive transfer of different lymphocyte subsets (CD4+ T cells, CD8+ T cells, CD19+ B cells, and dual combinations). Figure and Figure Legend Modified and Adapted from Dittmer et. al, *Nature Medicine*. 1999[27].

Determination of the lymphocyte subsets required for vaccine-induced protection from FV.

[Appendix figure/panels redacted]

Adoptive transfer experiments demonstrate that all three major lymphocyte subsets (CD4+ T cells, CD8+ T cells, and B cells) are required for vaccine protection. Figure and Figure Legend Modified and Adapted from Hasenkrug and Dittmer et. al, *Virology*. 2000[24].

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Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1:
Remarks to the Author:
I appreciate the authors going to great lengths to demonstrate that antibody depletion preferentially removes myeloid-biased HSCs and supports rejuvenation of the HSC compartment marked by accumulation of more balanced HSCs. However, the refusal of the authors to include transplantation experiments is somewhat puzzling. Transplantation has always been a key assay used (including by the Weissman group) to demonstrate the presence and cell intrinsic nature of lineage biased HSCs.

If the transplants are performed and confirm the results in primary mice treated with depleting antibodies, it will significantly strengthen the conclusions of the manuscript. However, I want to make it clear that in my opinion, if the transplants are performed and fail to demonstrate a rescue of the age-related myeloid bias, it will not preclude publication in Nature. Indeed, as the authors point out there may be effects of conditioning regimens that revert some of the effects observed in primary mice (although there are alternative conditioning regimens such as antibody depletion or busulfan that may minimize those effects), or more generally the effects may be diminished in transplantation. If this is the outcome, it does not completely invalidate results in the primary mice, but instead reveals a critical limitation of the study that should be thoroughly discussed by the authors in this manuscript and one that should probably be mechanistically investigated in future studies. The authors would be doing a massive injustice to their own work by not including these studies and clearly identifying these potential limitations if they exist.

Thus, I firmly believe that inclusion of these transplant experiments, regardless of the outcome, is absolutely critical to the rigor of the study. Omitting the transplant experiments jeopardizes the validity of the study as other independent groups will likely repeat the studies and perform these transplants. If other groups subsequently report that transplantation reveals persistent myeloid-biased HSCs after antibody depletion, it would immediately call into question the results of the present study. By including the transplant experiments, regardless of outcome, the authors enhance the rigor of the study and protect the integrity of their conclusions by clearly confirming the outcome or delineating critical limitations.

Referee #3:
Remarks to the Author:
This second revision by Ross et al. again only partially answers some of the key criticisms. Again, the authors have elected to not perform key experiments necessary to evaluate the significance of their observations. In my opinion, a major, concept-shifting manuscript should contain an original approach, demonstration of its mechanism(s) and demonstration of biological impact. While this work likely meets the first criterion, it falls short on the other two, and by a significant margin, in large part because authors refused to perform key discriminative experiments requested by myself and Reviewer 1.

The summary of the work by Ross et al. is as follows: the authors show some improvement in some parameters of immune function by rebalancing the HSC compartment. The conceptual novelty is in the approach of HSC rebalancing, which indeed has not been shown previously to improve immune aging. Even this part of the work, however, contains weaknesses and lacks convincing quantitative analyses. For example, Reviewer 1 asked for the demonstration that newly generated Bal-HSC are indeed balanced, which the authors elected not to analyze. I have asked for a quantitative explanation on why would one expect that a 6.2% reduction of Neo1+ My-HSC would be able to reduce signs of immune aging. The authors responded that this is a twofold, or over 200%, reduction, and that this is much more meaningful than the net % reduction. Perhaps. However, My-HSC of other phenotypes remain, and are presumably around and not affected by depletion of Neo1+ cells. Why are these other cells, or the residual Neo1+ My-HSC, no longer detrimental to the aging process? Quantitatively, I find this part difficult to understand.
Of the two other key criteria, the authors did not demonstrate the plausible mechanisms behind the rebalancing effect (no thymic cellularity increase, some but not consistent increase in circulatory naïve T cells). There is no data on whether there is any increase of naïve T and B cells in secondary lymphoid organs at a steady state, another experiment I have requested and the authors refused to performl; the authors contend that circulating naïve T and B cells are the relevant measure of increase, but these cells operate and are activated in secondary lymphoid organs (SLO), and it is supremely relevant to know whether SLO biology, and immune responses generated therein, may be improved by HSC rebalancing. There are other less burning issues, including why certain aspects of age-related immune dysfunction are improved and others not – particularly why only IL-1b is reduced and other cytokines are not, but they are not as critical relative to the ones listed above.

