nature portfolio

Peer Review File

Mutation-dependent responses to sleep and exercise in clonal hematopoiesis

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
I had the opportunity to review the manuscript submitted by Gerhardt et al. In this study, the authors employed sophisticated murine models to investigate how lifestyle factors—specifically sleep fragmentation and exercise—affect clonal hematopoiesis (CH) in the context of experimental atherosclerosis. They developed a chimeric Ldlr-/- mouse model by combining irradiation and reconstitution with a bone marrow (BM) cell mixture containing 90% wild-type (WT) CD45.1 cells and 10% CD45.2 cells harboring inducible CH-associated mutations: Jak2^V617Ffl/flMx1Cre, Tet2^fl/flMx1Cre, or p53^fl/flMx1Cre.
Their findings suggest that sleep fragmentation (SF) promotes CH expansion and accelerates atherosclerosis, whereas exercise mitigates CH and limits disease progression. Mechanistically, SF led to inflammasome activation, and neutralizing IL-1β with a monoclonal antibody abolished both CH expansion and atherosclerosis progression. The beneficial effects of exercise were attributed to catecholamine production, which activates β2-adrenergic receptors on vascular macrophages.

General Assessment:
The topic is timely and relevant. However, the novelty is somewhat limited. The pro-atherogenic effects of SF have already been reported by the same group (MacAlpine, Nature, 2019), and the association between clonal hematopoiesis and atherosclerosis is also well-established (e.g., Oren et al., JCI, 2024). While the experimental approaches are technically elegant, most of the data remain descriptive. The mechanistic depth of the analysis is insufficient. Although the authors characterized CH expansion in the blood and BM and conducted an in-depth analysis of atherosclerotic lesions using scRNA-seq, key mechanistic questions remain unanswered:
• What is the role of monocyte trafficking?
• Are neutrophils involved in the phenotype?
• Which cell types produce IL-1β?
• Why does IL-1β selectively promote CD45.2 mutant cell expansion ?
• Mechenisms driving atheroprotection after exercise are not convincing
Moreover, there are discrepancies between the scRNA-seq data and immunohistological analyses of plaque composition that require clarification.

Detailed Comments:
Macrophage Content and Monocyte Recruitment:
The impact of lifestyle factors on macrophage content in the lesions is superficially addressed, with an emphasis on proliferation. However, most lesional macrophages derive from circulating monocytes. Thus, monocyte trafficking and recruitment should be investigated more thoroughly.
Neutrophil Involvement:
Neutrophil infiltration is superficially analyzed, and transcriptomic data alone are insufficient to assess recruitment. Neutrophil stainings and depletion experiments would provide clearer insights into their role.
Timing and Source of IL-1β:
IL-1β and IL-6 were measured at the end of the experimental protocol, at which point the myeloid compartment was already significantly altered—potentially confounding results. Cytokine levels should be measured at earlier time points and during the course of disease progression. The source of IL-1β and the identity of IL-1β–responding cells need to be clarified.
Selective Expansion of CD45.2 Cells:
Although IL-1β is known to promote myelopoiesis, the authors show that it selectively expands CD45.2 mutant cells but not

CD45.1 WT cells. This raises important questions: Is this due to differential expression of the IL-1 receptor or differences in downstream signaling? Additional experiments are essential to address this point.
Figure 2:
The reported differences in IL-1β and IL-6 in the BM are modest, and their biological significance is unclear.
Figure 3:
The authors propose that sleep and exercise modulate CH-driven atherosclerosis via reprogramming of mutant macrophages. However, this claim is not directly supported by functional data. Additional histological analyses (e.g., macrophage and neutrophil staining) and monocyte/neutrophil depletion experiments would strengthen the conclusions.
Figure 4B:
Images are too small and difficult to interpret. In the SF group, a high degree of co-localization between macrophages and inflammasome markers (NLRP3, IL-1β) was reported, with IL-1β+ macrophages representing 80–85% of the total. This is inconsistent with scRNA-seq data, which indicate that inflammasome-positive macrophages form a minor subset.
Figure 4E:
CD64 staining is unconvincing and appears mislocalized. Its co-localization with Clec4a is also questionable.
Figure 4I:
Clec4a+ macrophages form a small subset in scRNA-seq data but appear overrepresented in histological images. This inconsistency requires explanation. Additionally, the images shown are not representative of plaque size and should be adjusted accordingly.
Figure 5 – Mechanism of Exercise Benefit:
The authors suggest that β2-adrenergic stimulation drives the beneficial effects of exercise via IL-1β suppression in macrophages, but the data are not fully convincing:
Figure 5C: Adrb2 mRNA levels appear increased in CH macrophages from exercised mice. Is this statistically significant? mRNA levels may not reflect protein expression due to post-transcriptional regulation. Protein-level validation is necessary because in vitro, the β2-adrenergic agonist salmeterol reduced IL-1β production in both WT and mutant macrophages.
Figure 5j-n: In vivo blockade of β2-adrenergic signaling reversed the protective effect of exercise in CH mice by shifting macrophages toward a more inflammatory phenotype, as indicated by increased IL1b expression. However, the mechanism remains unclear, since elevated numbers of $IL1b^{+}$ macrophages were observed in plaques from both the control and exercise groups following pharmacological treatment, while accelerated atherosclerosis was only seen in the exercise group. IL1b expression was quantified in all macrophages, but it is important to analyze expression separately in mutant (CD45.2) versus wild-type (CD45.1) macrophages. This distinction is especially relevant because, in Figure 5c, the authors reported that adrenergic receptor expression was higher in mutant cells, suggesting they may be more affected by pharmacological blockade.
Statistical Analysis:
The authors used repeated measures across four groups (Figures 1B and 1C), and in Figures 3 and 4, they appear to use the same control group for multiple comparisons. These aspects should be double-checked by a statistical expert.

Referee #2

(Remarks to the Author)
Reviewer Report for Manuscript on Mutation-Dependent CH Responses to Sleep and Exercise
Key Results: This manuscript investigates how lifestyle factors, particularly sleep fragmentation and voluntary exercise, modulate clonal hematopoiesis (CH) in a mutation-dependent manner. Using murine models and human cohort data, the study reveals that:
• Jak2 and Tet2 mutant clones expand under sleep fragmentation and are suppressed by exercise.
• p53 mutant clones are unresponsive to either intervention.
• These lifestyle effects selectively reprogram mutant hematopoietic stem cells and aortic macrophages.
• CLEC4E-inflammasome and norepinephrine-ADRβ2 signaling are key mutation-specific mechanisms.
• In human datasets, moderate-to-vigorous physical activity (MVPA) correlates with lower non-DNMT3A CH prevalence.

Validity: The experimental design is logically structured and spans multiple levels of analysis, from population-based human associations to cellular mechanisms in mouse models. The inclusion of multiple CH mutations provides important specificity, though the mechanistic depth is centered primarily on Jak2. The exclusion of a Dnmt3a model limits translational completeness, particularly since human data show MVPA does not reduce DNMT3A CH prevalence. Including a Dnmt3a mouse model is recommended and would substantially strengthen the work.

Originality and Significance: This is a novel and timely study that advances the understanding of how CH interacts with modifiable lifestyle factors in a mutation-dependent manner. It introduces new paradigms for personalized interventions and demonstrates that CH is not uniformly responsive to behavioral changes. The distinct signaling axes implicated—CLEC4E and norepinephrine-ADRβ2—further contribute to the originality of the work.

Methodological Rigor: The use of mixed bone marrow chimeras, scRNA-seq, metabolic assays, and behavioral interventions demonstrates exceptional technical breadth.
1. However, the lack of sex-stratified analyses is a critical omission given known sex differences in immune, metabolic, and neuroinflammatory responses. The authors state both sexes were used but provide no breakdown or stratified data. It appears that mixed sex controls were used, but only female mice were used in experimental groups. This is very unclear. The pooling justification is vague. While the authors note that results were “similar” in males and females, no sex-stratified data are shown to support this claim. Please clarify the sex used in each experiment and provide sex-stratified data where available, particularly for key outcome measures (e.g., clone expansion, lesion size, inflammatory markers, macrophage

clusters). If sexes were pooled based on the absence of statistically significant differences, summary statistics (e.g., p-values, direction of effect) should be provided in the supplementary materials. These additions will significantly enhance the rigor and reproducibility of the study. If sex was tested and found non-significant, a brief summary of those results should be shown. Given known sex-based differences in immune and metabolic responses, equal number of males and females should be used in the experiments if the findings are going to be interpreted in such a general context.
2. Details on number of animals used per group and per experiment are scattered and would benefit from consolidation in a supplemental table for transparency and reproducibility.
3. Randomization and blinding are not described for animal group assignments or data analysis. Were outcome assessments blinded? Were variables such as cage location and time of day controlled?

Statistical Analysis: The statistical approach used for analyzing murine data is insufficient given the complexity of the experimental design and the structure of the data. This significantly undermines the rigor and interpretability of several key findings.
1. The authors employ basic pairwise or group comparisons (e.g., ANOVA or t-tests), which do not adequately account for:
o Repeated measures (e.g., serial blood draws, longitudinal clone tracking)
o Nested data (cells within animals, animals within cohorts)
o Interactions between genetic background, intervention, and sex
2. The study involves a hierarchical structure. Use hierarchical mixed-effects models to account for repeated measures, nested data, and cohort variability. Include sex as a fixed effect and test for sex-by-mutation-by-intervention interactions.
3. Inappropriate treatment of zero-inflated or bounded data. Many endpoints (e.g., clone proportions, cytokine levels, lesion metrics) are bounded at 0, often right-skewed, or include excess zeros. These violate assumptions of normality and homoscedasticity. Apply appropriate transformations or use zero-inflated or beta regression models for bounded proportional data, and negative binomial models for over-dispersed count data (e.g., cell proliferation, lesion cell counts).
4. There is insufficient reporting of statistical details. Provide model coefficients, p-values, and confidence intervals.

Critique of "Healthy Sleep" Claims: The manuscript claims that "healthy sleep" reprograms CH dynamics. However, no direct sleep measures (e.g., EEG, telemetry, piezoelectric) were taken. This assumption is not valid. Mice not exposed to SF may still experience shortened total sleep time, reduced NREM/REM sleep, or poor sleep depth (e.g., delta power) due to environmental, inflammatory, or genetic factors. Critically, no direct measures of sleep quantity or architecture—such as EEG/EMG, telemetry recordings, or behavioral proxies—are reported. Thus, the conclusion that "healthy sleep" reprograms CH or modifies brain-body signaling pathways is unsupported by data. The authors should revise their language to reflect what was actually tested: e.g., “absence of tactile sleep fragmentation” rather than “healthy sleep.” Claims about the restorative or protective effects of sleep must be tempered unless direct sleep measures are included. It is recommended that sleep should be measured in at least a subset of animals to confirm the control animals for the SF experiments are sleeping as expected following the other experimental/genetic manipulations.

Jak2 Mechanistic Work: The deep mechanistic insights are largely derived from Jak2 models. While justified by clinical relevance, this limits generalizability. Broad claims about CH should be restricted and interpreted in the context of this mutation.
Translational Interpretation of Human Data: The cross-sectional associations in human cohorts do not establish causality. The authors should temper conclusions and acknowledge the potential for reverse causation and confounding.

Discussion of Clinical Implementation: The authors suggest genotype-guided lifestyle interventions for CH but do not discuss feasibility. Considerations such as patient genotyping, comorbidities, and real-world adherence need to be addressed. Most patients with CH are older adults with comorbidities that may impair sleep or physical activity capacity.

Brain-Body Mechanisms: While PAC1+ neuron activation and NE signaling are intriguing, these findings are largely correlative. Without circuit tracing or causal manipulation (e.g., optogenetics), claims should be framed as hypothesis-generating.

Figures and Language Suggestions:
• Improve clarity and define all abbreviations in figure legends.
• Avoid ‘sacrifice’ which carried religious connotations in some cultures and use ‘euthanize’ instead.
• Add colorblind-friendly palettes. Using a color blind-friendly palette in research is crucial for making data visualizations accessible and ensuring accurate interpretation of research findings by a wider audience.
• Provide time points for all intervention phases on the schematics.

Overall Recommendation: This is a conceptually novel and mechanistically rich manuscript that addresses an important question in aging and cardiovascular disease. With improvements to the statistical rigor, sex-specific reporting, and tempered claims regarding sleep and neuroimmune circuits, it will make a significant contribution to the field. Inclusion of a Dnmt3a mouse model would elevate the translational impact further. Acceptance is recommended pending major revision.

Referee #3

(Remarks to the Author)
In this study, McAlpine et al address the important question of whether lifestyle factors such as exercise and sleep impact the emergence of clonal hematopoiesis in mouse models and human epidemiologic data. The authors use a mouse model of common CH mutant BM transplanted into Ldlr-/- mice and layered on sleep deprivation or exercise to evaluate the impact on

clone expansion. Interestingly, they demonstrated that exercise mitigated clone expansion whereas sleep disruption promoted clone expansion particularly for Jak2-mutant clones but not for p53 mutant clones. Exercise also appeared to protect against CH in human cohorts such as the UKB. The authors hypothesize that exercise promotes a beta-adrenergic signal from the brain that counteracts a disease-driving hyperinflammatory gene program in macrophages; blocking beta adrenergic signaling was found to be sufficient to counteract the protecting effects of exercise in JAK2-driven CH associated atherosclerosis.

Overall this is a very expansive study with an interesting hypothesis and potentially impactful conclusions. However what the study offers in breadth it lacks in depth and rigor. Critical details are missing without consideration of alternative hypotheses. Experiments are not repeated. The data are not sufficient to support the claims in many instances, precluding the publication of the study in its current form.

Specific Comments:
1) It is unclear why Ldlr-/- mice were used in Figure 1 of this study as opposed to WT recipients. Ldlr-/- mice have been used in studies of the impact of CH on cardiovascular disease. However one of the points that the authors are trying to make here is that the effects of exercise and sleep deprivation on CH occur independently of weight, obesity, or glucose levels. Have the authors determined if lipid dysregulation is required for these effects to occur? Are similar effects of exercise and sleep disruption also observed in WT mice?

2) Figure 1: This sentence is not clear:

"all mice were fed a high cholesterol diet (HCD) and provided running wheels for voluntary exercise (Extended Data Fig 1a)31, subjected to tactile sleep fragmentation (SF) with automated cages30,36,37, or allowed to remain sedentary for 12 weeks."

What mice got what treatments and in what combinations?! This is only apparent after studying the figure in great detail.

3) What is the effect of sleep fragmentation or exercise alone on cytokine concentration and myeloid cell counts? These data need to be provided – not just for the Tet2 mosaics. Were IL1b and IL6 the only cytokines measured?

4) What is meant by “CD45.2 fraction growth”? These numbers could be influenced by the starting mosaicism which should be shown for all mice. Absolute fractional growth of 2% is different if the starting mosaicism is 1% versus 10%.

5) What were the weights and cholesterol levels of mice on SF and exercise? BMI and cholesterol levels for all groups should be provided. What about differences in WT for weights and lipid levels? Is dyslipidemia necessary to observe the beneficial effects of exercise?

6) Only female mice were used in the study. This should be acknowledged as a limitation of the study and all conclusions should specify that these effects are true for female mice. Human studies should include subset analysis of women and men separately to determine if there is a sex-specific effect.

3. The mouse experiments were only done once, which is not the scientific standard.

4. Uneven numbers of mice were used in the various groups, which biases the results to show differences in the larger groups.

6. No rationale is provided for the choice of Tet2, Jak2, and p53 for the murine experiments. The paper might be more logical if the human epidemiologic data are presented first and then the mouse studies are presented next, as the human data can used as rationale for the choice of these mutations.

7. Gene specific effects of environment on CH have been studied in other contexts (reviewed in Florez Cell Stem Cell) and should be cited.

8. The authors claim that: “Consistent with these dynamics, BM IL-1β and IL-6 were elevated in Ldlr-/-Jak2V617FBM mice exposed to SF and tended to be lowered by exercise (Fig 2c).” However the data are not significant and therefore the data do not support this conclusion. This again points to the question of whether other cytokines or other factors are being affected.

9. For the human data, the authors should do a subset analysis of the association between exercise and CH by mutation type. Or an association of the combined people with Tet2 and Jak2 CH and exercise.

10. How were CMPs and GMPs defined? How did the authors account for Sca shifts induced by inflammation that influence the definition of these populations?

11. The differences in IL1 levels with exercise or SF were 1-3 ng/mL --- how were these levels measured? Most ELISA and bead array methods do not measure cytokines at these levels at a sufficient level of precision to detect such small differences. Such small differences are unlikely to be physiologically relevant. Can the authors confirm IL1b or IL6 regulated genes were different between the groups?

12. Does KO or inhibition of CLEC4A rescue the expansion of Jak2 or Tet2 mutant clones in the setting of SF?

13. The authors did not fully address the question of whether expansion of macrophages versus reprogramming of macrophages is critical for CH expansion and progression of atherosclerosis. They imply that reversing the effect by reversing the reprogramming works; however what happens if mutant macrophage cell numbers are controlled – is this sufficient to abrogate atherosclerosis exacerbation?

14. Salmeterol appeared to be sufficient to replicate the b2-agonist effects of exercise. Do patients with asthma and frequent use of salmeterol or albuterol experience protective effects against CH-associated atherosclerosis?

Minor comments:

1. In the methods, the killing of mice should be described as “euthanasia” not “sacrifice”

2. Seahorse data provided in Extended Data Figure 4g should be quantified.

Referee #4

(Remarks to the Author)
Mutation-dependent responses to sleep and exercise in clonal hematopoiesis
Gerhardt et al. investigated the mutation-dependent effects of the lifestyle interventions sleep and exercise on clonal hematopoiesis and further examined how these interventions influence atherosclerosis and its underlying mechanisms. Through a combination of bone marrow transplantation experiments, single-cell RNA sequencing, in vivo chemical delivery, and physiological interventions—including sleep fragmentation and exercise—the authors demonstrated that clonal hematopoiesis can be exacerbated or mitigated in vivo depending on lifestyle intervention. They also leveraged large human cohort datasets, including the UK Biobank and All of Us Research Program, to propose that physical activity may attenuate non-DNMT3A-associated clonal hematopoiesis. The study addresses an important area of biological research, is thorough, and an impressive technical tour de force. The findings showing that exercise modulates clonal hematopoiesis (CH) and macrophage function via norepinephrine-mediated signaling is intriguing and a major scientific advance for the field. The role of sleep in promoting CH and influencing atherosclerosis had already been established by this same group in prior work (McAlpine et al., Nature, 2019; Yates et al., Nature Communications, 2025). Moreover, considering the literature that has identified IL-1β and inflammasome activation as central drivers of atherosclerosis, the current study's contribution to advancing our mechanistic understanding of the link between sleep and atherosclerosis may be somewhat incremental and needs to be better defined by the authors.
Comments
1. Overall, this study demonstrates that sleep fragmentation and exercise do not affect wild-type hematopoiesis but selectively modulate hematopoiesis derived from mutant myeloid progenitors, including Jak2 and Tet2 mutants. These are important finds but the mechanisms by which sleep and exercise exert mutation-specific regulation of clonal hematopoiesis is not fully explored at this stage.

2. The total amount of exercise performed by each mouse line/group over the 12- or 16- week training periods must be presented. The different strains and lines may have performed vastly different quantities of exercise, which could be a mechanism for differences between groups and treatments.

3. In Figure 1, the use of UK Biobank and All of Us datasets to examine associations between physical activity and DNMT3A vs. non-DNMT3A clonal hematopoiesis is intriguing. However, since the mutant models employed in this study are Tet2, Jak2, and p53, it would be more appropriate and aligned with the paper’s main focus to stratify the human cohort analyses based on these specific mutations.

4. In Figure 2a, exercise reduced the proliferation of CD45.2-positive common myeloid progenitors (CMPs) and granulocyte macrophage progenitors (GMPs) in Ldlr–/– Jak2V617F bone marrow. However, in Figure 2m, no notable differences in proliferation markers were observed between the Ldlr–/–Jak2V617F and Ldlr–/–Jak2V617F+Exercise groups. Does this suggest that exercise reduces proliferation in CMPs and GMPs without altering proliferation-related gene expression?

5. Furthermore, in CD45.1-derived cells, are the proliferation- and energy metabolism-related transcriptional changes observed in CD45.2 cells under sleep fragmentation or exercise conditions? A more in-depth scRNA-seq comparison between CD45.1 and CD45.2 populations would provide valuable insights into the mutation-dependent effects of sleep fragmentation and exercise.