Third, the authors demonstrate a reduction of Friend virus (FV) titers in the spleen following vaccination (I apologize for the misuse of Theiler's virus, that was a linguistic lapsus and not a conceptual permutation). They, however, do not, and did not attempt to, demonstrate by which mechanism (innate, NK cells, or adaptive, CD8, CD4 or B cells) this reduction occur. They elected not to perform incisive experiments to evaluate immune function of major arms of immunity (they now show that there is an increase in tetramer-positive, FV-specific CD8 T cells following vaccination); functional characteristics of these cells, their effector differentiation by expression of T-bet, IFNγ, TNFα, Granzyme B, all known to be reduced in aging, are not analyzed; CD4 T cells, B cells or NK cells are not tested at all. Supplementary figure in the rebuttal, from the Nature Medicine 1999 paper, shows that all adaptive immune cell subsets are involved in vaccine-mediated protection, so one would reasonably want to know what has been improved and by how much, following HSC rebalancing. Most importantly, the key question for this study is how much the immune function has been improved. Older adults and older mammals in general exhibit massively increased vulnerability to NEW, previously not encountered, infection. That is why primary responses to lethal challenge are so important in evaluating improvement of immune function with aging. The authors did not perform such challenge experiments in naïve mice to demonstrate whether and to what extent mice are indeed more resistant to infectious challenge. The authors replied in their rebuttal "We did not claim our experiments on vaccine-induced immune protection are "supposed to support the improvement in naive immune cell function and in immune defense". As stated in our manuscript, our experiments were designed to test immune function after vaccination, a clinically relevant experiment in a well-documented model of viral infection. For example, older subjects require higher doses of vaccines such as influenza in the hope that can make a protective immune response

not seen with doses given to younger subjects." -essentially lowering the bar relative to the experiments I have requested. However, that bar is not enough to evaluate efficacy of immune rejuvenation, including the one achieved here by HSC rebalancing. We know that higher doses of vaccines, as well as various types of recent vaccines (mRNA, Shingrix), can improve immune protection in older adults. Indeed, while enormous numbers of unvaccinated older adults died from COVID-19, there was practically no demonstrable difference in mortality between younger and older adults following mRNA vaccination. Thus, with the advent of contemporary vaccines that are much more efficacious in older adults, immune rejuvenation, including that by rebalancing the HSC, is not needed to achieve improved vaccinal immunity. Immune rejuvenation is needed to defend against new infections for which vaccines are not available. Therefore, it is imperative to know if HSC rebalancing can produce an improvement in innate or adaptive immune responses that will yield increased protective immunity against new, primary infections.
Based on the above, I cannot recommend this revision for publication.

Referees' comments:

Referee #1 (Remarks to the Author):

I appreciate the authors going to great lengths to demonstrate that antibody depletion preferentially removes myeloid-biased HSCs and supports rejuvenation of the HSC compartment marked by accumulation of more balanced HSCs. However, the refusal of the authors to include transplantation experiments is somewhat puzzling. Transplantation has always been a key assay used (including by the Weissman group) to demonstrate the presence and cell intrinsic nature of lineage biased HSCs.

If the transplants are performed and confirm the results in primary mice treated with depleting antibodies, it will significantly strengthen the conclusions of the manuscript. However, I want to make it clear that in my opinion, if the transplants are performed and fail to demonstrate a rescue of the age-related myeloid bias, it will not preclude publication in Nature. Indeed, as the authors point out there may be effects of conditioning regimens that revert some of the effects observed in primary mice (although there are alternative conditioning regimens such as antibody depletion or busulfan that may minimize those effects), or more generally the effects may be diminished in transplantation. If this is the outcome, it does not completely invalidate results in the primary mice, but instead reveals a critical limitation of the study that should be thoroughly discussed by the authors in this manuscript and one that should probably be mechanistically investigated in future studies. The authors would be doing a massive injustice to their own work by not including these studies and clearly identifying these potential limitations if they exist.

Thus, I firmly believe that inclusion of these transplant experiments, regardless of the outcome, is absolutely critical to the rigor of the study. Omitting the transplant experiments jeopardizes the validity of the study as other independent groups will likely repeat the studies and perform these transplants. If other groups subsequently report that transplantation reveals persistent myeloid-biased HSCs after antibody depletion, it would immediately call into question the results of the present study. By including the transplant experiments, regardless of outcome, the authors enhance the rigor of the study and protect the integrity of their conclusions by clearly confirming the outcome or delineating critical limitations.

Response

We appreciate the Reviewer's feedback. We performed transplant experiments with purified HSCs to test the hypothesis functionally that changes in HSC potential are altered after antibody-conditioning. HSCs isolated from antibody-treated aged mice demonstrated a significantly decreased myeloid-to-lymphoid cell ratio compared to mice reconstituted with HSCs isolated from aged control mice (**new Fig. 2i–k**). Together, our results demonstrate that antibody-conditioning depletes my-HSCs based on cell-surface, molecular, and functional phenotypes.

Referee #1 (additional remarks to the Author – after presentation of transplantation data):

Was happy to recommend acceptance with the transplant data included.

Response

We thank the Reviewer for feedback that transplantation of HSC from mice receiving myHSC targeting antibodies should reveal depletion of myHSC relative to balHSC, which the results verify.

Referee #3 (Remarks to the Author):

This second revision by Ross et al. again only partially answers some of the key criticisms. Again, the authors have elected to not perform key experiments necessary to evaluate the significance of their observations. In my opinion, a major, concept-shifting manuscript should contain an original approach, demonstration of its mechanism(s) and demonstration of biological impact. While this work likely meets the first criterion, it falls short on the other two, and by a significant margin, in large part because authors refused to perform key discriminative experiments requested by myself and Reviewer 1.

Response
As detailed below, we have addressed the issues raised by Reviewer 1 and the manuscript was found acceptable by Reviewer 2, whose summary we believe conveys the "original approach, demonstration of its mechanism(s) and demonstration of biological impact" of our work:

Reviewer 3 acknowledges our original approach but questions that we demonstrated mechanism or biological impact. We strongly disagree. We directly demonstrate that the mechanism behind the restoration of the protective immunity demonstrated in our paper is the depletion of My-HSC, which allows Bal-HSC to repopulate circulating naïve lymphocytes that were able to respond to a novel infection. We not only showed increases in circulating naive T cells and B cells, but as requested by Reviewer #3, showed increases in virus-specific T cells in the spleen, (the secondary lymphoid tissue draining i.v. infection, which is also the site of genesis of adaptive immune responses to iv infections via lymphocyte content in the blood trafficking to spleen rather than the small lymphocyte content in the spleen), following primary infection with a novel live virus, Friend leukemia helper virus, which we previously showed to generate protective immune responses against lethal challenge with Friend leukemia virus complex [1]. We then challenged the mice with the lethal virus complex and showed significantly improved recovery, a finding that demonstrates regeneration of protective immunity in mice with re-balanced HSCs. While the biological significance of these results appears quite obvious to us and the other reviewers, it is questioned by Referee 3. We further address this issue below.

Reviewer 3:
The summary of the work by Ross et al. is as follows: the authors show some improvement in some parameters of immune function by rebalancing the HSC compartment.

Response
The reviewer downplays the age-related parameters improved by rebalancing the HSC compartment. We demonstrate that depletion of my-HSCs in aged animals increased lymphocyte progenitors, naïve T cells, and B cells, and decreased age-related markers of inflammation and immune decline. These are the key parameters of immune decline, not just "some improvement". Furthermore, modulating these parameters of HSC biology is of general and broad importance, as summarized by a recent review on the topic, "Given that age-related changes in HSCs are thought to be key contributing factors to infectious susceptibility, reduced vaccination efficacy, leukemic emergence, and even solid tissue senescence, a focus has been placed on mitigating or reversing these changes for systemic functional benefit" [2]. Recent studies from independent groups have demonstrated the resistance of aged HSCs to many functional rejuvenation strategies [3-6], including

from heterochronic parabiosis, exercise, and diet [7]. A recent review summarizes 20 independent attempts to functionally rejuvenate HSCs [2], using systemic, microenvironmental, epigenetic, and metabolic approaches, none of which achieve the extent of functional rejuvenation of old HSCs in our study. Thus, our demonstration of *in vivo* rejuvenation of lymphopoiesis in old mice, including increasing the ratio of balHSC to myHSCs, complimented with phenotypic, molecular, and functional evidence, represents a major advance to the field.

Reviewer 3:
The conceptual novelty is in the approach of HSC rebalancing, which indeed has not been shown previously to improve immune aging. Even this part of the work, however, contains weaknesses and lacks convincing quantitative analyses. For example, Reviewer 1 asked for the demonstration that newly generated Bal-HSC are indeed balanced, which the authors elected not to analyze.