6. In Figure 4, the authors suggest that sleep fragmentation induces CLEC4E-dependent inflammasome activation, thereby promoting atherosclerosis, as predicted by single-cell RNA-seq and supported by in vitro treatment of BMDMs with a CLEC4E agonist, which increased Il-1 , Nlrp3, and Aim2 expression. However, agonist stimulation of BMDMs alone is insufficient to establish that CLEC4E mediates atherosclerosis in the sleep fragmentation context. It would be more convincing to treat Ldlr–/– Jak2V617F mice with the CLEC4E agonist and evaluate its impact on atherosclerosis progression in vivo.

7. In Figure 5, the authors propose that exercise suppresses atherosclerosis via norepinephrine-mediated signaling. However, as shown in Figure 5l, Adrb2 antagonism failed to alter CMP and GMP proliferation. This result would suggest that the observed effect of exercise may be more directly related to atherosclerosis modulation, rather than the regulation of clonal hematopoiesis per se. Thus, the mechanistic link between norepinephrine signaling and mutant clone suppression remains speculative and warrants further clarification.

8. The mouse studies are only performed in female mice. No rationale is stated for the choice of female mice, or why both sexes were not studied. This is a significant limitation of the work. Given that the literature shows marked sexual dimorphism in exercise training responses in terms of transcriptional, proteomic, metabolomic, and phenotypic responses adaptations, the current results are only applicable to females. Thus, the summary, results and conclusions should acknowledge this fact, and the term female should be included where appropriate.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I carefully evaluated the revised manuscript submitted by Gerhardt et al.

As previously stated, the overall novelty of the study remains limited. Indeed, the pro-atherogenic effects of sleep fragmentation have already been reported by the same group (MacAlpine et al., Nature, 2019), and the association between clonal hematopoiesis and atherosclerosis is now well established (e.g., Oren et al., JCI, 2024).

That said, the mechanistic investigations have been clearly improved. The authors performed a large number of additional and convincing experiments that substantially clarify the mechanisms by which lifestyle factors modulate atherosclerosis in the context of clonal hematopoiesis. The identification of increased IL-1β production by mutant bone-marrow–derived macrophages, together with the specific activation of IL-1β–related pathways in mutant progenitors, is particularly interesting. In addition, the authors performed a more in-depth analysis of macrophage subsets within atherosclerotic lesions.

One methodological issue should nevertheless be addressed :

Extended Data Figure 8a. To evaluate the contribution of neutrophils to the vascular phenotype, the authors used an anti-Ly6G depleting antibody during the last six weeks of the protocol. While this approach is appropriate, neutrophil depletion was assessed by flow cytometry using a gating strategy that includes the Ly6G marker. This represents a major confounding factor, as it is not appropriate to use the same target for both depletion and flow-cytometric identification, since epitope masking by the depleting monoclonal antibody may lead to inaccurate assessment of neutrophil numbers.

Referee #2

(Remarks to the Author)
In the revised manuscript, the authors have addressed many of the concerns raised in the initial review. Several issues that were unclear in the first round, particularly regarding experimental design and animal sex, have now been clarified, and overall transparency has improved.

That said, I still have some reservations about aspects of the statistical approach, which, while acceptable, do not fully incorporate the most rigorous hierarchical or cohort-level modeling strategies that might strengthen confidence in certain conclusions. In addition, although the authors now clearly state that all murine experiments were conducted in female mice and have acknowledged this as a limitation, the lack of male data in experimental groups continues to constrain the generalizability of the findings.

These remaining issues temper the strength of the conclusions but do not fundamentally undermine the study’s core findings. I am not certain that further rounds of revision would substantially resolve these issues.

Referee #3

(Remarks to the Author)

Gerhardt et al have revised the manuscript in response to prior reviews. Most of our prior comments have been addressed but we do have some remaining questions:

1. Is the metric of moderate-to-vigorous activity comparable between the UK biobank and All of Us cohorts to perform a meta-analysis?
2. The authors write that" "Whether the speed and extent of clonal expansion is strictly inscribed by mutation-directed intracellular signaling or if external cues also play a role remains unclear. " This statement from the text is not completely true – there have been many publications that you have cited in your manuscript that have shown that external cues have a significant role in promoting the expansion of specific CH clones.
3. All of the mosaic mice were treated with PIPC after transplant to induce the mutation; so the model was entirely PIPC exposed. Also all had a high cholesterol diet – these confounders should be discussed as potential limitations of the applicability of the model to other conditions.
4. The reported change in VAF is described as fold change from baseline and would be more clearly labeled as such instead of "CD45.2 fraction growth.
5. For the serum IL-1b time course data (figure 1f-h), are the same mice being serially sampled? If not, this graph is misleading by connecting the lines. It would be better suited to show as a bar graph or box-and-whisker over time.
6. We had previously asked if other cytokines besides just these two were measured and this question was not answered by the authors. Either way, it is an important caveat to note that other cytokines were not broadly surveyed; this should be mentioned as a limitation since people have tended to focus on these two without specific evidence that other cytokines might not be involved.
7. The authors claim that exercise influences atherosclerotic lesion in LdlrKO mice but extended figure 3d shows that exercise has no effect on lesion area or volume.
8. The authors note shifts in the types of Macrophages among the Jak2 mutant cells with SF v exercise. Are there any changes in the phenotype of the WT macrophages with SF v exercise?
The use of the word "reprogramming" suggests a durable change, whereas the changes noted here are directly after acute exposure. The word "redirect" or "promote" would be more appropriate. Or "shift the transcriptomic landscape" as the authors describe.

9. "In the aortic root, however, anti-CLEC4E completely abrogated SF-induced accelerated atherogenesis and necrotic core formation, but did not influence lesion size in sedentary mice (Fig 4k-l), revealing atherogenic regulation in the setting of SF independent of peripheral clone dynamics."
a. How do we know that the CLEC4E pathway is unique to Jak2 mutations? It could also be for relevant for Tet2 where there is an increase in IL-1b signaling. Is this receptor activated by the increased bacterial translocation known to occur with Tet2? Have the authors measured bacterial DNA in blood of SF v non SF mice?

10. The authors advance the hypothesis that exercise induces b-adrenergic signaling that protects against atherosclerosis by inducing an anti-inflammatory phenotype in macrophages. This hypothesis is opposite of a recent publication that shows that b adrenergic signaling amplified macrophage inflammatory responses: https://pubmed.ncbi.nlm.nih.gov/40466637/
Also, b adrenergic signaling promotes atherosclerosis https://pubmed.ncbi.nlm.nih.gov/39082138/
How do the authors address these contradictions?

Minor comments:
1. "In line with these clonal dynamics, SF raised while exercise lowered plasma IL-1β and IL-6 in Ldlr-/-Jak2V617FBM mice (Fig 1g and Extended Data 2c)." in this part of the manuscript, I believe the figure being referenced is Fig 1F not G for Jak2 IL-1b levels.
2. Is the endpoint for extended figure 3a/b different than figure 1e? Were the plasma serum levels taken at different times?
3. "In the blood, the presence of Jak2V617F cells raised hemoglobin, hematocrit, red blood cells, and reticulocytes, but lifestyle did not influence these parameters (Extended Data Fig 1f). Importantly, the influence of sleep and exercise on Jak2V617F CH evolution was not restricted to the setting of atherosclerosis, as SF expanded and exercise diminished Jak2V617F cell expansion in non-atherogenic chow-fed WT (Ldlr+/+) mice as well (Extended Data Fig 2g)." I think you mean Extended data fig 2F as there is no extended figure 1F…
4. "Exercise, meanwhile, significantly repressed clone expansion in Ldlr-/-Tet2-/-BM mice (Fig 1h and Extended Data Fig. 2h)." I think you mean Figure 1g as figure 1h refers to p53.
5. In the plasma of Ldlr-/-Tet2-/-BM mice, SF raised while exercise lowered IL-1β and IL-6 (Fig. 1g and Extended Data Fig 2i). it is misleading to claim that exercise lowered IL-6 as the comparison is between sedentary v exercise and there is no statistical significance (per extended fig2i). Relevant non-significance bars should be noted.
6. "Consistent with these dynamics, BM IL-1β and IL-6 were elevated in Ldlr-/-Jak2V617FBM mice exposed to SF and tended to be lowered by exercise (Fig 2c and Extended Data Fig 4b)." this is misleading as IL-1b and IL6 are not lower with exercise compared to sedentary Jak2. In fact, IL-6 is the same between SF and exercise!!
7. "In p53 CH, sedentary Ldlr-/-p53BM mice had accelerated atherosclerosis, but despite sleep not influencing clone expansion in the blood or BM (Fig 1), SF enhanced lesion area and volume, as well as p53-/-, but not WT, macrophage abundance in the aorta of Ldlr-/-p53BM mice (Fig 3d and Extended Data Fig 6i)." What is Fig 1 referring to? Figure 1h?

Referee #4

(Remarks to the Author)
The authors have adequately addressed my comments. The summary needs to state that the mouse studies The authors have done an excellent job addressing my comments. However, the summary needs to state that the mouse studies were done in females. This is an impressive study.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
No more comment

Referee #2

(Remarks to the Author)
The authors have adequately addressed my comments.

Referee #3

(Remarks to the Author)
Gerhardt et al have revised the manuscript in response to prior reviews. They have addressed most comments. In other cases, they agreed with our criticism but did not change the paper to reflect our suggestions. The items that still need to be revised are listed below:

1. The authors write that" "Whether the speed and extent of clonal expansion is strictly inscribed by mutation-directed intracellular signaling or if external cues also play a role remains unclear. " This statement from the text is not completely true – there have been many publications that you have cited in your manuscript that have shown that external cues have a significant role in promoting the expansion of specific CH clones.

We agree with the reviewer. Indeed, emerging evidence suggests external factors modify clone expansion. However, this idea is not universally accepted as there are studies suggesting cardiovascular risk factors and disease do not alter clone expansion and promote the idea that the behavior of mutant cells is not impacted by external cues (References 22 and 23). Thus, uncertainty remains. Further, the impact of sleep and exercise have not been tested and the underlying mechanisms remain unclear. Our study significantly advances this concept and identifies new therapeutically relevant mechanisms and biology. Moreover, it has remained unclear whether mutant cells and WT cells, existing in the same context, are similarly responsive to external cues. Our findings suggest that mutant cells are selectively influenced by sleep and exercise in unique ways.

Since the authors agree, they should modify their statement to acknowledge that while studies exist to demonstrate the importance of external cues, the impact of sleep and exercise have not been studied.

2. The reported change in VAF is described as fold change from baseline and would be more clearly labeled as such instead of "CD45.2 fraction growth.
We want to ensure that the data represent the growth of the CD45.2 cell fraction and avoid confusion with overall cell count or the CD45.1 fraction. In these graphs, the white data points represent WT CD45.2 cells where are all the other groups (orange, red, blue) are mutant CD45.2 cells. Therefore, we want to make it clear that these graphs show CD45.2 cell fraction growth over times, whether the CD45.2 cells are WT or mutant.
To achieve their stated goals, the authors should then label the Y axis as CD45.2 fold change. "Fraction growth" is arithmetic whereas "fold change" is geometric.

3. For the serum IL-1b time course data (figure 1f-h), are the same mice being serially sampled? If not, this graph is misleading by connecting the lines. It would be better suited to show as a bar graph or box-and-whisker over time.
4. We had previously asked if other cytokines besides just these two were measured and this question was not answered by the authors. Either way, it is an important caveat to note that other cytokines were not broadly surveyed; this should be mentioned as a limitation since people have tended to focus on these two without specific evidence that other cytokines might not be involved.

Thank you for this suggestion, we fully agree. While our data point to specific signaling mechanisms and contribution of IL-1β, other cytokines are likely to be involved and modified by lifestyle. Such investigations will certainly be the subject of future manuscripts. Identifying if sleep or exercise modify the level of other cytokines in CH will be valuable data, demonstrating that such changes causally and mechanistically influence clone expansion or atherosclerosis will also be next steps. In this study, our focus is on cytokines previously implicated in and undergoing active therapeutic targeting trials to mitigate CH and CVD, i.e. IL-1b and IL-6. This choice was intentional, allowing us to assess how lifestyle factors modify established CH-associated inflammatory pathways rather than broadly surveying cytokines without a specific mechanistic framework. It also adds translational value and clinical application to our findings.

Given that they agree, the authors should add this as a limitation to the discussion.

5. The authors claim that exercise influences atherosclerotic lesion in LdlrKO mice but extended figure 3d shows that exercise has no effect on lesion area or volume.

That is correct. In our hands, voluntary exercise has the most profound effect on atherosclerosis in the setting of CH. There can be several reasons for this. This may be due to different forms of exercise (voluntary v.s forced), timing and length of exercise exposure, or the sex of the mice (our studies were performed in females while prior work has been done in males e.g. reference 33). Alternatively, a “two-hit” model could be envisioned where exercise has the most profound effect on atherosclerosis in the setting of an already highly inflammatory and atherogenic context. Our data support this concept.

If there is no effect of exercise on lesion area or volume, then none should be claimed.

6. The authors note shifts in the types of Macrophages among the Jak2 mutant cells with SF v exercise. Are there any changes in the phenotype of the WT macrophages with SF v exercise?
The use of the word “reprogramming” suggests a durable change, whereas the changes noted here are directly after acute exposure. The word “redirect” or “promote” would be more appropriate. Or “shift the transcriptomic landscape” as the authors describe.

The transcriptional changes we observe suggest molecular reprogramming. We do not use this term to infer or suggest permanent effects. Indeed, cellular reprogramming is plastic. At the timepoints we evaluate, our data demonstrate that cells are functionally and transcriptionally restructured and altered as several signaling pathways are either promoted or repressed. Further studies will have to evaluate the durability of these changes, perhaps by assessing the epigenome in experiments where the lifestyle stimulus is ceased.

According to many definitions, cellular reprogramming involves not only transcriptional changes but also metabolic, epigenetic, and translational changes: PMID: 40140235. The authors should not use the word reprogramming when they are simply referring to changes in transcriptional states.

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Mutation-dependent responses to sleep and exercise in clonal hematopoiesis

We thank the Editors and Reviewers for their comprehensive, thoughtful, and positive review. To respond to reviewers' comments, we have performed several new experiments and data analyses in mice and humans. We have responded to the Reviewers' comments point-by-point and provide specific details of text and figure revisions below. Critically, the conclusions of the manuscript have not changed; rather, our new data strengthen our conclusions by deepening and expanding our mechanisms, models, and analyses. In the revised version of the manuscript and in this point-by-point reply, all new data and text are indicated in **red**.

Referee #1

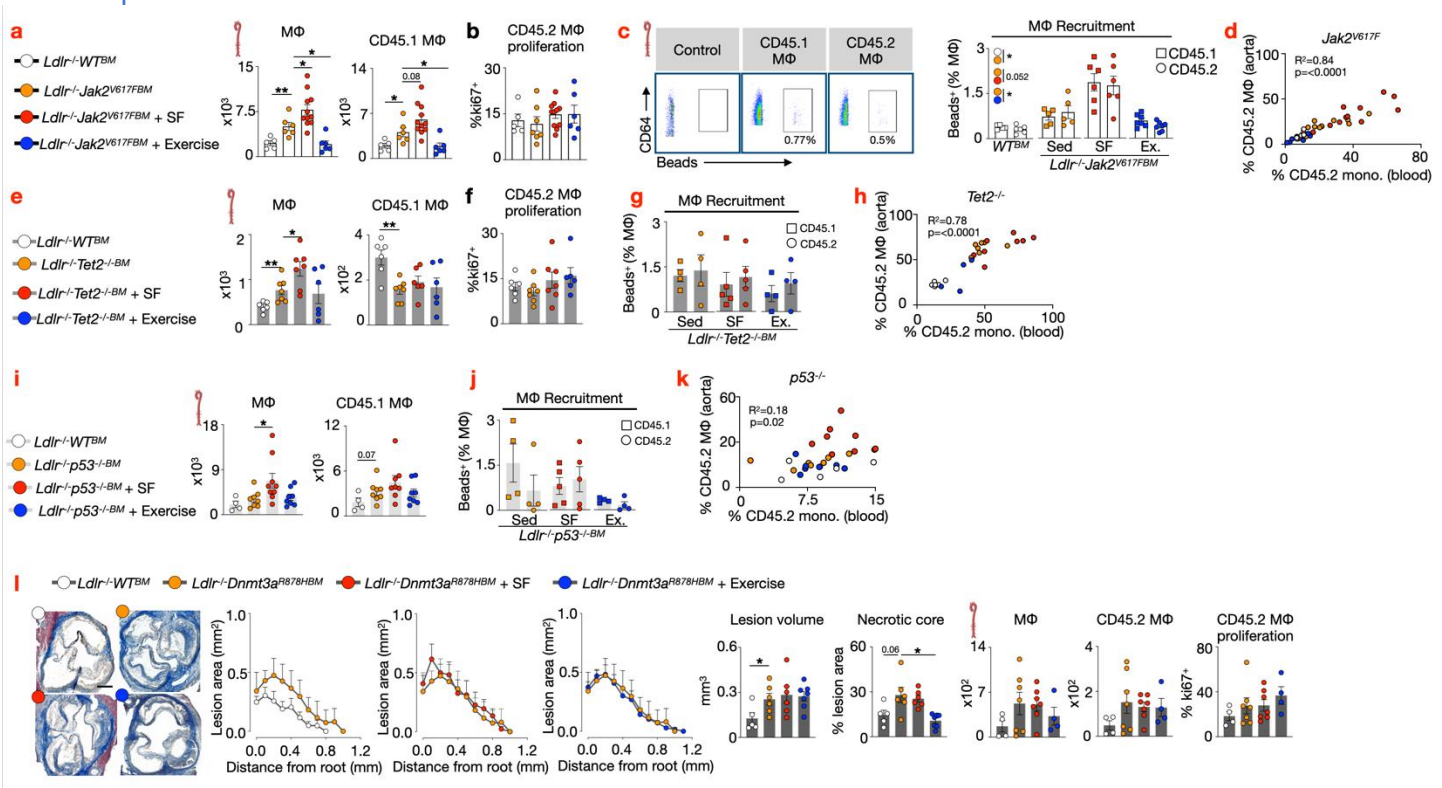
I had the opportunity to review the manuscript submitted by Gerhardt et al. In this study, the authors employed sophisticated murine models to investigate how lifestyle factors—specifically sleep fragmentation and exercise—affect clonal hematopoiesis (CH) in the context of experimental atherosclerosis. They developed a chimeric Ldlr-/- mouse model by combining irradiation and reconstitution with a bone marrow (BM) cell mixture containing 90% wild-type (WT) CD45.1 cells and 10% CD45.2 cells harboring inducible CH-associated mutations: Jak2^V617Ffl/flMx1Cre, Tet2^fl/flMx1Cre, or p53^fl/flMx1Cre. Their findings suggest that sleep fragmentation (SF) promotes CH expansion and accelerates atherosclerosis, whereas exercise mitigates CH and limits disease progression. Mechanistically, SF led to inflammasome activation, and neutralizing IL-1β with a monoclonal antibody abolished both CH expansion and atherosclerosis progression. The beneficial effects of exercise were attributed to catecholamine production, which activates β2-adrenergic receptors on vascular macrophages.

General Assessment:
The topic is timely and relevant. However, the novelty is somewhat limited. The pro-atherogenic effects of SF have already been reported by the same group (MacAlpine, Nature, 2019), and the association between clonal hematopoiesis and atherosclerosis is also well-established (e.g., Oren et al., JCI, 2024).
While the experimental approaches are technically elegant, most of the data remain descriptive. The mechanistic depth of the analysis is insufficient. Although the authors characterized CH expansion in the blood and BM and conducted an in-depth analysis of atherosclerotic lesions using scRNA-seq, key mechanistic questions remain unanswered:
• What is the role of monocyte trafficking?
• Are neutrophils involved in the phenotype?
• Which cell types produce IL-1β?
• Why does IL-1β selectively promote CD45.2 mutant cell expansion?
• Mechanisms driving atheroprotection after exercise are not convincing
Moreover, there are discrepancies between the scRNA-seq data and immunohistological analyses of plaque composition that require clarification.
We thank the reviewer for their helpful comments and suggestions for further improvements to our manuscript. We have greatly expanded mechanistic detail, including novel signaling mechanisms in the BM and aorta, and answered the reviewer's questions and comments with new experiments. We have addressed each comment in detail below.

Detailed Comments:
Macrophage Content and Monocyte Recruitment:
The impact of lifestyle factors on macrophage content in the lesions is superficially addressed, with an emphasis on proliferation. However, most lesional macrophages derive from circulating monocytes. Thus, monocyte trafficking and recruitment should be investigated more thoroughly.
We thank the reviewer for raising this important point. To address this, we have conducted several new experiments and analysis. First, we quantified total, CD45.1, and CD45.2 macrophage burden in aortic plaques in all CH models and lifestyle manipulations. In Jak2, Tet2, and p53 CH, but not Dnmt3a CH, sleep fragmentation (SF) increased, whereas exercise reduced, total and CD45.2 mutant lesional macrophage content (**Fig 3 a, c, d** and **Extended Data Fig 6 a, e, i, l**). Intriguingly, SF and exercise had more modest or

non-significant effects on CD45.1 WT aortic macrophage in all CH models, suggesting specific sensitivities by mutant cells to lifestyle factors (**Extended Data Fig 6 a, e, i**).