Response:
The Referee's view that our manuscript "lacks convincing quantitative analyses" is not shared by Reviewer 1 who commented, "the authors going to great lengths to demonstrate that antibody depletion preferentially removes myeloid-biased HSCs and supports rejuvenation of the HSC compartment marked by accumulation of more balanced HSCs". Our manuscript demonstrates that rejuvenated aged HSCs generate immune systems with less myeloid bias and more naïve lymphocytes than non-rejuvenated aged HSCs (**Fig. 2i–k**). Reviewer 3 is unaware that we have done the transplant experiment requested by Reviewer 1 using purified HSCs to functionally validate that changes in HSC potential are altered after antibody-conditioning. HSCs isolated from antibody-treated aged mice demonstrated a significantly decreased myeloid-to-lymphoid cell ratio compared to mice reconstituted with HSCs isolated from aged control mice and showed rather dramatic increases in the lymphoid compartment (**new Fig. 2i–k**). Together, our results demonstrate that antibody-conditioning depletes my-HSCs based on cell-surface (**Fig. 2a–c**), molecular (**Fig. 2g–h**), and functional (**Fig. 2i–k**) phenotypes.

Reviewer 3:
I have asked for a quantitative explanation on why would one expect that a 6.2% reduction of Neo1+ My-HSC would be able to reduce signs of immune aging. The authors responded that this is a twofold, or over 200%, reduction, and that this is much more meaningful than the net % reduction. Perhaps.

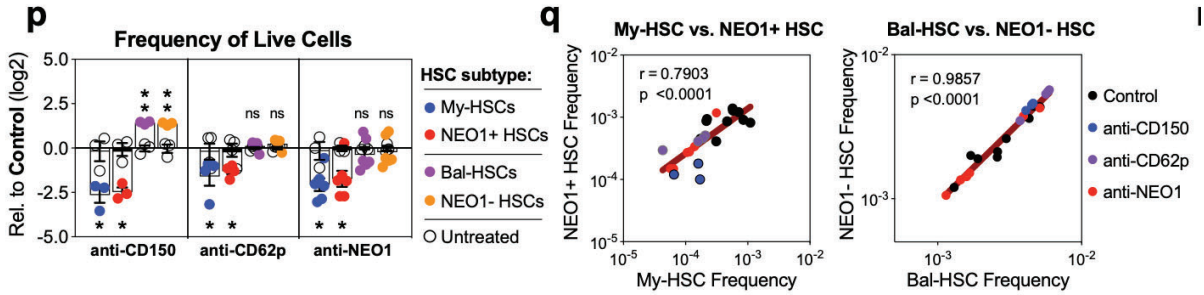
Response
The Reviewer asks for a "quantitative explanation" for why a greater than twofold reduction in my-HSCs would be able to reduce signs of immune aging. We pointed out that a reduction from 13% to 5.8% of myHSC is not a 6.2% reduction, but a 2:1, or ~200% reduction, or half as many myHSC. A 6.2% reduction from 13% would be to 12.2% by our calculation. Put simply, a twofold decrease in my-HSCs would be expected to produce at least twofold changes in parameters of immune aging. Since the stem cell is upstream of immune defects caused by differentiated myeloid cells, the effects could be amplified well above two-fold. In fact, this is what we observed in the lymphoid compartment in the new transplant experiments (**Fig. 2**).

Reviewer 3:

However, My-HSC of other phenotypes remain, and are presumably around and not affected by depletion of Neo1+ cells. Why are these other cells, or the residual Neo1+ My-HSC, no longer detrimental to the aging process? Quantitatively, I find this part difficult to understand.

Response

The Reviewer's statement that "My-HSC of other phenotypes remain and are presumably around and not affected by depletion of Neo1+ cells" is inconsistent with the data presented. We demonstrate that anti-Neo1 results in decreased frequencies of $CD150^{Hi}$ HSCs, as well as $Neo1^{+}$ HSCs (**Ex. Data Fig. 4p–q**, copied below). This is also true of anti-CD150 treatment and anti-CD62p treatment. The depletion of my-HSCs marked by two independent surface antigens with antibodies to one marker demonstrates that a common My-HSC subset is depleted regardless of the antigen targeted. We have additional data that my-HSCs marked by CD41 are also depleted, which we can provide if requested. The depletion of functional my-HSCs is also supported by our new transplant data (**Fig. 2i–k**). We had countered this issue first by showing the loss of neogenin1 by anti CD150 or CD62 proves this is loss of cell types, not simply single molecule antigenic modulation; but strong negative opinions seemingly can outweigh data, as shown again in reviewer 3's claims in the face of **p** below.



Response

The Reviewer asks why the "residual Neo1+ My-HSC, no longer detrimental to the aging process?". My-HSCs and bal-HSCs are both present in youth, but my-HSCs substantially expand with age relative to bal-HSCs. We demonstrate this imbalance can be sufficiently reduced to improve deficits of HSC aging, supporting the expansion of my-HSCs contributing to immune decline.