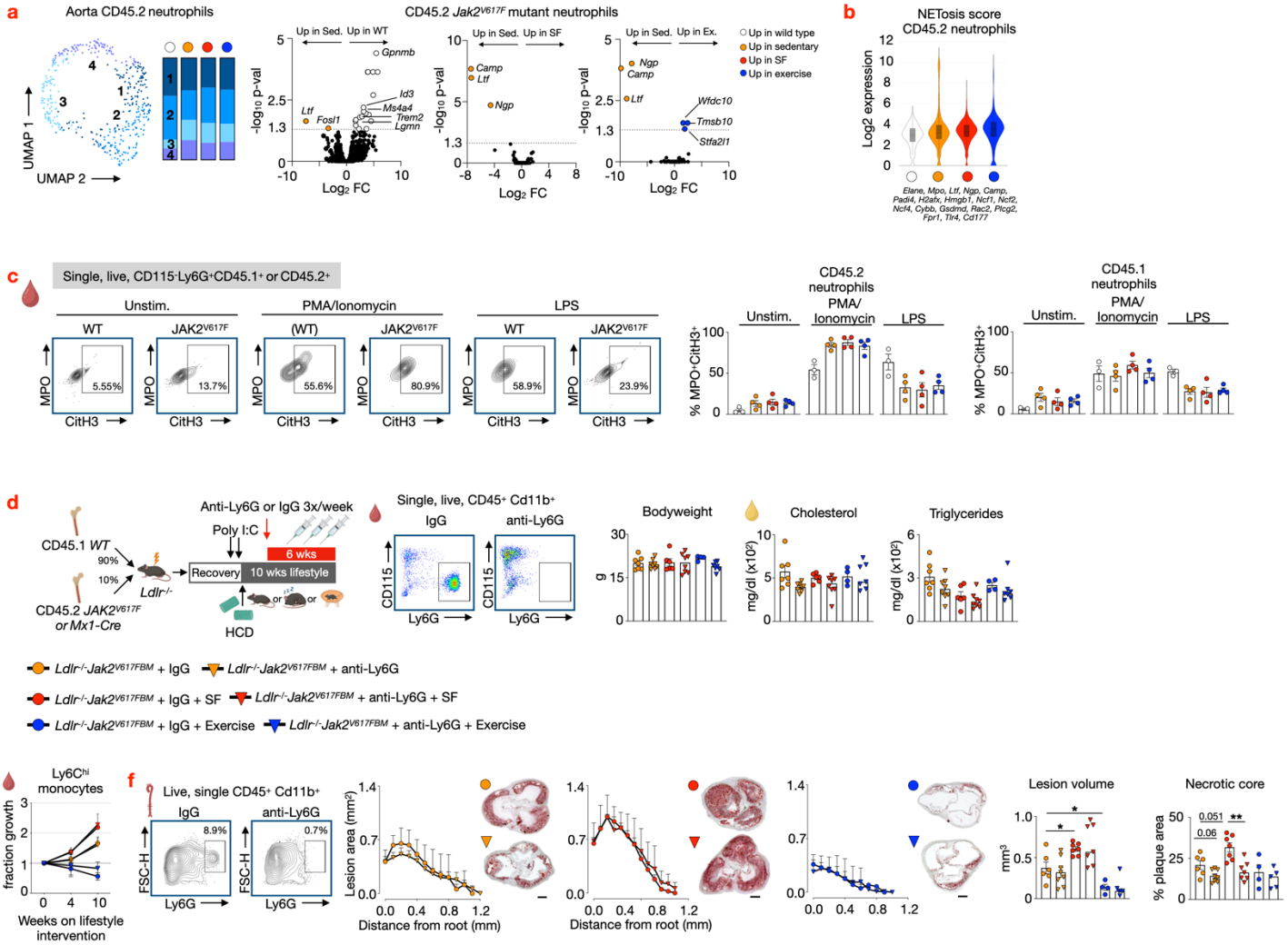
To directly address the contribution of recruitment to changes in aortic macrophage content elicited by lifestyle, we performed recruitment assays. *Ldlr$^{-/-}$Jak2^{V617FBM}*, *Ldlr$^{-/-}$Tet2$^{-/-BM}$*, and *Ldlr$^{-/-}$p53$^{-/-BM}$* mice exposed to sedentary, SF, or exercise were i.v. injected with fluorescent beads after 10 weeks of HCD and lifestyle and the proportion of bead+ CD45.1 and CD45.2 macrophages was quantified in the aorta 24 hours later. We found that in sedentary *Ldlr$^{-/-}$Jak2^{V617FBM}* mice, CD45.1 and CD45.2 cells were recruited to the aorta equivalently but both populations were recruited at higher proportions relative to sedentary *Ldlr$^{-/-}$WT^{BM}* mice (**Extended Data Fig 6c**), consistent with prior data (PMID: 33731931). In *Ldlr$^{-/-}$Jak2^{V617FBM}* mice, SF increased while exercise decreased macrophage recruitment, however it did so equivalently among both WT (CD45.1) and mutant (CD45.2) cells (**Extended Data Fig 6c**), suggesting lifestyle-controlled cell recruitment programs are not genotype-dependent and reflect blood chimerism. In *Ldlr$^{-/-}$Tet2$^{-/-BM}$*, and *Ldlr$^{-/-}$p53$^{-/-BM}$* mice, we also found that WT and mutant cells were recruited to the aorta equivalently, however lifestyle did not impact macrophage recruitment in these CH models (**Extended Data Fig 6g and j**). Alongside our macrophage proliferation data (**Extended Data Figure 6b, f and Fig 3d**), these new recruitment findings demonstrate that in *Ldlr$^{-/-}$Jak2^{V617FBM}* mice, SF promotes plaque growth, at least in part, by promoting the recruitment of both mutant and non-mutant macrophages equivalently into the lesion, whereas exercise restrains plaque growth by modestly limiting mutant and non-mutant macrophage recruitment. If this hypothesis is correct, the proportion of mutant cells in the blood and aorta should therefore be directly and positively correlated. To test this directly, we correlated the number of blood mutant CD45.2 Ly6C^{hi} monocytes with lesional CD45.2 mutant macrophages across all individual mice in all our models. In *Jak2* and *Tet2* chimeras, this analysis revealed a strong, positive (R^2=0.8), linear, and statistically significant (p<0.001) relationship, such that the fraction of mutant monocytes in blood was closely mirrored by the fraction of mutant macrophages in the aorta (**Extended Data Fig 6d and h**). I.e. in sedentary, SF, and exercise, the change in mutant cells in the blood was directly reflected in the aorta. On the other hand, in p53 mutants, the same directional trend was observed, however the correlation was much weaker (R^2=0.18), far less significant (p=0.02 vs. <0.0001), and more variable (**Extended Data Fig 6k**), affirming the role of local macrophage proliferation in this model (see **Fig 3d**). Together these data offer new nuanced and gene-specific insights into the contribution of CH mutations and lifestyle on macrophage/monocyte recruitment to lesions. All of these new data are contained in the revised manuscript.



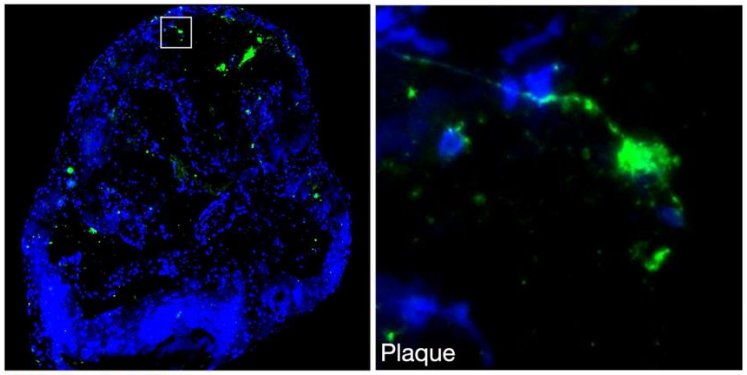
Extended Dat Fig 6. a. Counts for all macrophages and CD45.1 WT macrophages in the aorta of *Ldlr*$^{-/-}$*Jak2*^{V617FBM} and *Ldlr*$^{-/-}$*WT*^{BM} mice measured by flow cytometry at the time of euthanasia (n = 5, 5 wt; 6, 6 sed; 10, 11 SF and 6, 6 exercise). **b.** CD45.2^{+} aortic macrophage proliferation measured by flow cytometry in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 5 wt, 7 sed, 11 SF, 6 exercise). **c.** Representative flow cytometry plots for bead uptake into aortic macrophages in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice with quantification of percent of bead-positive macrophages across all lifestyle groups (n = 5, 6 wt; 5, 5 sed; 6, 6 SF and 7, 7 exercise for CD45.1 and CD45.2, respectively). **d.** Correlation between the fractions of CD45.2 monocytes in the blood and CD45.2 macrophages in the aorta of *Ldlr*$^{-/-}$*Jak2*^{V617FBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 41, 41 for blood and aorta, respectively). **e.** Counts for all macrophages and CD45.1 WT macrophages in the aorta of *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ and *Ldlr*$^{-/-}$*WT*^{BM} mice measured by flow cytometry at the time of euthanasia (n= 6, 6 wt; 7, 7 sed; 6, 7 SF and 6, 6 exercise). **f.** CD45.2^{+} aortic macrophage proliferation measured by flow cytometry in *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n= 6 wt, 7 sed, 7 SF and 6 exercise). **g.** Quantification of bead uptake into aortic macrophages in *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ mice across all lifestyle groups (4, 4 sed; 5, 5 SF and 4, 4 exercise). **h.** Correlation between the fractions of CD45.2 monocytes in the blood and CD45.2 macrophages in the aorta of *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 26, 26 for blood and aorta, respectively). **i.** Counts for all macrophages and CD45.1 WT macrophages in the aorta of *Ldlr*$^{-/-}$*p53*$^{-/-BM}$ and *Ldlr*$^{-/-}$*WT*^{BM} mice measured by flow cytometry at the time of euthanasia (n = 4, 4 wt; 8, 8 sed; 9, 8 SF and 8, 8 exercise). **j.** Quantification of bead uptake into aortic macrophages in *Ldlr*$^{-/-}$ *p53*$^{-/-BM}$ mice across all lifestyle groups (4, 4 sed; 5, 5 SF and 4, 4 exercise for CD45.1 and CD45.2, respectively). **k.** Correlation between the fractions of CD45.2 monocytes in the blood and CD45.2 macrophages in the aorta of *Ldlr*$^{-/-}$*p53*$^{-/-BM}$ and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 29, 29 for blood and aorta, respectively). **l.** Lesion size, volume and necrotic core area (n= 5 wt, 6 sed, 6 SF, 7 exercise) with representative images of aortic roots stained with Masson's Trichrome stain, quantification of total and CD45.2 aortic macrophage number, and CD45.2 macrophage proliferation in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 4 wt, 7 sed, 7 SF, 4 exercise); scale bar=200 μm. Data are mean ± s.e.m; *p<0.05, **p<0.01. P-values were obtained from independent, two-tailed, Welch's t-tests (for parametric data) or Mann–Whitney U tests (for non-parametric data). Details on numbers of animals per group and exact p values are provided in source data file_extended data F6. CD=cluster of differentiation; MΦ=macrophage; mono.=monocyte; SF=sleep fragmentation; WT=wild type.

Taken together, these new findings clarify changes in macrophage abundance in lesions. The results show that (i) local proliferation of mutant macrophages is not modified by lifestyle and (ii) monocyte/macrophage recruitment is modulated by lifestyle but occurs proportionally between WT and mutant cells (i.e., is not mutant-selective). Thus, because mutant BM hematopoietic progenitor cells have heightened sensitivity to lifestyle interventions (see new findings in **Figure 2** and **Extended Data Figure 5**) resulting in lifestyle-dependent changes in the circulating mutant monocyte pool (**Figure 1**), these systemic changes are proportionally reflected in the plaque via recruitment.

Neutrophil involvement:
Neutrophil infiltration is superficially analyzed, and transcriptomic data alone are insufficient to assess recruitment.
We thank the reviewer for this helpful suggestion. We significantly expanded our analysis of neutrophils, including functional assays and depleting the cells *in vivo*. First, we assessed CD45.1 and CD45.2 neutrophils in our aortic scRNAseq data. We found that SF and exercise did not impact the proportion of *Jak2*^{V617F} neutrophil clusters and only modestly changed the expression of a small number of genes (**Extended Data Fig 8a**). To build on this and because *Jak2*^{V617F} neutrophils can modulate atherosclerosis by releasing of neutrophil extracellular traps (NETs) (PMID:39531316), we evaluated a NETosis score and found that lifestyle did not alter this parameter among aortic *Jak2*^{V617F} neutrophils (**Extended Data Fig 8b**). To expand these transcriptomic data with functional and *in vivo* experiments, we isolated whole blood from sedentary, SF, and exercised *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice and activated neutrophils *ex vivo* with PMA/ionomycin or LPS. Intriguingly, the proportion of MPO^{+}CitH3^{+} NETing *Jak2*^{V617F} or WT neutrophils was not different in SF and exercised mice relative to sedentary controls (**Extended Data Fig 8c**). We also looked for MPO+DAPI+ NETing neutrophils by immunofluorescent imaging inside plaques, but the abundance of these cells was rare, preventing their accurate quantification (**Reviewer Figure 1**). Finally, we directly assessed the contribution of neutrophils to lifestyle-elicited atherogenic phenotypes in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice by depleting the cells *in vivo*. *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice were exposed to the lifestyles and received anti-Ly6G or control IgG antibodies for the final 6 weeks which did not impact body weight or circulating lipid levels (**Extended Data Fig 8d**). This strategy successfully depleted neutrophils from the blood and aorta, however it did not alter the SF or exercise induced changed in *Jak2*^{V617F} cell clonal expansion in the blood nor atherosclerotic lesion size (**Extended Data Fig 8d-f**). These observations demonstrate that neutrophils do not substantially contribute to the SF or exercise elicited changes in *Jak2*^{V617F} CH or its associated atherosclerosis.



Extended Data Figure 8. a. UMAP and cluster proportions of reclustered aortic CD45.2 neutrophils across lifestyle groups in *Ldlr*$^{-/-}$ *Jak2*^{V617FBM} mice. Volcano plots of DEGs comparing sedentary and WT, sedentary and SF, and sedentary and exercise CD45.2 neutrophils. **b.** NETosis score in CD45.2 neutrophils across lifestyle groups (*Elane, Mpo, Ltf, Ngp, Camp, Padi4, H2afx, Hmgb1, Ncf1, Ncf2, Ncf4, Cybb, Gsdmd, Rac2, Plcg2, Fpr1, Tlr4, Cd177*). **c.** Representative flow cytometry plots of MPO and CitH3 positivity in CD45.1 WT and CD45.2 *Jak2*^{V617F} neutrophils exposed to either no stimulation, PMA/Ionomycin, or LPS. Quantification of MPO^{+}CitH3^{+} CD45.2 WT or *Jak2*^{V617F} neutrophils exposed to either no stimulation, PMA/Ionomycin, or LPS across the 4 lifestyle groups (n= 3 wt, 4 sed, 4 SF and 4 exercise for all three interventions and both CD45.1 and CD45.2 neutrophils, respectively. **d.** Experimental schematic; exemplary flow cytometry plots showing neutrophil depletion in the blood in the anti-Ly6G group compared to IgG injected controls. Bodyweight, plasma cholesterol, and plasma triglyceride levels in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice injected with IgG or anti-Ly6G across lifestyle groups at the time of euthanasia. **e.** Expansion of blood CD45.2 *Jak2*^{V617F} monocytes in mice injected with IgG or anti-Ly6G across lifestyle groups relative to baseline (n= 3 sedentary + IgG, 8 sedentary + anti-Ly6G, 4 SF + IgG, 8 SF + anti-Ly6G, 4 exercise + IgG and 6 exercise + anti-Ly6G). **f.** Representative flow cytometry plots for aortic neutrophils after injection of IgG control or anti-Ly6G antibody. Lesion area, volume, and proportion of the necrotic core in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to sedentary lifestyle, SF, exercise plus either IgG or anti-Ly6G with representative aortic root images stained with Oil-red-O (n= 6 sedentary + IgG, 8 sedentary + anti-Ly6G, 7 SF + IgG, 7 SF + anti-Ly6G, 4 exercise + IgG and 5-6 exercise + anti-Ly6G). Scale bars are 200 μm. Data are mean ± s.e.m; *p<0.05, **p<0.01.



Reviewer Figure 1. Representative image of MPO+DAPI+ NETing neutrophil in the plaque.

Timing and Source of IL-1β:
IL-1β and IL-6 were measured at the end of the experimental protocol, at which point the myeloid compartment was already significantly altered—potentially confounding results.

Cytokine levels should be measured at earlier time points and during the course of disease progression. The source of IL-1β and the identity of IL-1β–responding cells need to be clarified.
We appreciate the reviewer's point and we agree. We have significantly extended our analysis of IL-1β producing cells in the BM and aorta, performed new IL-1 signaling experiments, and in the revised manuscript provide a novel IL-1β mediated mechanism in the BM through which mutant cells are selectively expanded by lifestyle.

First, as the reviewer suggested, we measured plasma IL-1β in *Jak2*, *Tet2*, and *p53* CH mice and our new *Dnmt3a* CH model throughout the 12 weeks of lifestyle intervention. We find that in *Jak2* and *Tet2* CH, SF increases while exercise decreases plasma IL-1β over time (**Fig 1 f and g**). In *p53* and *Dnmt3a* CH, we find that SF and exercise do not impact plasma IL-1β (**Fig 1h and Extended Data Figure 2q,** in line with unchanged mutant cell dynamics).

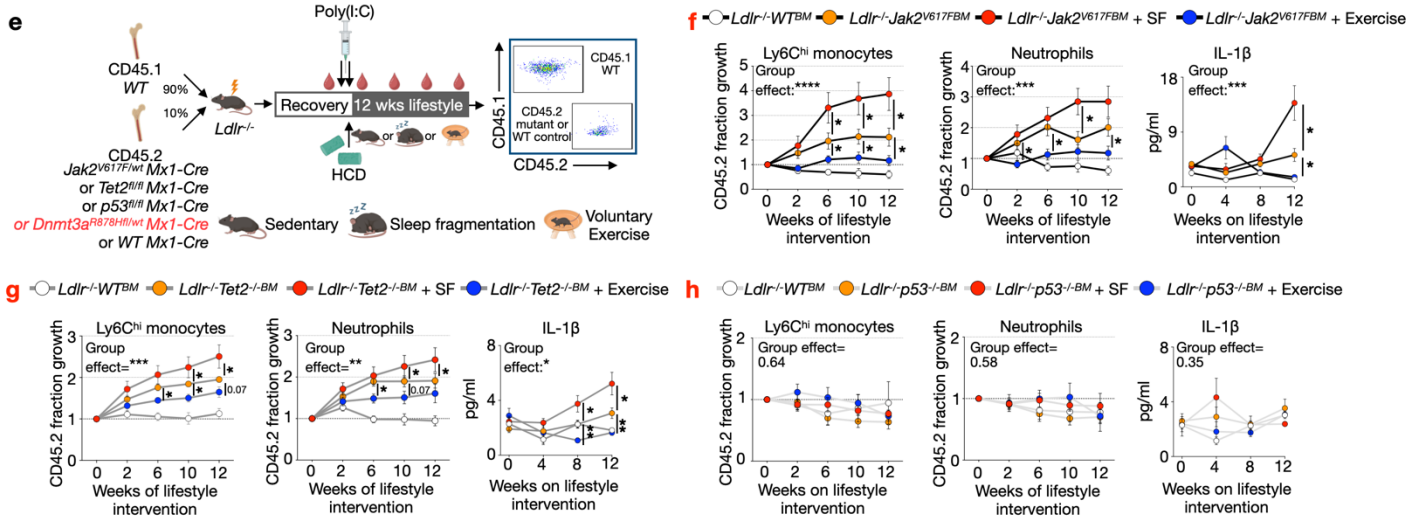


Figure 1. e. Schematic of experimental design. **f.** Expansion relative to baseline of blood CD45.2 *Jak2*^{V617F} or *WT* Ly6C^{hi} monocytes (n=10,18,16,16 for *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM}Sedentary, SF, and Exercise, respectively) and neutrophils (n=11, 18, 16, 16). Time course of plasma IL-1β levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM}Sedentary, SF, and Exercise (n=8,13,9,10; 4,4,4,4; 6,8,8,9 and 7,9,10,6 at baseline, 4,8 and 12 weeks respectively). **g.** Expansion relative to baseline of blood CD45.2 *Tet2*$^{-/-}$ or *WT* Ly6C^{hi} monocytes and neutrophils (both n=7,7,6,6 for *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Tet2*$^{-/-}$ Sedentary, SF, and Exercise, respectively). Time course of plasma IL-1β levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Tet2*$^{-/-}$ Sedentary, SF, and Exercise (n=8,5,6,5; 4,5,5,5; 6,7,5,5, and 6,6,7,6 at baseline, 4,8 and 12 weeks respectively). **h.** Expansion relative to baseline of blood CD45.2 *p53*$^{-/-}$ or *WT* Ly6C^{hi} monocytes and neutrophils (both

n=5,9,10,12 for *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*p53*$^{-/-}$ Sedentary, SF, and Exercise, respectively). Time course of plasma IL-1β levels (n=8,5,5,5; 4,5,5,5; 6,5,5,5 and 4,9,9,7 at baseline, 4,8 and 12 weeks in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*p53*$^{-/-}$ Sedentary, SF, and Exercise, respectively).