Reviewer 3:

Of the two other key criteria, the authors did not demonstrate the plausible mechanisms behind the rebalancing effect

Response

Presumably, the reviewer is referring to the rebalancing effect on the immune system. In the revised manuscript we provide a direct explanation in the discussion as it appears to have been missed. As noted above, the depletion of My-HSC allows Bal-HSC to rebalance the hematopoietic system by increasing lymphocyte progenitors (**Fig. 3d**) and circulating naïve lymphocytes (**Fig. 3e**), which we further demonstrate are capable of responding to a novel infection in a secondary lymphoid organ (SLO). This mechanism is not only "plausible", it is evident.

Reviewer 3:
(no thymic cellularity increase,

Response
In response to Reviewer 1 in the first review, we demonstrated that the thymus cells necessary for T cell education are present in the aged thymus (**Ex. Data Fig. 6m, n**). Interrogation of thymic cellularity was asked by Reviewer 1, who found our response acceptable. As noted in the previous rebuttal, newly emigrated lymphocytes are found in the circulation, not necessarily in all SLO [8]. The spleen, but not other SLO organs such as lymph nodes and Peyers patches, is the drainage site for antigens in the blood, and as Ford showed decades ago [9-11], the splenic response to blood borne antigens is dependent on blood lymphocytes, not lymphocytes in the spleen when antigen enters. The homing receptors for PP high endothelial venules and for LN high endothelial venules are different. Furthermore, Leins et al. states, "We report that both phenotypic and functional changes in the immune system on aging are primarily a consequence of changes in the function of HSCs on aging and, to a large extent, i**ndependent of the thymus**" [12]. Thus, our findings are consistent with the known literature and biology of naïve T lymphocytes. Furthermore, My-HSC overproduce myeloid cells, including proinflammatory cells and factors.

Reviewer 3:
some but not consistent increase in circulatory naïve T cells).

Response
The statement that we observed "some but not consistent increase in circulatory naïve T cells" is inconsistent with the results. We observed a consistent increase in circulating naïve ($CD44^{-}CD62^{+}$) T cells measured by both frequency (**Fig. 3e**) and by absolute number (**Ex. Data Fig. 7a**) in all experiments. The "not consistent increase" in T cells referred to by this Reviewer might relate to our computational analysis of circulating T cells, which was conducted to further characterize these subpopulations in an unbiased manner (**Ex. Data Fig. 7h**). We performed this analysis to address the question by Reviewer 3 related to what happens to T cell populations other than naïve T cells. This 12-marker cluster-based analysis confirmed the increase in circulating naïve T cells we observed based on the gold-standard $CD44^{-}CD62^{+}$ phenotype. The multiple-hypothesis adjusted p-value for this UMAP-defined naïve T cell cluster was 0.057 (the unadjusted p-value for this comparison is 0.0463 by one-tailed parametric t-test with Welch's correction), and so the Reviewer might be referring to this lower-powered experiment to suggest that our results are not consistent. This additional post-hoc analysis does impact the original validation using the gold-standard to measure naïve T cells.

Reviewer 3:
There is no data on whether there is any increase of naïve T and B cells in secondary lymphoid organs at a steady state, another experiment I have requested and the authors refused to performl; the authors contend that circulating naïve T and B cells are the relevant measure of increase, but these cells operate and are activated in secondary lymphoid organs (SLO),

Response
One of the classic experiments in immunology and lymphocyte physiology was the the demonstration that inserting cannulas in the splenic artery and splenic vein allowed the placement

of cells that initiate, and those that participate in anti-SRBC antibody responses. Spleens from thoracic duct lymphocyte depleted rats could only initiate anti-SRBC responses after introduction of SRBC via the arterial cannula, while cannulae containing selectively lymphocyte-depleted blood circulating through spleens from lymphocyte replete animals could not [9-11]. Sadly, the investigator died in a car accident before he could test T vs B cells for the initiator and the participator functions just described. Now, 50 years later, to our knowledge it remains unsolved. But it contradicts the above assertion *"the authors contend that circulating naïve T and B cells are the relevant measure of increase, but these cells operate and are activated in secondary lymphoid organs (SLO)."* This statement by the reviewer is simply incorrect. No doubt there are numerous parameters of SLO biology that could be further studied, but the ideas that certain thymic and SLO parameters must be restored in order for rejuvenation to occur are unproven hypotheses. As stated in a recent 2023 review on this subject by Sonar, Watanabe and Nikolich [13], "the direct mechanistic evidence that age-related changes in the SLO stromal cells underlie defective immunity with aging is still limited". As described above, we have performed the examination of naïve lymphocytes in the correct tissue. Providing data to determine if there is "any increase of naïve T and B cells in secondary lymphoid organs at a steady state" is not indicated or necessary to demonstrate improved functional immunity. We made clear in the revised manuscript that we demonstrate improved T cell function in in primary and secondary immune responses to live viral infection in secondary lymphoid organs (SLO). Examining the SLO at steady-state will not change the result.