We then evaluated the source of IL-β in the BM and the signals that mediate lifestyle-regulated *Jak2*^{V617F} progenitor proliferation and expansion. IL-1β response genes, including *Il1r1* and *IL1r2*, were elevated by SF and repressed by exercise in *Jak2*^{V617F} CMPs and GMPs but not in their WT counterparts (**Fig 2n and Extended Data Fig 5e**). To identify the source of IL-1β in the BM, we measured pro-IL-1β among all BM hematopoietic cells and found that BM macrophages were the highest producers (**Fig 2o and Extended Data Fig 5f**). *Jak2*^{V617F} BM macrophages produced more pro-IL-1β relative to WT cells (**Fig 2p**) and SF increased while exercise decreased pro-IL1β producing *Jak2*^{V617F} BM macrophages (**Fig 2q**). To test if IL-1β preferentially promotes the proliferation of *Jak2*^{V617F} progenitors, we injected IL-1β protein into WT mice that had been irradiated and reconstituted with 10% CD45.2 *Jak2*^{V617F} BM and 90% CD45.1 WT BM. While IL-1β induced WT BM CMP proliferation, the IL-1β-induced proliferative increase among *Jak2*^{V617F} CMPs was greater (**Extended Data Fig 5g**), demonstrating enhanced responsiveness. Finally, to directly test if SF expedites *Jak2*^{V617F} clone expansion via IL-1β, we exposed *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice to either antibody-mediated IL-1β blockade or IgG control throughout sedentary and SF lifestyles (**Fig 2r**). Anti-IL-1β treatment did not influence body weight or plasma lipid levels (**Extended Data Fig 5h**). We quantified clone expansion in the blood and discovered that in sedentary *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice, IL-1β blockade did not alter the expansion of *Jak2*^{V617F} monocytes and neutrophils (**Fig 2r**). In *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to SF, meanwhile, IL-1β blockade strongly diminished SF-induced expedited expansion of *Jak2*^{V617F} clones (**Fig 2r**). In the BM, IL-1β blockade did not alter WT or *Jak2*^{V617F} CMP proliferation in sedentary mice, but it repressed the proliferation of *Jak2*^{V617F}, but not WT, CMPs in SF animals (**Fig 2s and Extended Data Fig 5i**). Altogether, these findings show that uninterrupted sleep and exercise selectively reprogram *Jak2*^{V617F} myeloid progenitor cells towards less proliferative and more metabolically active phenotypes by modulating the abundance of IL-1β-producing *Jak2*^{V617F} BM macrophages and the IL-1β sensitivity of *Jak2*^{V617F} hematopoietic progenitors, a new and previously unknown lifestyle mediated signaling mechanism in CH.

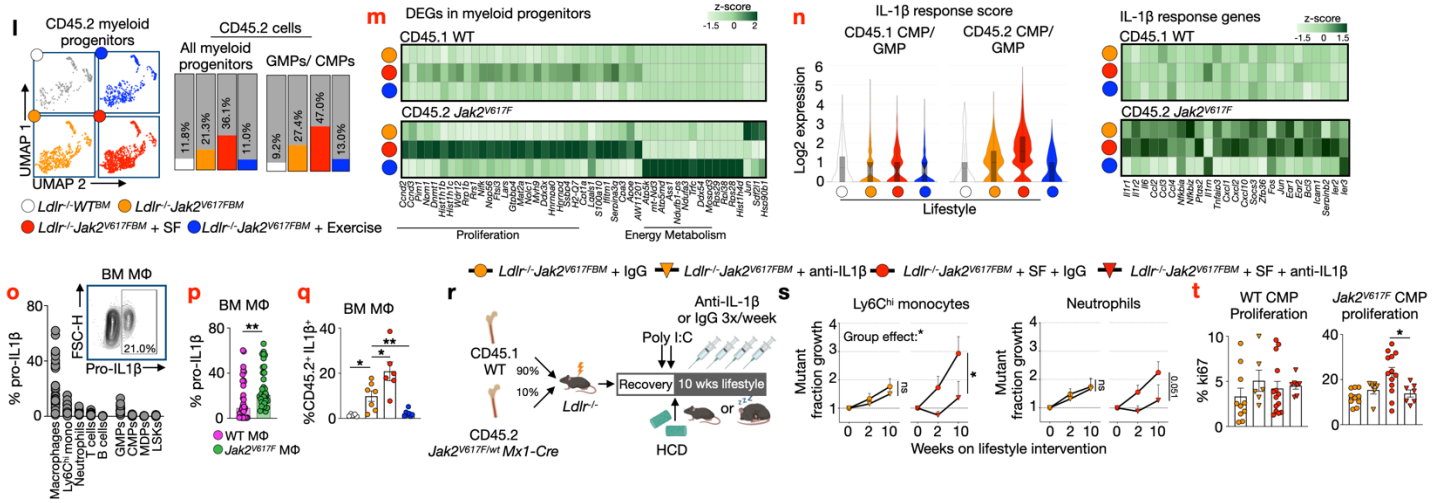
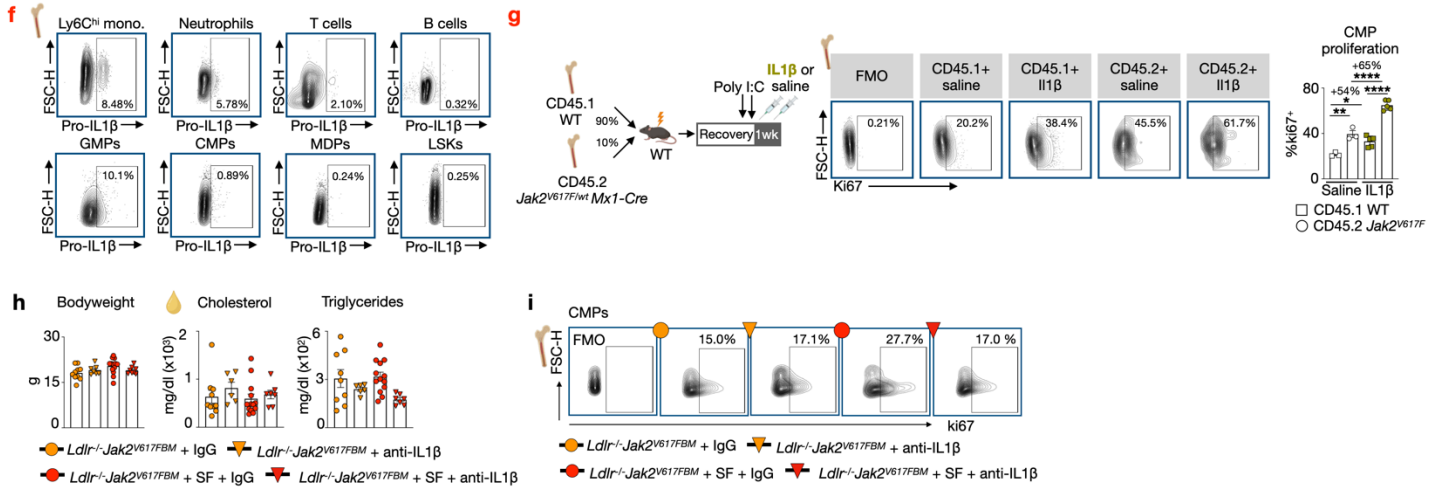
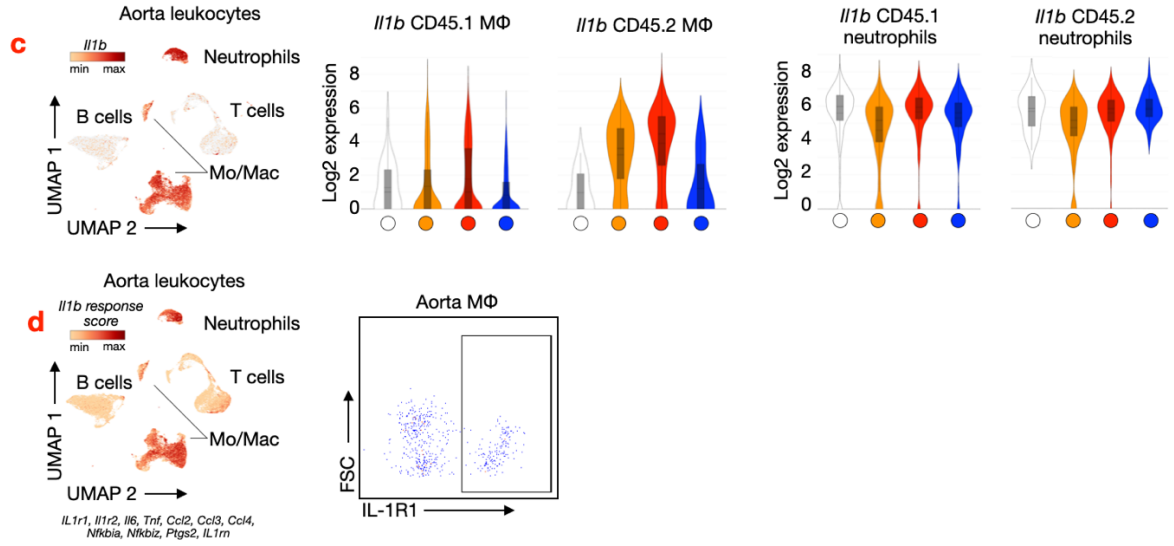


Figure 2. l. UMAP of CD45.2 myeloid progenitors split by experimental group and proportion of CD45.2^{+} cells within the indicated populations. **m**. Heatmap of all genes significantly upregulated in *JAK2*^{V617F} myeloid progenitors for Sed, SF and exercise across CD45.1 WT and *JAK2*^{V617F} myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition. **n**. IL-1β response scores for CD45.1 and CD45.2 CMP/GMPs. Response scores represent composite Log2 expression of *Il1r1, Il1r2, Il6, Tnf, Ccl2, Ccl3, Ccl4, Nfkbia, Nfkbiz, Ptgs2,* and *Il1rn.* Heatmap of *IL1r1*, *IL1r2* and select IL-1β response genes in CD45.1 WT and *JAK2*^{V617F} mutant myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition. **o.** pro-IL-1β expression measured by flow cytometry across different hematopoietic cell types with representative flow plot in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} chimeras (n=36 mature cell types, n=20 progenitors). **p.** pro-IL-1β expression in CD45.1 WT and CD45.2 *Ldlr*$^{-/-}$*Jak2*^{V617FBM} BM-macrophages (n=35). **q.** pro-IL-1β expression in CD45.2 BM-macrophages by lifestyle group (n=5,7,6,7; groups as in (**a**)). **r.** Schematic of experimental design. **s.** Expansion of blood *Jak2*^{V617F} Ly6C^{hi}monocytes (n=19,6,16,10 for Sedentary+IgG, Sedentary+anti-IL-1β, SF+IgG and SF+anti-IL-1β, respectively) and neutrophils (n=19,7,16,9; same groups) relative to baseline. **t.** Proliferation of WT CMPs (n=10,6,14,7; groups as in (**s**)) and *Jak2*^{V617F} CMPs (n=10,6,13,7; groups as in (**s**)). **j-n.** n= 5 pooled mice per group.



Extended Data Fig. 5. f. Representative flow cytometry plots showing pro-IL-1β expression in various cell types from the BM of *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice. **g.** Experimental setup, and representative flow cytometry plots showing ki67 expression in bone marrow CMPs in WT*Jak2*^{V617FBM} mice injected with saline or IL-1β, as well as quantification of %ki67 positivity in CMPs. **h.** Bodyweight, plasma cholesterol, and plasma triglyceride levels in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β at the time of euthanasia. n= 10, 10, 9 no SF + IgG; 7, 6, 6 no SF + anti-IL-1β; 13, 13, 13 SF + IgG and 8, 7, 7 SF + anti-IL-1β for bodyweight, cholesterol and triglycerides, respectively. **i.** Flow cytometry plots for ki67 in CMPs from *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β. Data are mean ± s.e.m; ns= not significant, *p<0.05, **p<0.01,****p<0.0001.

We then turned our attention to the aorta and IL-1β producing cells at that location. In Jak2 CH mice, macrophages were a dominant hematopoietic source of IL-1β in the aorta and expressed high levels of the cytokine (**Extended Data Fig 7c**). Moreover, SF increased while exercise reduced *Il1b* expression in *Jak2*^{V617F}, but not in WT, aortic macrophages but did not alter *Il1b* in WT or *Jak2*^{V617F} neutrophils (**Extended Data Fig 7c**). This finding demonstrates that lifestyle only modifies IL-1b production by mutant macrophages and not WT cells, demonstrating the selective sensitivity of the mutant cells to lifestyle modification. Aortic macrophages respond to IL-1β as they express IL1-R1 and genes associated with an IL-1β response (**Extended Data Fig 7d**). Together, these findings are in line with our inflammatory profiling of aortic macrophages and position the cells as the predominant hematopoietic sources and responding cells in the aorta.



Extended Data Fig 7. c. UMAP of *Il1b* expression across all aortic leukocyte populations with Log2 expression of *Il1b* in CD45.1 and CD45.2 macrophages and neutrophils. **d.** UMAP of *Il1b* response score (*IL1r1, Il1r2, Il6, Tnf, Ccl2, Ccl3, Ccl4, Nfkbia, Nfkbiz, Ptgs2, IL1rn)* across all aortic leukocyte populations. Representative flow cytometry plot showing IL-1R1 expression in aortic macrophages.

Selective Expansion of CD45.2 Cells:

Although IL-1β is known to promote myelopoiesis, the authors show that it selectively expands CD45.2 mutant cells but not CD45.1 WT cells. This raises important questions: Is this due to differential expression of the IL-1 receptor or differences in downstream signaling? Additional experiments are essential to address this point. This is a critical point and we have performed several new experiments to assess IL-1β sensitivity and signaling in mutant and WT hematopoietic progenitors. We have discovered that the expression of IL-1β response genes, including *Il1r1* and *IL1r2*, are elevated in *Jak2*^{V617F} CMPs and GMPs relative to WT cells (**Fig 2n and Extended Data Fig 5e**). Further, the expression of *Il1r1* and *IL1r2* and IL-1β response genes is enhanced by SF and repressed by exercise in *Jak2*^{V617F} CMPs and GMPs but not in WT cells (**Fig 2n and Extended Data Fig 5e**). These findings demonstrate that lifestyle selectively reprograms the sensitivity and IL-1β response capacity of *Jak2*^{V617F} progenitor cells. To build on this and directly functionally test if SF expedites *Jak2*^{V617F} clone expansion via IL-1β, we exposed *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice to either antibody-mediated IL-1β blockade or IgG control throughout sedentary and SF lifestyles (**Fig 2r**). We quantified clone expansion in the blood and discovered that in sedentary *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice, IL-1β blockade did not alter the expansion of *Jak2*^{V617F} monocytes and neutrophils (**Fig 2s**). In *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to SF, meanwhile, IL-1β blockade strongly diminished SF-induced expedited expansion of *Jak2*^{V617F} clones (**Fig 2s**). In the BM, IL-1β blockade did not alter WT or *Jak2*^{V617F} CMP proliferation in sedentary mice, but it specifically repressed the proliferation of *Jak2*^{V617F}, but not WT, CMPs in SF animals (**Fig 2t and Extended Data Fig 5i**). These data support the notion that anti-IL-1β therapy has a selective effect on mutant cells, likely because of their increased IL-1β sensitivity. Finally, to further address this point with additional experiments, we injected IL-1β protein into WT mice that had been irradiated and reconstituted with 10% CD45.2 *Jak2*^{V617F} BM and 90% CD45.1 WT BM. As expected, IL-1β induced WT BM CMP proliferation, however the proliferative increase among *Jak2*^{V617F} CMPs was greater (**Extended Data Fig 5g**) again demonstrating the selectively heightened sensitivity of mutant cells to IL-1β.

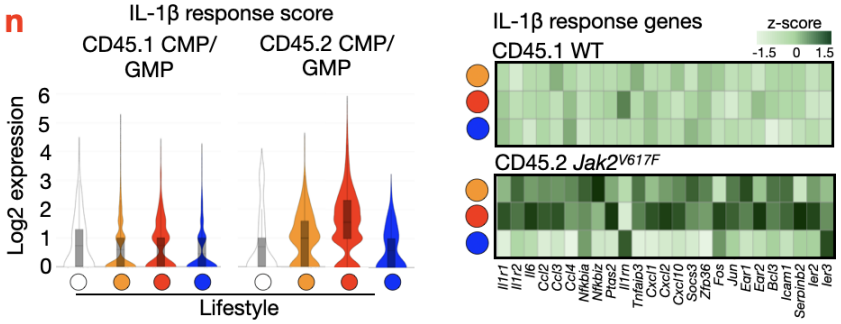


Fig 2. n. IL-1β response scores for CD45.1 and CD45.2 CMP/GMPs. Response scores represent composite Log2 expression of *Il1r1, Il1r2, Il6, Tnf, Ccl2, Ccl3, Ccl4, Nfkbia, Nfkbiz, Ptgs2,* and *Il1rn.* Heatmap of *IL1r1*, *IL1r2* and select IL-1β response genes in CD45.1 WT and *JAK2*^{V617F} mutant myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition.

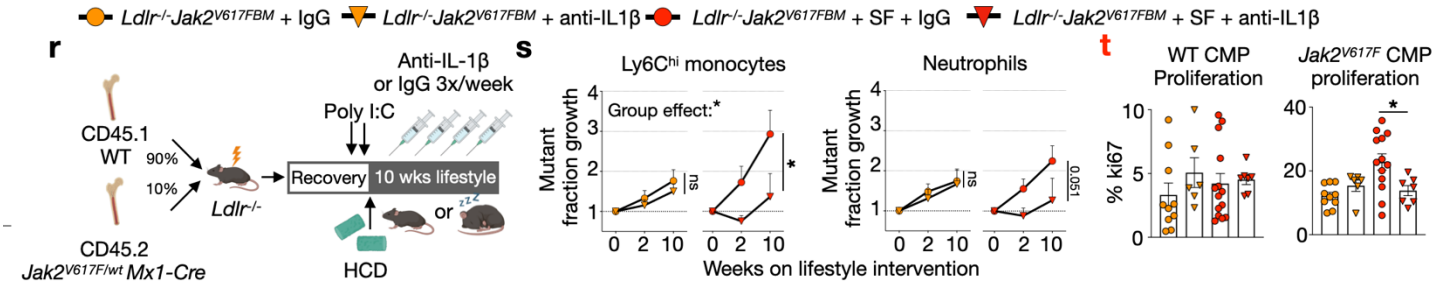
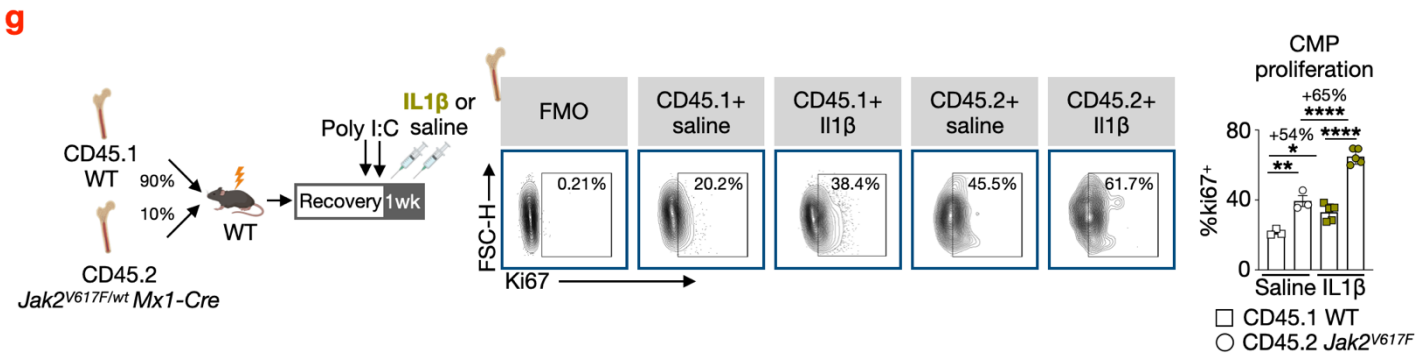


Fig 2 r-t. r. Schematic of experimental design. **s.** Expansion of blood *Jak2*^{V617F} Ly6C^{hi}monocytes (n=19,6,16,10 for Sedentary+IgG, Sedentary+anti-IL-1β, SF+IgG and SF+anti-IL-1β, respectively) and neutrophils (n=19,7,16,9; same groups) relative to baseline. **t.** Proliferation of WT CMPs (n=10,6,14,7; groups as in (**s**)) and *Jak2*^{V617F} CMPs (n=10,6,13,7; groups as in (**s**)). **j-n.** n= 5 pooled mice per group.