Reviewer 3:

and it is supremely relevant to know whether SLO biology, and immune responses generated therein, may be improved by HSC rebalancing.

Response

In the revised manuscript, we better explained that the "vaccine" we use is a replication competent virus that infects the spleen but is only pathogenic in neonatal or immune incompetent animals. The spleen is an SLO where we show virus control and demonstrate virus-specific CD8+ T cell staining for both primary and secondary responses. The new data in our revised manuscript clearly demonstrate an "immune responses generated therein".

Reviewer 3:

There are other less burning issues, including why certain aspects of age-related immune dysfunction are improved and others not – particularly why only IL-1b is reduced and other cytokines are not, but they are not as critical relative to the ones listed above.

Response

As shown in **Figure 3i, j**, IL-1a and CXCL5 were the most elevated proinflammatory cytokines in our cohort of mice and were significantly reduced upon my-HSC depletion. Numerous independent groups have demonstrated that IL-1a/b are critical and central regulators of HSC aging [14-21]. In humans, IL-1b is known to be a pro-inflammatory cytokine with levels associated with cardiovascular disease, cancer, functional decline, and various degenerative diseases [22], so its reduction in our rejuvenated mice is of high import. The Reviewer makes the general assumption that other cytokines such as TNFa should have been reduced, but TNFa was not elevated in our aged cohort. Furthermore, TNFa blockade has been shown to have no effect on HSC aging [23].

Reviewer 3:

Third, the authors demonstrate a reduction of Friend virus (FV) titers in the spleen following vaccination (I apologize for the misuse of Theiler's virus, that was a linguistic lapsus and not a conceptual permutation).

Response

We drew attention to the Referee's misuse of the virus name because the pathogen we studied – Friend retrovirus – infects the spleen, which is a secondary lymphoid organ (SLO). The Referee has commented on numerous occasions that we have not examined the impact on SLO, which is not correct – every infection experiment conducted in this study involved the spleen.

Reviewer 3:

They, however, do not, and did not attempt to, demonstrate by which mechanism (innate, NK cells, or adaptive, CD8, CD4 or B cells) this reduction occur. They elected not to perform incisive experiments to evaluate immune function of major arms of immunity (they now show that there is an increase in tetramer-positive, FV-specific CD8 T cells following vaccination); functional characteristics of these cells, their effector differentiation by expression of T-bet, IFNγ, TNFα, Granzyme B, all known to be reduced in aging, are not analyzed; CD4 T cells, B cells or NK cells are not tested at all. Supplementary figure in the rebuttal, from the Nature Medicine 1999 paper, shows that all adaptive immune cell subsets are involved in vaccine-mediated protection, so one would reasonably want to know what has been improved and by how much, following HSC rebalancing.

Response

The original request that we demonstrate functional immunity did not state that we must address every aspect of every immune response and we feel this is beyond the scope of this new discovery paper. We had already obtained some data on the frequency of dextramer+ T cells in the spleens, which in the second revision we demonstrated virus-specific CD8+ T cell responses to vaccination with a live-attenuated virus, and also virus-specific CD8+ T cell responses to challenge, a response that directly correlated with virus control. This new data is acknowledged by the reviewer, who stated "they now show that there is an increase in tetramer-positive, FV-specific CD8 T cells following vaccination". In our view, we provide sufficient mechanistic explanation by demonstrating that depletion of my-HSCs increases lymphocyte progenitors and circulating naïve T cells, resulting in improved antigen-specific T cell response to primary infection, followed by improved functional immunity to a secondary lethal infection, which is correlated with the degree of antigen-specific T cells. While more detailed analyses seem beyond the scope of this manuscript to us, if required by the Editor, we can include markers of cytolytic function for the CD8+ T cells and markers of activation for CD4+ T cells and B cells in a revised manuscript. In the revised manuscript, we have added the data on the direct correlation of virus-specific CD8+ T cell responses to infectious challenge (**new Ex. Data Fig. 9k**), which was included in the prior Response Letter.