Extended Data Fig 5. g. Experimental setup, and representative flow cytometry plots showing ki67 expression in bone marrow CMPs in WT*Jak2*^{V617FBM} mice injected with saline or IL-1β, as well as quantification of %ki67 positivity in CMPs.

Figure 2:
The reported differences in IL-1β and IL-6 in the BM are modest, and their biological significance is unclear.
IL-1β is a powerful, prototypical inflammatory cytokine and marker of adverse outcomes that is clinically and therapeutically relevant (e.g. CANTOS trial and ongoing trails on IL-6, a downstream product of IL-1β signaling). Even small changes in IL-1β are biologically meaningful and have important and significant implications. For example, we find that IL-1β protein levels are increased in the BM, lifestyle modulates BM macrophage-derived IL-1β (see new mechanistic data in **Fig 2m-t**), and, crucially, that mutant progenitors are selectively sensitized to IL-1β signaling: IL-1β response scores were increased in CD45.2 CMP/GMP with SF and reduced with exercise (**Fig 2n**). Together, these data support a comprehensive picture of both ligand and receptor/sensitivity signaling modulation by CH and lifestyle, which together amplify IL-1β responses. Most importantly, however, and to show biological significance, we demonstrate that blocking IL-1β with an anti- IL-1β antibody reduced proliferation of mutant CD45.2, but not CD45.1 WT, cells in the BM of Jak2 CH mice (**Fig 2t**). Indeed, IL-1β blockade completely abrogated the expedited clonal expansion of mutant cells in SF mice (**Fig 2s**). These new findings indicate that even modest elevations in IL-1β, and concomitant rises in receptor expression, can drive a clone-restricted proliferative program in mutant progenitors which has important biological significance. Because it is not the central target of this new mechanism, data on IL-6 has been moved to the supplement.

Figure 3:
The authors propose that sleep and exercise modulate CH-driven atherosclerosis via reprogramming of mutant macrophages. However, this claim is not directly supported by functional data. Additional histological analyses (e.g., macrophage and neutrophil staining) and monocyte/neutrophil depletion experiments would strengthen the conclusions.
We agree with the reviewer that functional experiments are important to substantiate our mechanistic model. We conducted the additional analyses suggested by the reviewer. An important strength of our manuscript is the disentanglement and disassociation of BM clonal dynamics peripherally from local macrophage programming/function in the lesions and how lifestyle can modulate each of these aspects of CH independently. Using new experiments and staining, we demonstrate this for both SF and exercise which each rely on different mechanisms. In new experiments, we injected sedentary and SF *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with anti-Clec4e antibodies throughout atherogenesis to block this macrophage PRR that is upregulated by SF only in *Jak2*^{V617F} aortic macrophages (**Fig 4i and Fig 4c-d**). Critically, anti-Clec4e did not alter the expansion of *Jak2*^{V617F} cells in the BM or blood of SF or sedentary mice (**Fig 4j**). In the aorta, however, anti-Clec4e completely abrogated the SF-induced accelerated atherosclerosis in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice but did not change lesion size in sedentary mice (**Fig 4k**). Along with this, anti-Clec4e repressed IL-1β only in *Jak2*^{V617F} aortic macrophages of SF mice and decreased NLRP3 and AIM2 in CD45.2 *Jak2*^{V617F} aortic macrophages only in SF mice as well (**Fig 4m-p**). These findings show that SF-induced Clec4e macrophage signaling locally reprograms mutant macrophages in the aorta without impacting peripheral clonal dynamics or trajectories.

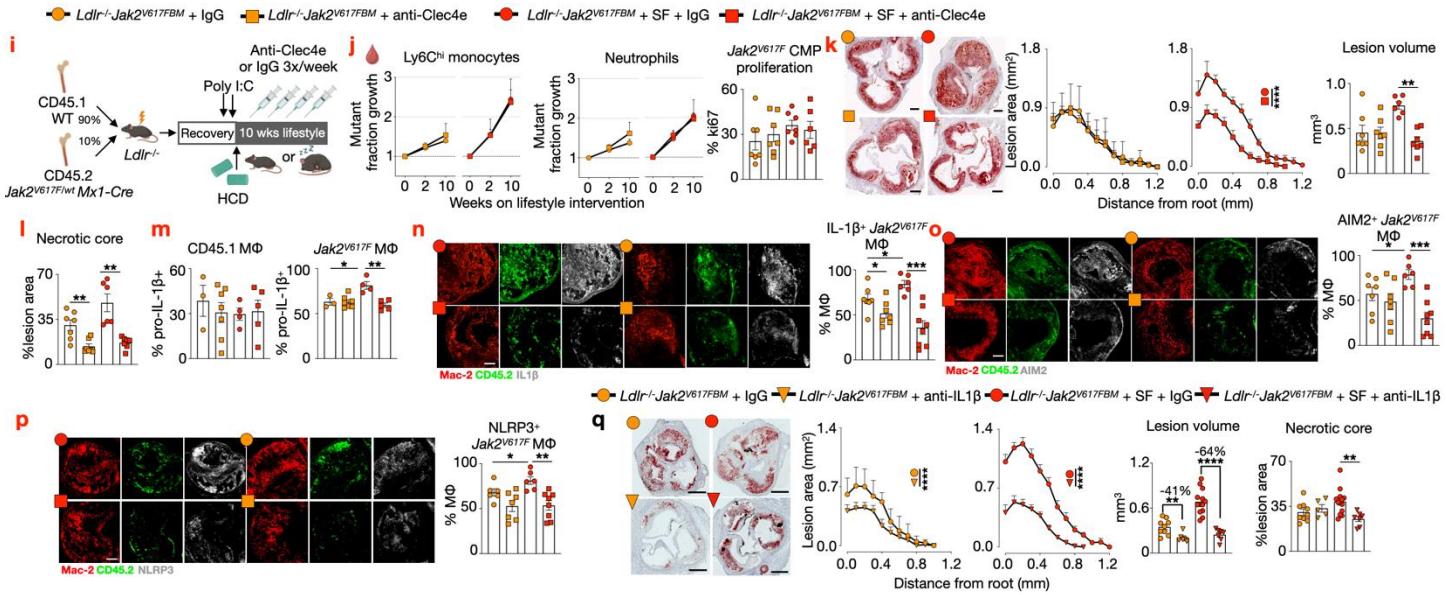


Figure 4. i. Experimental outline. **j**. Expansion of *Jak2*^{V617F} blood Ly6C^{hi} monocytes (n=6,7,5,8 for Sedentary+IgG, Sedentary+anti-Clec4e, SF+IgG, SF+anti-Clec4e) and neutrophils (n= 6,7,5,7) relative to baseline. Proliferation of *Jak2*^{V617F} CMPs (n=7,7,7,6; same groups) **k**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size and volume (both n=7,7,6,7; groups as in (**j**)). Scale bars are 200µm. **l.** Proportion of the necrotic core relative to plaque area (n= 7,7,6,7; groups as in (**j**)). **m.** pro-IL1β expression in CD45.1 WT and *Jak2*^{V617F} macrophages (both n=3,7,4,5; groups as in (**j**)) by flow cytometry. **n.** MAC-2, CD45.2, and IL-1β immunofluorescence and quantification of CD45.2, and IL-1β pixel overlap (n=7, 7,6,7, groups as in (**j**)). Scale bar=100 µm **o.** MAC-2, CD45.2, and AIM2 immunofluorescence and quantification of CD45.2 and AIM2 pixel overlap (n=7,7,6,7; groups as in (**j**)). Scale bar=100 µm. **p.** MAC-2, CD45.2, and NLRP3 immunofluorescence and quantification of CD45.2 and NLRP3 pixel overlap (n=6,7,6,7; groups as in (**j**)). Scale bar=100 µm**. q**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size (n=10, 6, 13, 7) and volume (n=9, 6, 13, 7) in sequential sections and proportion of the necrotic core (n=9, 5, 13, 7) relative to plaque area; groups as in (**j**).

Similarly, in the setting of exercise, ADRβ2 antagonism did not impact peripheral clonal dynamics in sedentary or exercised *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice (**Fig 5k-m**). However, it strongly increased lesion volume (**Fig 5n**) and elevated *Jak2*^{V617F} aortic macrophage IL-1β (**Fig 5o**) only in exercised mice. All together, these data allow us to disassociate clonal trajectories in the periphery from local aorta macrophage functional programming using IF for CD45.2 and IL-1β, NLRP3, and AIM2. Altogether, using new causal interventional experiments, we demonstrate how SF (via macrophage CLEC4E) and exercise (via macrophage ADRβ2) modulate mutant aortic macrophage inflammatory functions to consequently impact CH-driven atherosclerosis without modifying clonal expansion in the BM or blood.

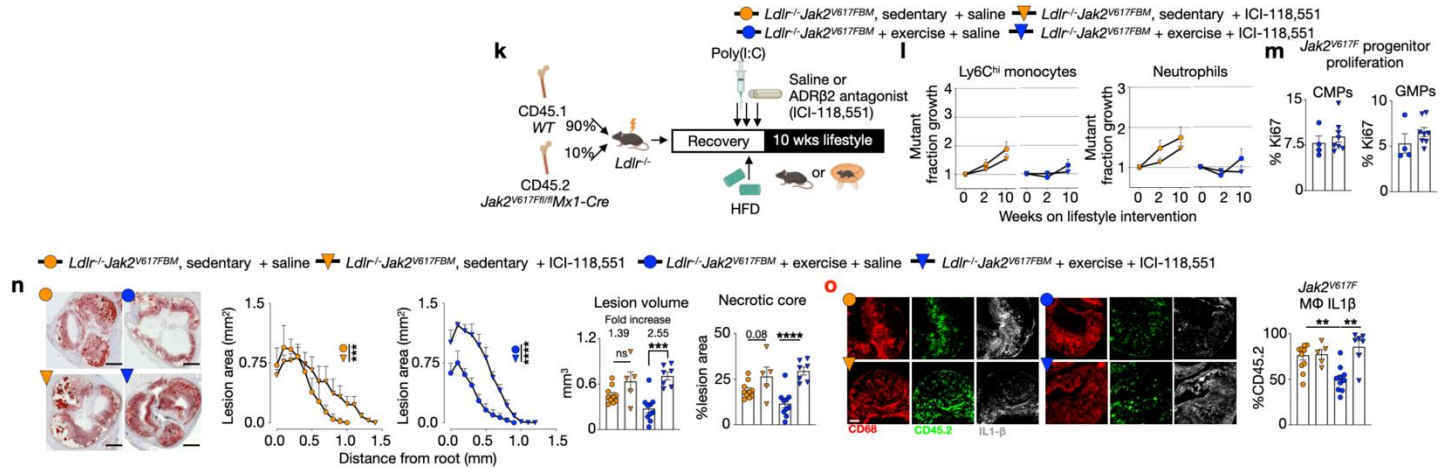
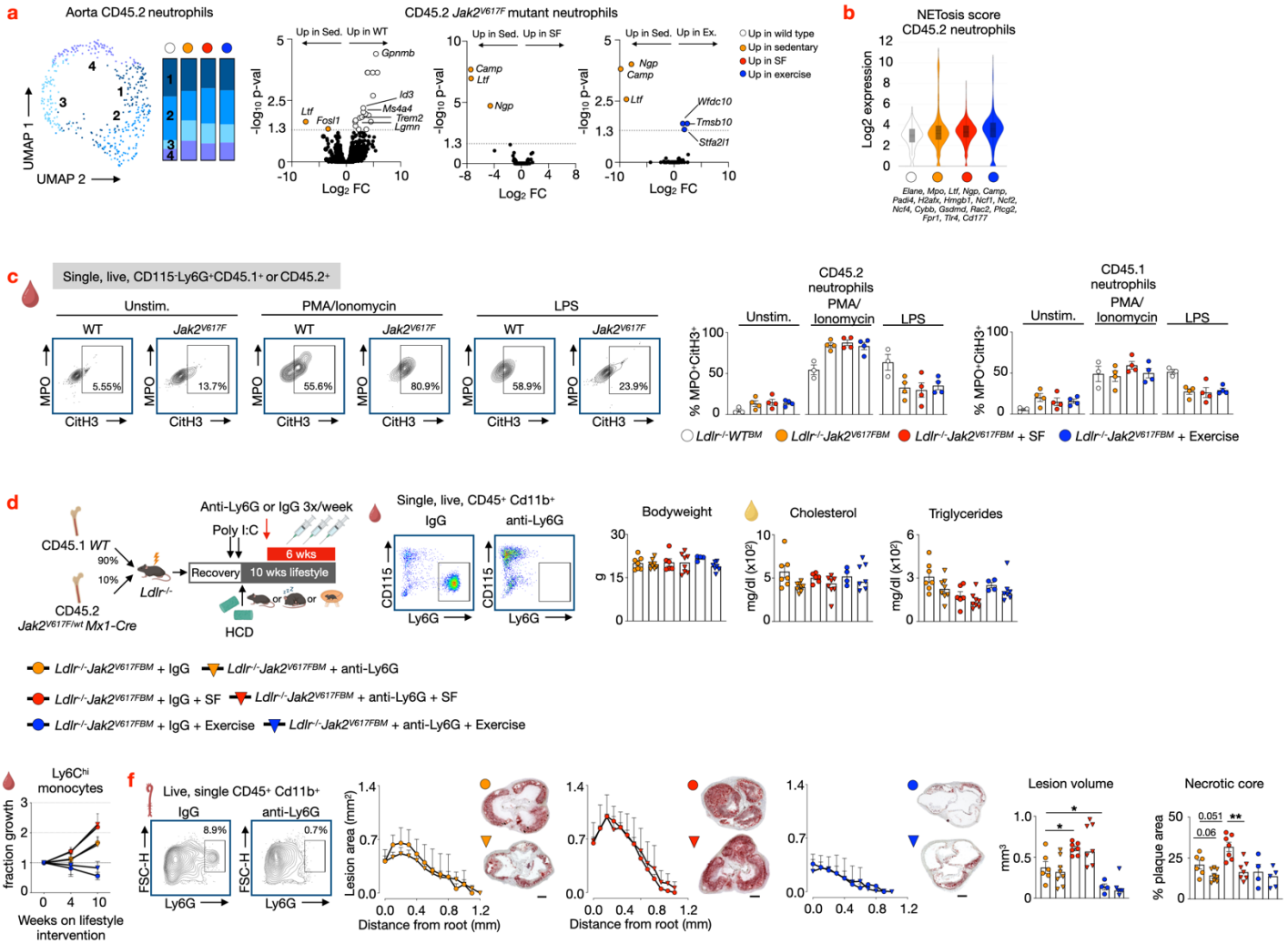


Figure 5. **k**. Experimental outline. **l**. Expansion of *Jak2*^{V617F} blood Ly6C$_{hi}$ monocytes (n=21, 5, 18, 8) and neutrophils (n=19, 5, 14, 10) relative to baseline. Groups are Sedentary, Sedentary+ICI-118,551, Exercise, Exercise+ICI-118,551) **m**. Proliferation of *Jak2*^{V617F} bone marrow CMPs and GMPs measured by Ki67 expression in flow cytometry. n=5, 8 for Exercise+Saline and Exercise+ICI-118,551,

respectively. **n**. Representative aortic root cross-sections stained with Oil-Red O and quantification of lesion size and volume (both n=10,5,10,7) in sequential sections. Proportion of the necrotic core relative to plaque area (n=7,5,4,7). Groups as in (**l**). **o**. IL-1β and CD45.2 immunofluorescence and quantification of pixel overlap in the aortic root (n=10,5,10,7). Groups as in (**l**). Scale bar=100µm.

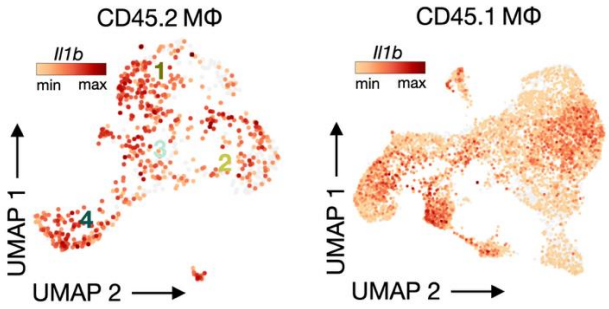
Finally, we performed the experiment suggested by the reviewer and depleted neutrophils. We found that lifestyle did not alter the NETing potential of mutant neutrophils (**Extended Data Fig 8a-c**) and that neutrophil depletion with anti-Ly6G did not alter atherosclerosis in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to exercise or SF (**Extended Data Fig 8d-f**). These new data reaffirm the important contribution of aortic mutant macrophages to CH atherogenesis modulation by lifestyle.



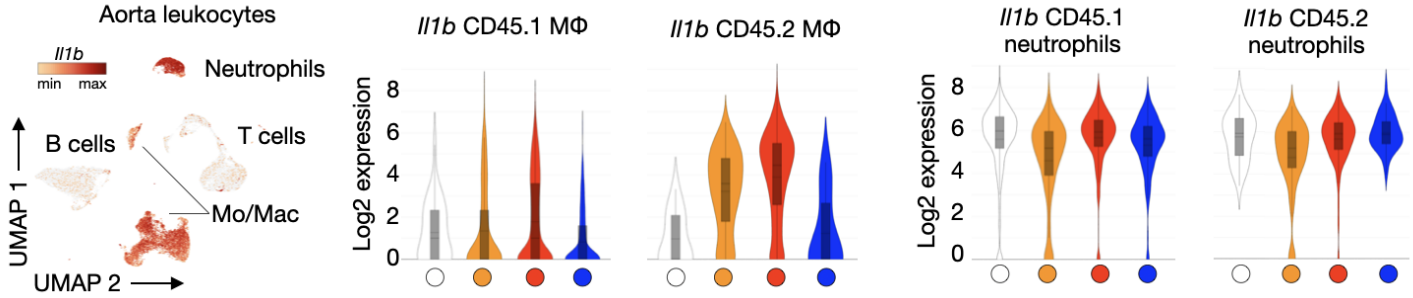
Extended Data Fig 8. a. UMAP and cluster proportions of reclustered aortic CD45.2 neutrophils across lifestyle groups in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice. Volcano plots of DEGs comparing sedentary and WT, sedentary and SF, and sedentary and exercise CD45.2 neutrophils. **b.** NETosis score in CD45.2 neutrophils across lifestyle groups (*Elane, Mpo, Ltf, Ngp, Camp, Padi4, H2afx, Hmgb1, Ncf1, Ncf2, Ncf4, Cybb, Gsdmd, Rac2, Plcg2, Fpr1, Tlr4, Cd177*). **c.** Representative flow cytometry plots of MPO and CitH3 positivity in CD45.1 WT and CD45.2 *Jak2*^{V617F} neutrophils exposed to either no stimulation, PMA/Ionomycin, or LPS. Quantification of MPO^{+}CitH3^{+} CD45.2 WT or *Jak2*^{V617F} neutrophils exposed to either no stimulation, PMA/Ionomycin, or LPS across the 4 lifestyle groups (n= 3 wt, 4 sed, 4 SF and 4 exercise for all three interventions and both CD45.1 and CD45.2 neutrophils, respectively. **d.** Experimental schematic; exemplary flow cytometry plots showing neutrophil depletion in the blood in the anti-Ly6G group compared to IgG injected controls. Bodyweight, plasma cholesterol, and plasma triglyceride levels in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice injected with IgG or anti-Ly6G across lifestyle groups at the time of euthanasia. **e.** Expansion of blood CD45.2 *Jak2*^{V617F} monocytes in mice injected with IgG or anti-Ly6G across lifestyle groups relative to baseline (n= 3 sedentary + IgG, 8 sedentary + anti-Ly6G, 4 SF + IgG, 8 SF + anti-Ly6G, 4 exercise + IgG and 6 exercise + anti-Ly6G). **f.** Representative flow cytometry plots for aortic neutrophils after injection of IgG control or anti-Ly6G antibody. Lesion area, volume, and proportion of the necrotic core in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to sedentary lifestyle, SF, exercise plus either IgG or anti-Ly6G with representative aortic root images stained with Oil-red-O (n= 6 sedentary + IgG, 8 sedentary + anti-Ly6G, 7 SF + IgG, 7 SF + anti-Ly6G, 4 exercise + IgG and 5-6 exercise + anti-Ly6G). Scale bars are 200 µm. Data are mean ± s.e.m; *p<0.05, **p<0.01.

Figure 4B:
Images are too small and difficult to interpret. In the SF group, a high degree of co-localization between macrophages and inflammasome markers (NLRP3, IL-1β) was reported, with IL-1β+ macrophages representing 80–85% of the total. This is inconsistent with scRNA-seq data, which indicate that inflammasome-positive macrophages form a minor subset.

Thank you for pointing this out, we have made these adjustments. In the scRNA-seq dataset, *Il1b* transcripts are most enriched in Cluster 1 but are detectable at varying levels in all aortic macrophages (**Reviewer Figure**). In the revised manuscript, we include new UMAPs and quantification showing *Il1b* gene expression across CD45.1 and CD45.2 aortic macrophages and neutrophils (**Extended Data Fig 7c**). This indicates that *Il1b* is not confined to a rare subset but broadly expressed across the macrophage compartment, with highest expression in cluster 1.

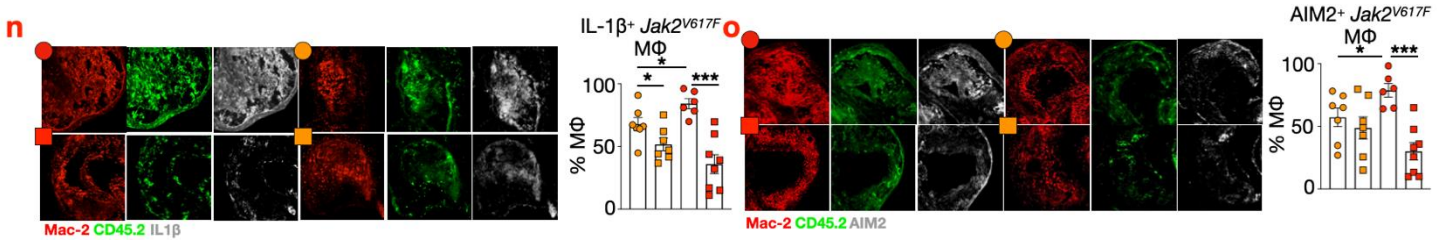


Reviewer Figure. Il1b expression in aortic CD45.1 and CD45.1 macrophages of *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice.



Extended Data Figure 7c. UMAP of *Il1b* expression across all aortic leukocyte populations with Log2 expression of *Il1b* in CD45.1 and CD45.2 macrophages and neutrophils.

Moreover, we have updated our aorta histological analysis to now include the mutant cell marker CD45.2 allowing us to specifically quantify IL-1β in CD45.2 (mutant) aortic macrophages by IF. We assessed IL-β, NLRP3, and AIM2 specifically in CD45.2 cells by IF and conducted the appropriate quantification (**Fig 4n-p and Fig 5o**). This new analysis, in contrast to scRNAseq data, identifies IL-1β protein–positive macrophages and quantification of staining intensity once it surpasses a threshold, thereby capturing the fraction of cells with higher protein abundance. The observed range of ~50-70% IL-1β+ CD45.2 macrophages reflects an increasing share of mutant macrophages with strong IL-1β protein expression.



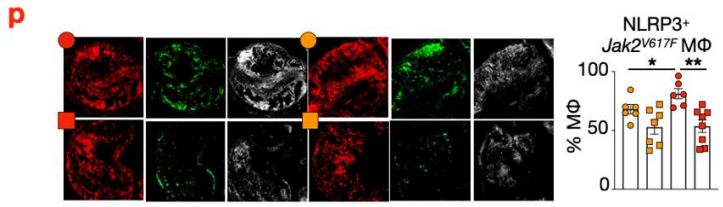


Fig 4. n. MAC-2, CD45.2, and IL-1β immunofluorescence and quantification of CD45.2, and IL-1β pixel overlap (n=7, 7,6,7, groups as in (**j**)). Scale bar=100 μm **o.** MAC-2, CD45.2, and AIM2 immunofluorescence and quantification of CD45.2 and AIM2 pixel overlap (n=7,7,6,7; groups as in (**j**)). Scale bar=100 μm. **p.** MAC-2, CD45.2, and NLRP3 immunofluorescence and quantification of CD45.2 and NLRP3 pixel overlap (n=6,7,6,7; groups as in (**j**)). Scale bar=100 μm**.**

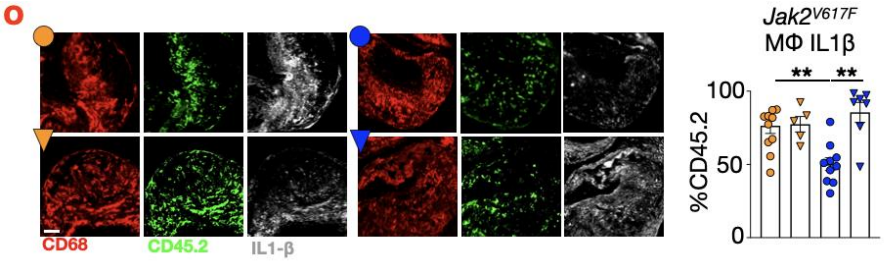


Fig 5o. IL-1β and CD45.2 immunofluorescence and quantification of pixel overlap in the aortic root (n=10,5,10,7). Groups as in (**l**). Scale bar=100μm.

Taken together, these findings resolve the apparent discrepancy, refine and specify our analysis to CD45.2 mutant cells. In sum, scRNA-seq highlights that nearly all macrophages express *Il1b* to some degree, while histology emphasizes the proportion with high-level protein expression in mutant cells, which rises with SF and decreases with exercise.

Figure 4E:
CD64 staining is unconvincing and appears mislocalized. Its co-localization with Clec4a is also questionable.
We thank the reviewer for this helpful suggestion, we agree. To strengthen the macrophage identification and clarify co-localization, we repeated the staining using an alternative macrophage marker (CD68), which improved specificity and localization within lesions. As shown in the new **Fig. 4e**, Clec4e signal clearly overlaps with CD68+ macrophages and is markedly enhanced in lesional macrophages from the SF condition compared to sedentary controls. These new data replace the prior CD64 images and reinforce the conclusion that Clec4e is expressed by lesion macrophages.

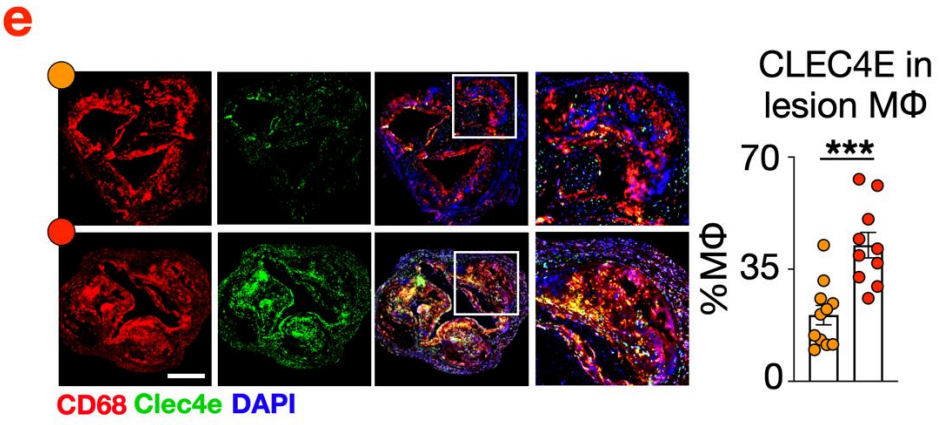


Fig 4e. CLEC4E and CD68 immunofluorescence in the aortic root and quantification of colocalization, n=11, 10 for Sedentary and SF. Scale bar=400 μm

Figure 4l:
Clec4e+ macrophages form a small subset in scRNA-seq data but appear overrepresented in histological images. This inconsistency requires explanation. Additionally, the images shown are not representative of plaque size and should be adjusted accordingly.

We agree with the reviewer that histological images should reflect both the abundance and spatial distribution of Clec4e+ macrophages. We therefore repeated the IF staining with CD68 and re-quantified Clec4e positivity across multiple plaques. The updated data (**Fig 4e**) demonstrate improved staining quality and clearer co-localization, with quantification values that are more consistent with the revised approach. While Clec4e^{+} macrophages form a relatively small subset in scRNA-seq data, they are nonetheless readily detected in lesions by immunofluorescence, particularly in the SF condition where Clec4e expression is consistently enriched. This apparent difference between scRNAseq and IF imaging likely reflects technical and biological factors: scRNA-seq provides a transcriptional snapshot with inherent dropout and thresholding limitations, whereas immunofluorescence detects protein that may persist longer or accumulate in cells, and can reveal biologically meaningful low-level expression not captured at the RNA level. In addition, UMAP clustering does not convey spatial distribution, whereas Clec4e^{+} macrophages may form focal accumulations within plaques, making them visually more prominent in situ. To address the reviewer's concern about representation, we have now included new images that reflect typical lesion size and morphology.

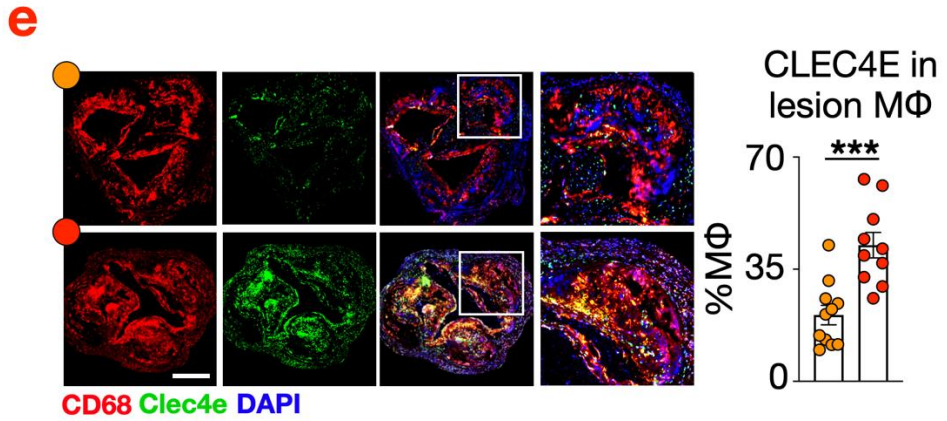


Fig 4e. CLEC4E and CD68 immunofluorescence in the aortic root and quantification of colocalization, n=11, 10 for Sedentary and SF. Scale bar=400 μm

Figure 5 – Mechanism of exercise benefit:
The authors suggest that β2-adrenergic stimulation drives the beneficial effects of exercise via IL-1β suppression in macrophages, but the data are not fully convincing: Figure 5C: Adrb2 mRNA levels appear increased in CH macrophages from exercised mice. Is this statistically significant? mRNA levels may not reflect protein expression due to post-transcriptional regulation. Protein-level validation is necessary because in vitro, the β2-adrenergic agonist salmeterol reduced IL-1β production in both WT and mutant macrophages.

We have performed the analyses suggested by the reviewer. First we conducted statistical analyses and indeed, the increased in Adrb2 expression in mutant macrophages of exercised mice is statistically significant (**Fig 5c**). Second, we repeated our qPCR analysis of WT vs Jak2 mutant BMDMs derived from sedentary and exercised mice and show a statistically significant rise in Adrb2 expression by exercise (**Extended Data 10g**). Finally, we performed immunofluorescent staining of atherosclerotic lesions from sedentary and exercised *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice for CD45.2 and ADRβ2. We found a significant increase in ADRβ2 protein in mutant (CD45.2) aortic macrophages of exercised mice (**Fig 5d**).

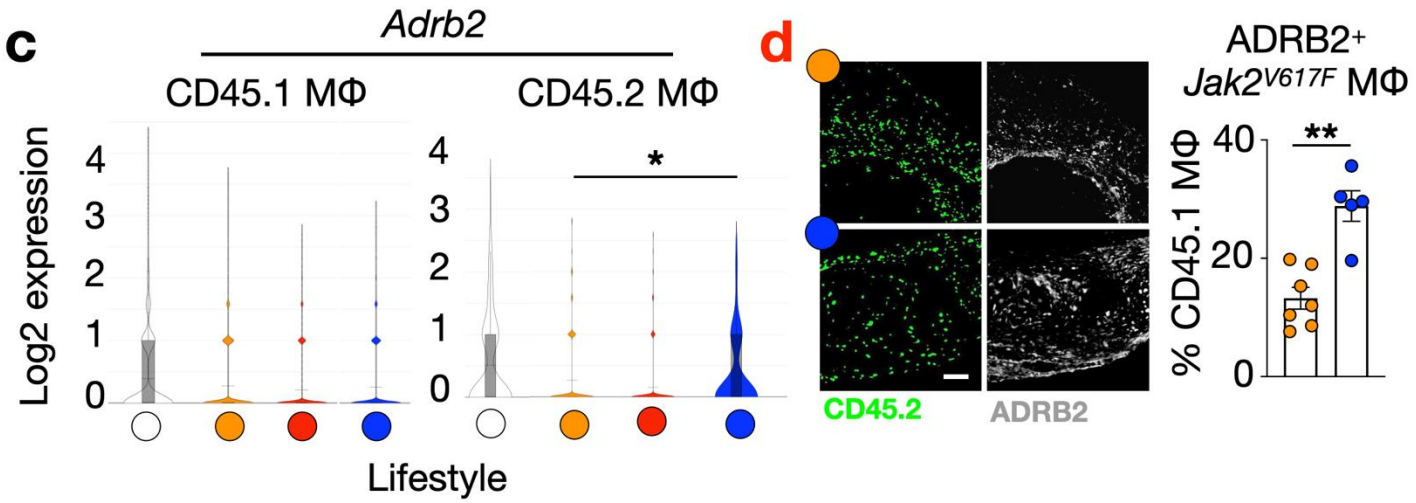
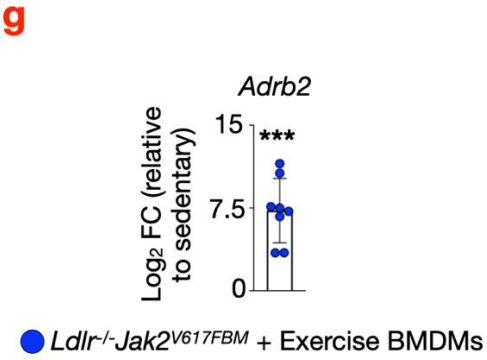


Fig 5. **c**. Log_2 expression of *Adrb2* in wild-type (CD45.1) and CD45.2 MΦ. n=5 pooled mice. **d**. CD45.2 and ADRB2 Immunofluorescence in the aortic root with quantification of pixel overlap. n=7,5 for sedentary and exercise, respectively. Scale bar=100µm.



Extended Data Figure 10g. *Adrb2* gene expression measured by qPCR in BMDMs from *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice subjected to exercise, expressed relative to values from sedentary *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice (n= 8).

Figure 5j-n: In vivo blockade of β2-adrenergic signaling reversed the protective effect of exercise in CH mice by shifting macrophages toward a more inflammatory phenotype, as indicated by increased IL1b expression. However, the mechanism remains unclear, since elevated numbers of IL1b^{+} macrophages were observed in plaques from both the control and exercise groups following pharmacological treatment, while accelerated atherosclerosis was only seen in the exercise group. IL1b expression was quantified in all macrophages, but it is important to analyze expression separately in mutant (CD45.2) versus wild-type (CD45.1) macrophages. This distinction is especially relevant because, in Figure 5c, the authors reported that adrenergic receptor expression was higher in mutant cells, suggesting they may be more affected by pharmacological blockade. We thank the reviewer for raising this important point. To directly address it, we quantified IL-1β specifically in CD45.2 *Jak2*^{V617F} aortic macrophages of the mice that received ADRβ2 blockade and were sedentary or exercised. As shown in the new **Fig 5o**, β2-adrenergic blockade had no effect on IL-1β *Jak2*^{V617F} mutant macrophages in the sedentary group, but it significantly increased IL-1β^{+} in Jak2^{V617F} mutant macrophages in exercised mice. This result is consistent with our scRNA-seq data (**Fig. 5c**), which showed higher *Adrb2* expression in mutant macrophages. Together these findings demonstrate that mutant macrophages in exercised mice are selectively sensitive to ADRβ2 pharmacological blockade.

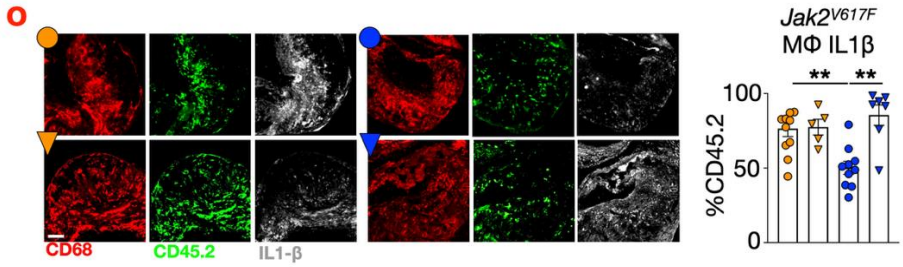


Fig 5. o. IL-1β and CD45.2 immunofluorescence and quantification of pixel overlap in the aortic root (n=10,5,10,7). Groups as in (**l**). Scale bar=100μm.

Statistical Analysis:
The authors used repeated measures across four groups (Figures 1B and 1C), and in Figures 3 and 4, they appear to use the same control group for multiple comparisons. These aspects should be double-checked by a statistical expert.
We thank the reviewer for this valuable comment, we have updated our statistical analysis. Because our dataset includes blood measurements within mice across multiple experimental groups, we employed mixed-effects modeling to appropriately account for within-subject correlation and occasional missing values.

For the analyses shown in Figure 1b and other longitudinal data, we fit linear mixed-effects models including fixed effects for time, intervention (sleep fragmentation or exercise), and their interaction. This approach provides a statistically robust estimate of group-level differences while controlling for repeated measures. Importantly, our primary goal was not to model individual temporal trajectories within a mouse (repeated measures) but to identify the time points at which group means diverged. Therefore, after confirming significant overall group effects in the mixed model, we performed between-group comparisons at individual time points to pinpoint when mutant clone expansion differed between interventions and controls. Critically, our all of our comparisons are between different experimental groups and not within a single group or mouse.

We appreciate the reviewer's note regarding the reuse of the same control group across Figures 3 and 4. We would like to note that each experiment had its own control cohorts (including having unique *Ldlr*$^{-/-WTBM}$ and sedentary mutant mice for every single experiment). We agree that if multiple related hypotheses within a single construct were tested against the same control, multiplicity corrections (e.g., Kruskal–Wallis with Steel's test) would be warranted. However, in our study, each comparison addresses a distinct, prespecified research question in a separate physiologic domain (e.g., sleep fragmentation vs sedentary or exercise vs sedentary). These are therefore independent analyses rather than repeated tests of the same hypothesis. We have clarified this distinction in the Methods and explicitly note a potential increase the perceived risk of Type I error.

Finally, we emphasize that the CH sleep-fragmentation and exercise groups were not directly compared to each other. Each intervention group was analyzed relative to a sedentary CH control cohort run in parallel under identical conditions, minimizing variability and considering ethical animal use. WT sedentary mice were included as an additional rigor control. Because only two a priori comparisons were prespecified (sleep fragmentation vs sedentary and exercise vs sedentary), additional multiple-testing correction was not required.

Referee #2

Reviewer Report for Manuscript on Mutation-Dependent CH Responses to Sleep and Exercise
Thank you for these encouraging comments. We have addressed each point below. New figures and data are marked in **red**.