Reviewer 3:

Most importantly, the key question for this study is how much the immune function has been improved. Older adults and older mammals in general exhibit massively increased vulnerability to NEW, previously not encountered, infection. That is why primary responses to lethal challenge

are so important in evaluating improvement of immune function with aging. The authors did not perform such challenge experiments in naïve mice to demonstrate whether and to what extent mice are indeed more resistant to infectious challenge. The authors replied in their rebuttal "We did not claim our experiments on vaccine-induced immune protection are "supposed to support the improvement in naive immune cell function and in immune defense". As stated in our manuscript, our experiments were designed to test immune function after vaccination, a clinically relevant experiment in a well-documented model of viral infection. For example, older subjects require higher doses of vaccines such as influenza in the hope that can make a protective immune response not seen with doses given to younger subjects." -essentially lowering the bar relative to the experiments I have requested. However, that bar is not enough to evaluate efficacy of immune rejuvenation, including the one achieved here by HSC rebalancing.

Response

Once again, we reiterate that we challenged naïve mice with a novel virus and demonstrated that they had an improved immune response. The insistence by this reviewer that we use a lethal primary infection is excessive, was not stipulated by the original Editor, and is not necessary to demonstrate that HSC rebalancing has significantly improved immune responses to infection. Furthermore, the improvement in antigen specific CD8+ T cells to naïve infection that we demonstrate exceeds experimental observations cited by Reviewer 3 in the first round by Surh and Nikolich-Žugich (Thompson, H.L. et al., Aging Cell, 2019) [24], whereby aged thymic-rejuvenated mice were unable to generate antigen specific CD8+ T cells to naïve infections. Thus, the improvement we demonstrate in the primary responses of aged mice is a significant advancement. We also observed substantial improvements in the secondary responses of rejuvenated mice challenged with lethal viral thereby demonstrating protective immunity.

Reviewer 3:

We know that higher doses of vaccines, as well as various types of recent vaccines (mRNA, Shingrix), can improve immune protection in older adults. Indeed, while enormous numbers of unvaccinated older adults died from COVID-19, there was practically no demonstrable difference in mortality between younger and older adults following mRNA vaccination. Thus, with the advent of contemporary vaccines that are much more efficacious in older adults, immune rejuvenation, including that by rebalancing the HSC, is not needed to achieve improved vaccinal immunity. Immune rejuvenation is needed to defend against new infections for which vaccines are not available.

Response

Reviewer 3 makes the broadly speculative statement that, "with the advent of contemporary vaccines that are much more efficacious in older adults, immune rejuvenation, including that by rebalancing the HSC, is not needed to achieve improved vaccinal immunity". This is not a fact-based assessment widely held by immunologists in the vaccine field or rejuvenation field, apparently including the other reviewers. First of all, most vaccines in use today are live-attenuated viruses. These include: measles, mumps, rubella, smallpox, chickenpox, rotavirus, yellow fever and BCG, one of the most widely used vaccines in the world. The reviewer faults us (wrongly) for looking at secondary responses but then cites the Shingrix vaccine, which is to re-stimulate memory cells in aged people carrying latent varicella zoster infections. It is not for primary vaccination. As we and the reviewer have noted, the primary problem in the aged is the lack of

naïve T cells required to mount immune responses to a novel infection. The mRNA vaccines for SARS-CoV-2 don't elicit immune responses in the aged equivalent to young people until after boosting (which is then a secondary response), and the long-term stability of protection of mRNA vaccines has not been established. While there is a single publication from the Nikolich-Zugich lab showing that the age-related reduction in immune responses to SARS-CoV-2 mRNA vaccination was "measurable but minimal" after boosting [25], the results were based on only 17 participants over the age of 55. Not only is this a small cohort, but 55 is not considered an aged population, vaccine boosting was required, no virus protection was shown, and this study conflicts with other studies. For example, a study from the Gupta lab showed significantly reduced mRNA vaccine responses in the elderly[26]. A study from the Lu lab demonstrated diminished antibody functions in aged populations following SARS-CoV-2 mRNA vaccination[27], and a study with a 655 SARS-CoV-2 mRNA vaccinees showed reduced serological and T cell immunity in the elderly[28]. None of those studies included vaccine efficacy trials. However, a study of over 75 million Brazilian vaccinees against COVID-19 (albeit with a vectored vaccine) demonstrated that vaccine efficacy was progressively lower with age for parameters of hospitalization, ICU admission and DEATH. Even if the Nikolich-Zugich study turned out to be correct, results from a single vaccine cannot be generalized to all vaccines and all viruses. In 2023, numerous papers have been published that address the importance of age-related vaccine responses. We quote from just a few:

<u>2023</u>
"Our study provides mechanistic insights for weaker response to inactivated vaccine by older adults and suggests the need for further vaccination optimization for the old population." [29]