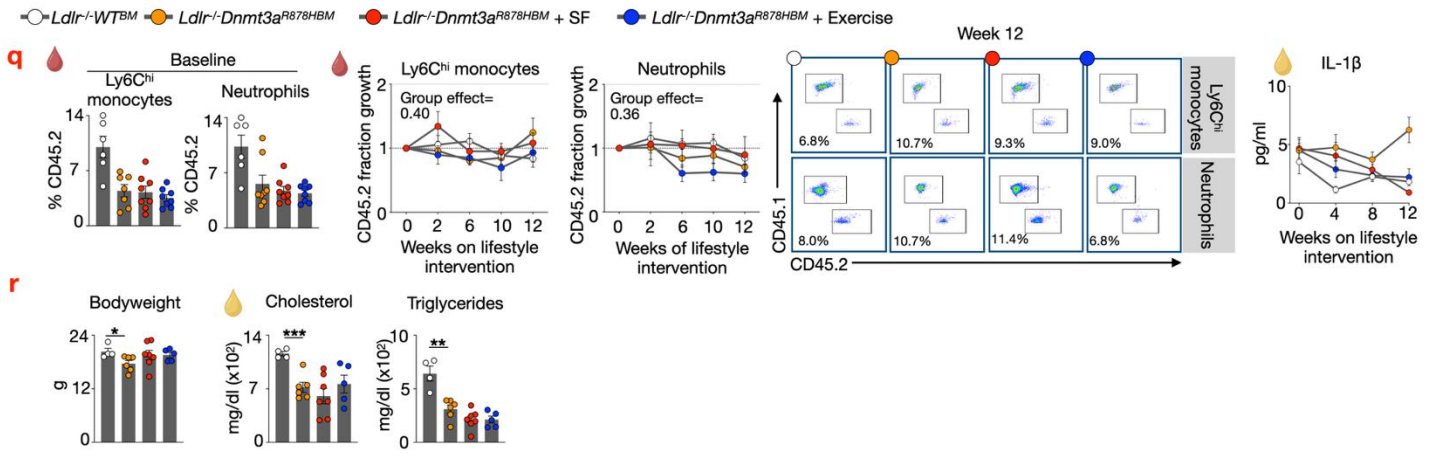
Key Results: This manuscript investigates how lifestyle factors, particularly sleep fragmentation and voluntary exercise, modulate clonal hematopoiesis (CH) in a mutation-dependent manner. Using murine models and human cohort data, the study reveals that:
• Jak2 and Tet2 mutant clones expand under sleep fragmentation and are suppressed by exercise.
• p53 mutant clones are unresponsive to either intervention.

• These lifestyle effects selectively reprogram mutant hematopoietic stem cells and aortic macrophages.
• CLEC4E-inflammasome and norepinephrine-ADRβ2 signaling are key mutation-specific mechanisms.
• In human datasets, moderate-to-vigorous physical activity (MVPA) correlates with lower non-DNMT3A CH prevalence.

Validity: The experimental design is logically structured and spans multiple levels of analysis, from population-based human associations to cellular mechanisms in mouse models. The inclusion of multiple CH mutations provides important specificity, though the mechanistic depth is centered primarily on Jak2. The exclusion of a Dnmt3a model limits translational completeness, particularly since human data show MVPA does not reduce DNMT3A CH prevalence. Including a Dnmt3a mouse model is recommended and would substantially strengthen the work.

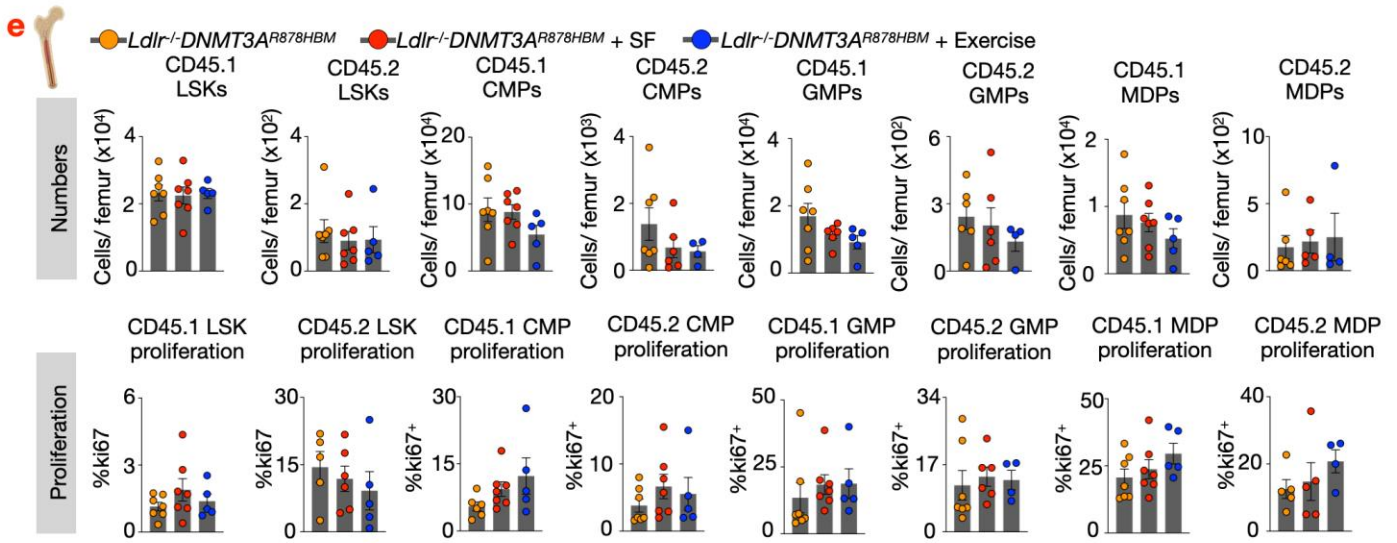
Originality and Significance: This is a novel and timely study that advances the understanding of how CH interacts with modifiable lifestyle factors in a mutation-dependent manner. It introduces new paradigms for personalized interventions and demonstrates that CH is not uniformly responsive to behavioral changes. The distinct signaling axes implicated—CLEC4E and norepinephrine-ADRβ2—further contribute to the originality of the work.

Including a Dnmt3a mouse model is recommended and would substantially strengthen the work.
We completely agree and have therefore added Dnmt3a^{R878H} CH mice, the fourth mouse model included in the manuscript. Briefly, as we did with our other CH models, we bred inducible Dnmt3a^{R878H} mice (Mx1Cre-Dnmt3a$^{R878Hwt/fl}$). We irradiated *Ldlr*$^{-/-}$ mice and reconstituted their BM with 90% WT (CD45.1) and 10% Mx1Cre- Dnmt3a$^{R878Hwt/fl}$ (CD45.2) BM cells. Alongside, a control cohort of *Ldlr*$^{-/-WTBM}$ mice was also included. All mice received Poly(I:C) to initiate the *Dnmt3a*^{R878H} mutation, fed an HCD, and exposed to sedentary, SF, or exercise interventions for 12 weeks. In agreement with our human data, sleep fragmentation or exercise did not influence the clonal expansion of *Dnmt3a*^{R878H} (CD45.2) monocytes or neutrophils in the blood nor plasma IL-1β (**Extended Data Figure 2q-r**). Moreover, SF and exercise did not impact the proliferation or abundance of WT or mutant hematopoietic progenitor cells in the BM of *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice (**Extended Data Figure 4e**). Finally, while sedentary *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice had larger atherosclerotic lesions than *Ldlr*$^{-/-WTBM}$ mice, we discovered that SF and exercise did not alter atherosclerotic lesion size, lesion macrophage content, or macrophage proliferation in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice (**Extended Data Figure 6l**). Together with our data on *Jak2*, *Tet2*, and *p53* CH, these new data add a fourth CH mutant model and mirror our human data suggesting mutation-dependent and nuanced influence of sleep and exercise on CH and its associated atherosclerosis.

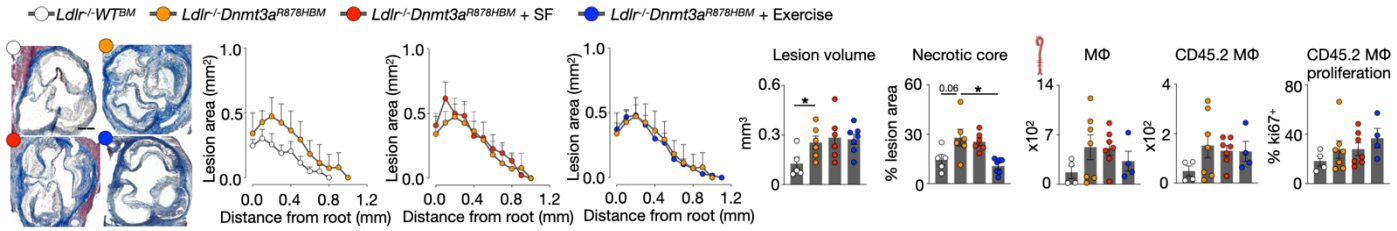


Extended Data Figure 2. q. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice at baseline (n= 6 wt, 7-8 sed, 8 SF, 8 exercise). Analysis of CD45.2 Ly6C^{hi} monocyte and CD45.2 neutrophil fraction growth in *Ldlr*$^{-/-}$*WT*^{BM} or *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice undergoing lifestyle intervention (n= 6 wt, 10 sed, 7 SF, 8 exercise) with representative flow cytometry plots. Time course of plasma IL-1β levels (n= 6 wt, 11 sed, 8 SF, 8 exercise). **r** Body weight and

plasma lipid levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice exposed to lifestyle at the time of euthanasia (4, 4 wt; 6, 6 sed; 7, 7, SF and 5, 5 exercise).



Extended Data Figure 4. Cell counts of CD45.2 mutant or CD45.1 wild-type LSKs, CMPs, GMPs, and MDPs in *Ldlr*$^{-/-}$*Dnmt3A*^{R878HBM} (**e**) mice. Proliferation of cell populations, quantified by the proportion of ki67-positive cells using flow cytometry.



Extended Data Figure 6. l. Lesion size, volume and necrotic core area (n= 5 wt, 6 sed, 6 SF, 7 exercise) with representative images of aortic roots stained with Masson's Trichrome stain, quantification of total and CD45.2 aortic macrophage number, and CD45.2 macrophage proliferation in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 4 wt, 7 sed, 7 SF, 4 exercise); scale bar=200 μm. Data are mean ± s.e.m; *p<0.05, **p<0.01.

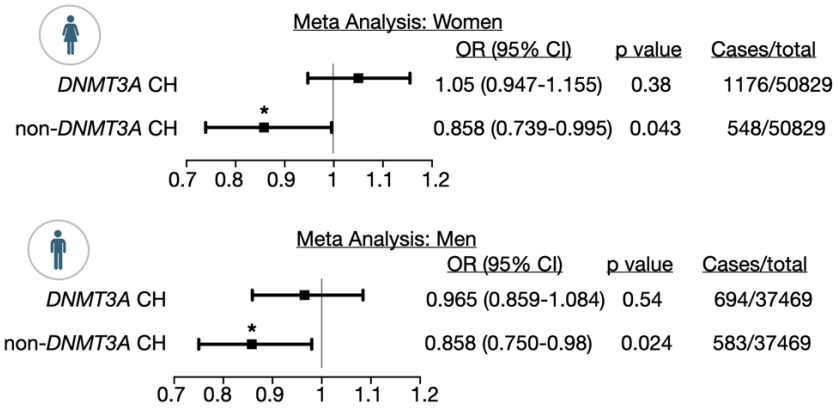
The lack of sex-stratified analyses is a critical omission given known sex differences in immune, metabolic, and neuroinflammatory responses. The authors state both sexes were used but provide no breakdown or stratified data. It appears that mixed sex controls were used, but only female mice were used in experimental groups. This is very unclear. The pooling justification is vague. While the authors note that results were "similar" in males and females, no sex-stratified data are shown to support this claim. Please clarify the sex used in each experiment and provide sex-stratified data where available, particularly for key outcome measures (e.g., clone expansion, lesion size, inflammatory markers, macrophage clusters). If sexes were pooled based on the absence of statistically significant differences, summary statistics (e.g., p-values, direction of effect) should be provided in the supplementary materials. These additions will significantly enhance the rigor and reproducibility of the study. If sex was tested and found non-significant, a brief summary of those results should be shown. Given known sex-based differences in immune and metabolic responses, equal number of males and females should be used in the experiments if the findings are going to be interpreted in such a general context.

We appreciate the emphasis on sex as an important biological variable. To clarify, all mouse experiments in this study were performed exclusively in female mice, using female recipient and donor mice, including experimental and control animals. No mixed-sex experimental or control groups were used. This design choice was deliberate and consistent with prior mechanistic studies of *Jak2*^{V617F} (PMID: 33731931) and *Tet2*$^{-/-}$ (PMID: 28104796) driven CH in atherosclerosis, which were also performed exclusively in female *Ldlr*$^{-/-}$ mice. This strategy was chosen so that our findings can be directly contrasted with prior data. Moreover, female *Ldlr*$^{-/-}$ mice exhibit higher and more stable LDL cholesterol levels and a higher, less variable atherosclerotic lesion burden than males (PMID: 29430260), reducing biological noise and variability in our atherosclerosis

experiments. Further, adequately addressing sex as an independent variable in bone marrow chimeras would require inclusion of all four sex-matched and sex-mismatched donor–recipient combinations, substantially increasing experimental complexity and animal use. Importantly, however, we fully agree with the reviewer as to the importance of sex in cardiovascular and inflammatory outcomes. Indeed, recent work has demonstrated that sex can significantly influence CH clone dynamics and inflammatory profiles (e.g., PMID: 40178977). This highlights the potential for sex-specific confounding when testing the impact of lifestyle on CH-driven inflammation. We have added new text to the discussion noting the limitation of only using females in mouse studies.

Nevertheless we appreciate the reviewer's suggestion, thus to better align our human and murine analyses and assess sex-specific confounding factors in our human data, we have performed a new sex-stratified analysis for the human analyses (**Extended Data Figure 1b**). This new analysis reveals that in both men and women, MVPA associated with reduced *DNMT3A* but not non-*DNMT3A* CH, without evidence of interaction by sex. These new data are now found in the new **Extended Data Figure 1b**.

b



Extended Dat Figure 1b. Meta-analysis of UK Biobank and All of Us on the association of moderate-to-vigorous physical activity with the prevalence of *DNMT3A* CH and non-*DNMT3A* CH, split by sex.

Details on number of animals used per group and per experiment are scattered and would benefit from consolidation in a supplemental table for transparency and reproducibility.

Thank you for this valuable suggestion. The number of animals used per group in every experiment is indicated in the figure legends. Moreover, each data point in bar graphs indicates an individual mouse. We are also following the reviewer's suggestion. In the revised version of the manuscript, we provide all of the source data in table files, which also indicate the number of animals in each group in every experiment. The number of animals varies because most of our mice were derived from in house breeding which generates uneven numbers of mice that are then randomized to interventions. Second, as expected in BM transplant experiments, some mice do not survive the BMT procedure before randomization to lifestyle intervention. Finally, some experimental readouts necessitate larger group sizes (like atherosclerosis quantification) while other cellular and molecular analyses can be conducted with smaller group sizes. This has been explained in the updated murine statistic section.

Randomization and blinding are not described for animal group assignments or data analysis. Were outcome assessments blinded? Were variables such as cage location and time of day controlled?

We have expanded and clarified these points in the updated methods section. In brief, yes, all mice were randomized to interventions and all outcomes were assessed blinded. Cage location did not differ as all mice were group housed in the same room. Finally, we are acutely aware of the influence of circadian rhythm and all mice, including all controls in every experiment, were euthanized at the exact same time of day: ZT 2. This has all been clarified in the updated methods. This has been explained in the updated animals section of the methods.

Statistical Analysis: The statistical approach used for analyzing murine data is insufficient given the complexity of the experimental design and the structure of the data. This significantly undermines the rigor and interpretability of several key findings.
Thank you for these helpful suggestions and comments. We have addressed each one and have conducted an updated statistical analysis as outlined below. Our updated murine statistical approach is also fully described in the methods section of the revised manuscript, please see red text in the revised manuscript. We address each point individually below.

1. The authors employ basic pairwise or group comparisons (e.g., ANOVA or t-tests), which do not adequately account for:
    - o Repeated measures (e.g., serial blood draws, longitudinal clone tracking)
    - o Nested data (cells within animals, animals within cohorts)
    - o Interactions between genetic background, intervention, and sex

We thank the reviewer for their careful attention to the statistical design of our study. For the longitudinal blood cell expansion curves (Figure 1), we used repeated-measures ANOVA or mixed-effects analyses, as indicated in the updated manuscript. This approach accounts for the non-independence of serial blood draws from the same animals and allows variance due to time, which is shared across groups, to be modeled explicitly while testing for differences in fractional CD45.2 growth between groups. Post-hoc Tukey's tests were then applied to identify specific pairwise differences. We have clarified these details in the figure legends and methods to ensure transparency. To be clear, all of our analyses, and thus our conclusions, relate to differences between different treatment groups (e.g. sedentary vs. SF and sedentary vs. exercise) at each timepoint and not repeated measures over time within an experimental group or individual mouse.

With respect to the reviewer's point about nested data, we would like to clarify that our analyses are conducted at the level of the animal, not the cell. This avoids pseudoreplication and ensures that each data point represents a biological replicate, i.e., an individual mouse. While we acknowledge that cohort effects can occur when experiments are repeated with different groups of mice, our study design does not involve linear modeling in which variables such as cohort or cage number could be included as predictors. Instead, experiments were pooled where appropriate to maximize statistical power, consistent with standard practice in animal studies and mindful of ethical constraints on animal use. We have added clarifications to the methods section to emphasize that our reported N reflects biological replicates (i.e. individual mice). In cases where qPCR or culture assays were used, technical replicates were included to improve intra-experiment reliability, but the unit of analysis remained at the level of the animal or the independently prepared culture.

Finally, regarding potential interactions between genotype, intervention, and sex, all of the mice used in this study were female Ldlr−/− animals (C57BL6 background). As such, sex and genetic background were controlled for and held constant across all experimental groups, making it impossible or inappropriate to test for genotype-by-sex interactions in our data. We have added a statement in the revised manuscript to make this point explicit.

This has all been explained in the updated murine statistic section.

2. The study involves a hierarchical structure. Use hierarchical mixed-effects models to account for repeated measures, nested data, and cohort variability. Include sex as a fixed effect and test for sex-by-mutation-by-intervention interactions.
We agree with the reviewer that hierarchical mixed-effects models are important in studies where there are multiple levels of nesting (e.g., cells within animals, animals within cages, cohorts within experiments) or where there is variability across sex or genotype. In our study, however, all experimental animals were female Ldlr-/- mice (C57BL/6 genetic background), so sex and genetic background were held constant by design. As such, including sex or genotype as fixed effects, or testing for sex-by-mutation-by-intervention interactions, is not applicable in this context. Cohort effects can be a concern in large-scale epidemiologic designs, but in our basic science animal experiments, mice were randomized within each experiment and repeated experiments

were pooled at the level of the animal. We therefore do not have hierarchical variability across sex or genotype that would justify inclusion of those terms in the models.

That said, we recognize the importance of accounting for within-animal correlation due to multiple measures. For our longitudinal outcomes, we used repeated-measures ANOVA or, when missing values occurred, mixed-effects models, which are the appropriate tools for capturing the non-independence of serial blood draws within animals. However, as stated above, for our longitudinal data, our comparisons remained between experimental treatments and not repeated measures within a cohort of mice. For cross-sectional outcomes we applied one-way ANOVA or its non-parametric alternatives, as detailed in the Methods. We have clarified this rationale in the revised text to avoid any ambiguity. This has all been explained in the updated murine statistic section.

3. Inappropriate treatment of zero-inflated or bounded data. Many endpoints (e.g., clone proportions, cytokine levels, lesion metrics) are bounded at 0, often right-skewed, or include excess zeros. These violate assumptions of normality and homoscedasticity. Apply appropriate transformations or use zero-inflated or beta regression models for bounded proportional data, and negative binomial models for over-dispersed count data (e.g., cell proliferation, lesion cell counts).
There is insufficient reporting of statistical details. Provide model coefficients, p-values, and confidence intervals.
We thank the reviewer for raising this point. To clarify, none of the variables we analyzed were zero-inflated, and our source data confirm that absolute zeros do not occur in these datasets. Because our experiments do not involve modeling count data with excess zeros, the use of zero-inflated models would not be appropriate. While some endpoints—such as clone proportions, lesion metrics, or cytokine levels—are bounded and occasionally right-skewed, we addressed this by using non-parametric tests (e.g., Mann–Whitney U) for nearly all comparisons. These rank-based methods do not assume normality or equal variance and are widely accepted in the context of preclinical animal studies with bounded biological data.