"Here, we examined the response of these two components to the SARS-CoV-2 vaccine in 655 Danish citizens. The response of both components was lower in people over 75 years old and with other diseases." [28]

"At present, the optimum number of vaccination boosters is uncertain, but the potential immune suppression by continuous use of SARS-CoV-2 vaccine boosters should raise concern and studies are needed to confirm whether this occurs in humans, especially in the older population due to age-related changes of the immune system" [30]

"As we age, the immune system undergoes immunosenescence, which is marked by a systemic process known as inflammaging along with a series of defects in immunological activity that results in poor responses to infectious agents, vaccination and cancer (6–8)." [31]

<u>2022</u>
"Aging is associated with a reduced magnitude of primary immune responses to vaccination" [25]

"The adaptive immune response is a major determinant of the clinical outcome after SARS-CoV-2 infection and underpins vaccine efficacy." [32]

<u>2021</u>
"The production of interferon-γ and interleukin-2 by SARS-CoV-2 spike-specific T cells was lower in older participants, and both cytokines were secreted primarily by CD4 T cells. We

conclude that the elderly are a high-risk population and that specific measures to boost vaccine responses in this population are warranted" [26]

"This diminished capacity of the aged immune system is clinically evident, as aging is associated with high morbidity and mortality rates for various infections and significant reductions in vaccine efficacy" [33]

Reviewer 3:
Therefore, it is imperative to know if HSC rebalancing can produce an improvement in innate or adaptive immune responses that will yield increased protective immunity against new, primary infections.

Response
This is precisely what we demonstrate in our manuscript. We evaluated a primary response to a completely novel pathogen by infecting animals with replication competent live virus (Friend murine leukemia helper virus [1]. At two weeks post-infection, we found significantly increased virus-specific CD8+ T cells from the aged/rejuvenated mice compared to aged controls (**Fig. 4b**). Thus, rejuvenation not only decreased levels of My-HSCs and increased the levels of circulating naïve T cells, but also produced an improvement in adaptive immune responses of naïve mice to a novel virus challenge. From an immunological perspective, protective immunity is achieved by either vaccination or prior exposure. To test whether rejuvenation and the associated improvement in primary immune responses yielded protective immunity, the mice were then challenged with a lethal, pathogenic virus that shares all antigens expressed by the vaccine virus [1]. Compared to aged controls, the aged/rejuvenated mice showed increased protection from pathogenesis (**Fig. 4c**), better control of virus (**Fig. 4d**) and increased virus-specific CD8+ T cell responses (**Fig. 4e**) that correlated with protection (**new Ex. Data Fig. 9k**). These findings conclusively establish that a single injection of My-HSC-depleting antibodies improves functional immunity and answer the initial request by the editor, "we would ask the revised manuscript to include an assessment of the effects of myeloid-biased HSC depletion on the response to infection, as requested by referee #3." Therefore, contrary to Reviewer 3's contention, rejuvenation resulted in improved protective immunity in both primary (naïve) and secondary (re-challenge) responses.

Reviewer 3:
Based on the above, I cannot recommend this revision for publication

Response
For the reasons enumerated above, we adamantly disagree with this assessment. We again stress that the primary import of our study is demonstrating that age-related changes to the HSC compartment can be significantly reversed by depleting my-HSCs. The demonstration of improved immunity was to provide functional validation of the downstream effects of HSC re-balancing and proof-of-principle to pursue studies on other age-related phenotypes driven by HSCs. On their own, the findings in our original manuscript already represented a major advance in HSC aging research, which we functionally validated with improved immune responses and immune protection in our revised manuscripts. Furthermore, our study does not claim to solve all aspects of immune system rejuvenation, but demonstrates that therapeutic elimination of aged HSCs restores more youthful hematopoiesis and improves functional immunity, placing HSCs as critical

upstream regulators of age-related immune dysfunction. It opens, rather than closes, the door to detailed improvements in the system, as well as providing evidence for seeking 'rejuvenation' protocols in humans. We feel that the concerns raised by Reviewer 3 are either wrong or off the main point. Furthermore, Reviewer 3's critique of our work on demonstrating enhanced immune function is not shared by Reviewer 1 or Reviewer 2, who stated in the second round of review:

Referee #1 (Remarks to the Author):
"The authors have significantly improved the manuscript and provided key new controls as well as new data on immune function after my-HSC depletion."

Referee #2 (Remarks to the Author):
"The new experiments and data supporting restored T cell immunity in aged mice following myeloid-biased HSC depletion are very exciting and support the overall themes of the paper."
"The work is original and significant, and addresses a critical health problem in the aging population."

References

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