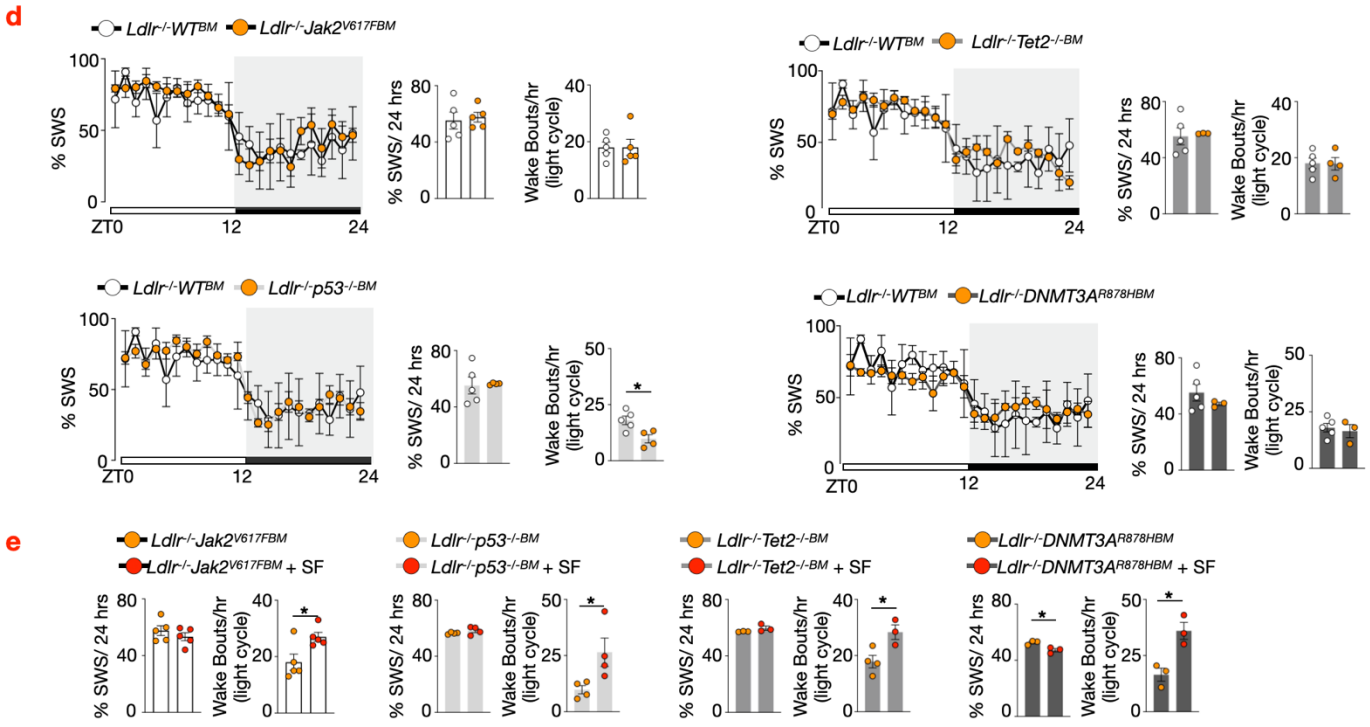
In the few instances where parametric tests (e.g., ANOVA) were employed, we first verified approximate normality (via Q–Q plots and Shapiro–Wilk) and equality of variance. When assumptions were not met, we cross-checked the results with non-parametric alternatives and obtained consistent outcomes. We agree that beta regression or negative binomial models can be powerful approaches, particularly in large-scale datasets or generalized linear modeling frameworks; however, given the scope and size of our animal studies, our non-parametric approach was well suited and adequately powered to detect biologically meaningful effects.

To provide full transparency, we include all of the raw source data spreadsheets with the revised manuscript so that readers can confirm the absence of zero inflation and evaluate the data distributions directly. We also provide exact p-values for all statistical comparisons in the figure legends, and all figures display error bars (mean ± SEM). This has all been explained in the updated murine statistic section.

Critique of "Healthy Sleep" Claims: The manuscript claims that "healthy sleep" reprograms CH dynamics. However, no direct sleep measures (e.g., EEG, telemetry, piezoelectric) were taken. This assumption is not valid. Mice not exposed to SF may still experience shortened total sleep time, reduced NREM/REM sleep, or poor sleep depth (e.g., delta power) due to environmental, inflammatory, or genetic factors. Critically, no direct measures of sleep quantity or architecture—such as EEG/EMG, telemetry recordings, or behavioral proxies—are reported. Thus, the conclusion that "healthy sleep" reprograms CH or modifies brain-body signaling pathways is unsupported by data. The authors should revise their language to reflect what was actually tested: e.g., “absence of tactile sleep fragmentation” rather than “healthy sleep.” Claims about the restorative or protective effects of sleep must be tempered unless direct sleep measures are included. It is recommended that sleep should be measured in at least a subset of animals to confirm the control animals for the SF experiments are sleeping as expected following the other experimental/genetic manipulations.

Thank you for raising this critical point. We fully agree. We have addressed this comment in two ways. First, we have revised and tempered our language by removing the term “healthy sleep” and replaced it with “uninterrupted sleep” or “absence of tactile sleep fragmentation”. Second, as suggested by the reviewer, we

have performed a comprehensive sleep analysis using wireless EEG/EMG telemetry across all four of our CH mutant models exposed to sedentary or SF (**Extended Data Figure 1d-e**). First, we find that in sedentary mice, the presence of the *Jak2*, *Tet2*, *p53*, or *Dnmt3a* CH mutation does not overtly alter slow wave sleep abundance, rhythmicity, or fragmentation (**Extended Data Figure 1d**). Indeed, we find that gross SWS architecture is intact and does not largely differ between the CH models and mutations. However, as expected, that our tactile SF system successfully fragments the sleep of CH mice and raises wake bouts (transitions from sleep to wake) during the light cycle when mice are naturally asleep and when the SF sweep bar operates (**Extended Data Fig 1e**). Indeed, each CH model maintains sleep rhythmicity and SWS and REM time. Nonetheless, we retain tempered language as a deeper sleep analysis assessing how CH might influence microarchitecture and power and spectra is beyond the scope.



Extended Data Figure 1EEG/EMG based quantification of slow-wave sleep and wake bout frequency 10 weeks after BM transplant in *Ldlr*$^{-/-}$*WT*^{BM}, *Ldlr*$^{-/-}$*Jak2*^{V617FBM}, *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$, *Ldlr*$^{-/-}$*p53*$^{-/-BM}$, and *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice in (**d**) a sedentary state and (**e**) during sleep fragmentation

Jak2 Mechanistic Work: The deep mechanistic insights are largely derived from Jak2 models. While justified by clinical relevance, this limits generalizability. Broad claims about CH should be restricted and interpreted in the context of this mutation.

We agree with the reviewer that the mechanistic analyses in this study primarily focus on Jak2^{V617F}-driven clonal hematopoiesis. To avoid overgeneralization, we have revised the manuscript to consistently frame mechanistic conclusions within the context of Jak2-mutant CH. Importantly, lifestyle-dependent effects on clonal expansion were also observed in Tet2 CH, while Dnmt3a and p53 mutants showed no such response, indicating mutation-specific sensitivity to environmental modulation. These points have been clarified and emphasized in the discussion. While Jak2-mutant CH serves as a mechanistically tractable model revealing new biological insights, our findings support the concept that lifestyle can differentially influence distinct CH genotypes. Language has been adjusted in the text.

Translational Interpretation of Human Data: The cross-sectional associations in human cohorts do not establish causality. The authors should temper conclusions and acknowledge the potential for reverse causation and confounding.

We agree and have tempered our language. The last two sentences describing the human data are now as follows: “These findings reveal that in humans, physical activity is associated with lower prevalence of non-

DNMT3A CH, but not *DNMT3A* CH. Although causality and directionality cannot be inferred in this cross-sectional analysis, our findings likely reflect changes in clone progression to the 2% VAF diagnostic threshold and not *de novo* mutagenesis." Moreover, in the discussion the following statement has been added: "Our human cross-sectional analysis demonstrating reduced non-*DNMT3A* CH in people with MVPA does not establish directionality or causation, but our murine studies address these gaps."

Discussion of Clinical Implementation: The authors suggest genotype-guided lifestyle interventions for CH but do not discuss feasibility. Considerations such as patient genotyping, comorbidities, and real-world adherence need to be addressed. Most patients with CH are older adults with comorbidities that may impair sleep or physical activity capacity.
Thank you, we agree. The following sentence has been added to the discussion: "While genotyping for CH diagnosis is being more broadly considered, interventional approaches should consider real-world adherence and comorbidities that may impact sleep and the ability to exercise."

Brain-Body Mechanisms: While PAC1+ neuron activation and NE signaling are intriguing, these findings are largely correlative. Without circuit tracing or causal manipulation (e.g., optogenetics), claims should be framed as hypothesis-generating.
Thank you, we have reworded the discussion to include limitations and alternatives including LC circuit tracing: "Deeper mechanistic investigations are warranted, including specific macrophage response to lesion environment, LC circuit tracing, and specific mechanisms by which lifestyle modifies the molecular programming of leukocytes harboring CH mutations beyond *Jak2*^{V617F}."

Figures and Language Suggestions:
- Improve clarity and define all abbreviations in figure legends.
- Avoid 'sacrifice' which carried religious connotations in some cultures and use 'euthanize' instead.
- Add colorblind-friendly palettes. Using a color blind-friendly palette in research is crucial for making data visualizations accessible and ensuring accurate interpretation of research findings by a wider audience. Are our colors not colorblind friendly?
- Provide time points for all intervention phases on the schematics.

Thank you for all of these suggestions. They have all been incorporated into the revised manuscript.

Overall Recommendation: This is a conceptually novel and mechanistically rich manuscript that addresses an important question in aging and cardiovascular disease. With improvements to the statistical rigor, sex-specific reporting, and tempered claims regarding sleep and neuroimmune circuits, it will make a significant contribution to the field. Inclusion of a Dnmt3a mouse model would elevate the translational impact further. Acceptance is recommended pending major revision.
Thank you for your helpful and thoughtful comments and suggestions, which have greatly improved our manuscript.

Referee #3

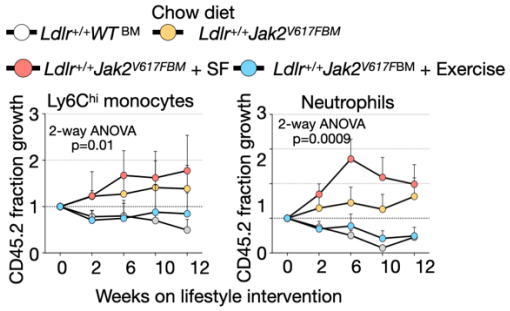
In this study, McAlpine et al address the important question of whether lifestyle factors such as exercise and sleep impact the emergence of clonal hematopoiesis in mouse models and human epidemiologic data. The authors use a mouse model of common CH mutant BM transplanted into Ldlr-/- mice and layered on sleep deprivation or exercise to evaluate the impact on clone expansion. Interestingly, they demonstrated that exercise mitigated clone expansion whereas sleep disruption promoted clone expansion particularly for Jak2-mutant clones but not for p53 mutant clones. Exercise also appeared to protect against CH in human cohorts such as the UKB. The authors hypothesize that exercise promotes a beta-adrenergic signal from the brain that counteracts a disease-driving hyperinflammatory gene program in macrophages; blocking beta adrenergic signaling was found to be sufficient to counteract the protecting effects of exercise in JAK2-driven CH associated atherosclerosis.
Overall this is a very expansive study with an interesting hypothesis and potentially impactful conclusions. However what the study offers in breadth it lacks in depth and rigor. Critical details are missing without

consideration of alternative hypotheses. Experiments are not repeated. The data are not sufficient to support the claims in many instances, precluding the publication of the study in its current form. Specific comments:

Thank you for the helpful and constructive comments. Each comment has been addressed below.

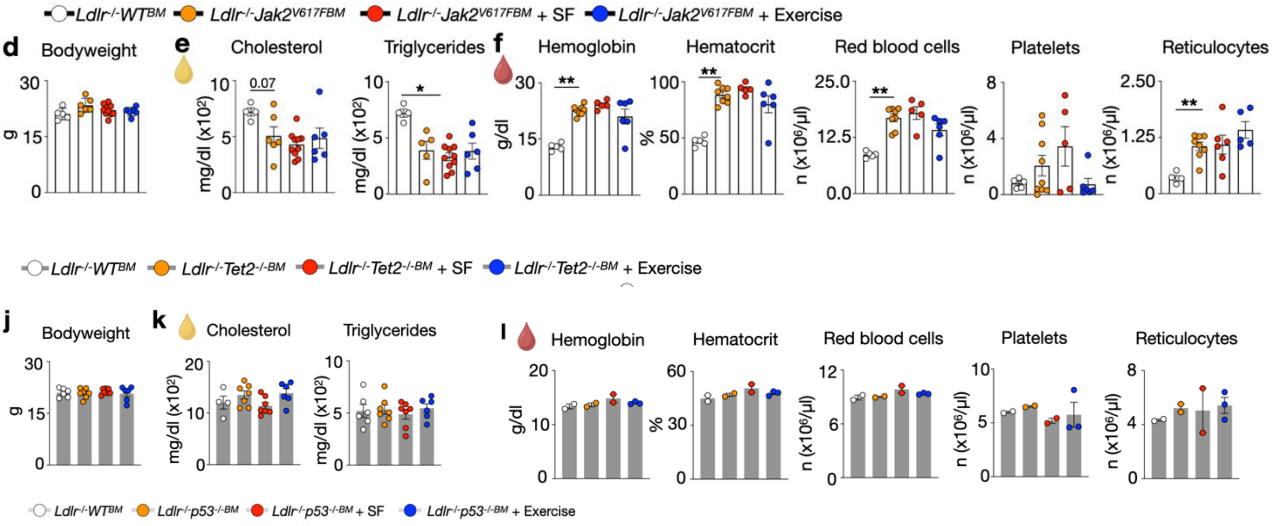
Specific Comments:
It is unclear why Ldlr-/- mice were used in Figure 1 of this study as opposed to WT recipients. Ldlr-/- mice have been used in studies of the impact of CH on cardiovascular disease. However one of the points that the authors are trying to make here is that the effects of exercise and sleep deprivation on CH occur independently of weight, obesity, or glucose levels. Have the authors determined if lipid dysregulation is required for these effects to occur? Are similar effects of exercise and sleep disruption also observed in WT mice?

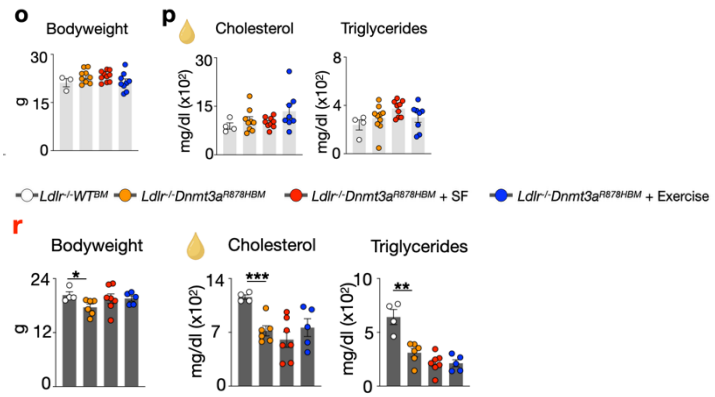
Thank you for this important point. Ldlr-/- mice are a reliable and broadly used model of atherosclerosis and our primary focus is on the influence of sleep and exercise on CH in the setting of atherosclerosis. However, as the reviewer correctly points out, we hypothesize that the influence of lifestyle on CH is independent of weight, obesity, lipid levels. To address this, we have conducted experiments in WT recipient mice (i.e. without atherosclerosis or hyperlipidemia). Briefly, WT mice were irradiated and reconstituted with 90% CD45.1 WT BM cells and 10% *Jak2*^{V617F}-Mx1-Cre cells. Recipient mice were fed a chow diet and exposed to sedentary, SF, or exercise lifestyles. Consistent with our data in our HCD fed *Ldlr*$^{-/-}$ mice, *Jak2*^{V617F} clone expansion was accelerated by SF and repressed by exercise in WT mice (**Extended Data Fig 2g**).



Extended Data Fig 2g. Analysis of CD45.2 Ly6C^{hi} monocyte and CD45.2 neutrophil fraction growth in *Ldlr*$^{+/+}$ mice with *WT*^{BM} or *Jak2*^{V617F} CH exposed to SF or exercise.

Furthermore, we have conducted an extensive analysis of bodyweight, cholesterol, triglycerides, hemoglobin, hematocrit, red blood cells, platelets, and reticulocytes in our Ldlr-/- mice with CH and exposed to lifestyles (**Extended Data Fig 2d, e, f, j, k, l, o, p, r**). Crucially, SF or exercise does not impact these parameters in HCD fed Ldlr-/- mice with Jak2, Tet2, p53, or Dnmt3a CH.





Extended Data Fig 2. Body weight (**d**), plasma lipids (**e**), and blood parameters (**f**) in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to lifestyle at the time of euthanasia. Body weight (**j**), plasma lipids (**k**), and blood parameters (**l**) in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ mice exposed to lifestyle at the time of euthanasia. Body weight (**o**) and plasma lipids (**p**) in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*p53*$^{-/-BM}$ mice exposed to lifestyle at the time of euthanasia. **r** Body weight and plasma lipid levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice exposed to lifestyle at the time of euthanasia

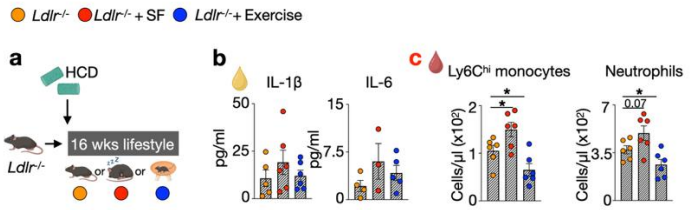
Figure 1: This sentence is not clear: "all mice were fed a high cholesterol diet (HCD) and provided running wheels for voluntary exercise (Extended Data Fig 1a)31, subjected to tactile sleep fragmentation (SF) with automated cages30,36,37, or allowed to remain sedentary for 12 weeks." What mice got what treatments and in what combinations?! This is only apparent after studying the figure in great detail.

Thank you for pointing this out. To clarify, mice were randomized to a single intervention. Mice either remained sedentary, were subjected to SF, or were provided running wheels for exercise. No mice received multiple or a combination of interventions. The sentence has been reworded as follows: “Beginning at the same time as the Poly(I:C) injections, all mice were fed a high cholesterol diet (HCD) and randomized to either voluntary exercise by being provided running wheels[32], tactile sleep fragmentation (SF) with automated cages[31,37,38], or allowed to remain sedentary for 12 weeks.”

What is the effect of sleep fragmentation or exercise alone on cytokine concentration and myeloid cell counts? These data need to be provided – not just for the Tet2 mosaics. Were IL1b and IL6 the only cytokines measured?

Thank you for these important questions. We have conducted a new deep and temporal analysis of how SF and exercise alter cytokine levels and myeloid cell counts across all of our CH models and in mice without CH.

First, we evaluated the impact of SF and exercise on cytokine levels and myeloid cell counts in *Ldlr*$^{-/-}$ without CH. We find that, in the absence of CH, SF and exercise don’t overtly impact plasma IL-1b and IL-6. SF increases and exercise decreases blood monocytes;SF does not alter blood neutrophils, but exercise decreases neutrophils (**Extended Data Figure 3a-c**).



Extended Data Figure 3. a. Experimental setup. **b.** Plasma levels of IL-1β and IL-6 at the time of euthanasia (n = 5, 4 sed; 6, 3 SF; 6, 5 exercise). **c.** Blood counts of Ly6C^{hi} monocytes and neutrophils at the time of euthanasia (all n= 6).

Moreover, we have performed an extensive analysis of blood myeloid cells and cytokine concentrations in Jak2, Tet2, p53, and Dnmt3a mice exposed to SF, exercise or that remained sedentary. First, in *Ldlr*$^{-/-}$

Jak2^{V617FBM} mice, we performed a new analysis of plasma IL-1β over the course of lifestyle exposure which revealed temporal kinetics (**Fig 1f**).

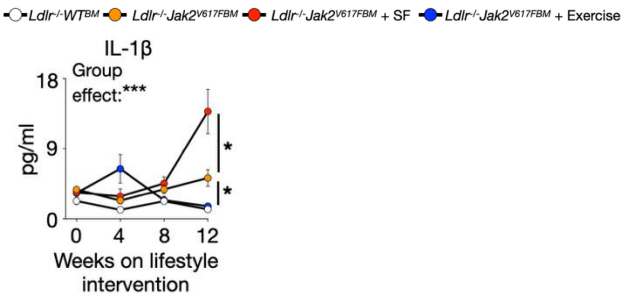
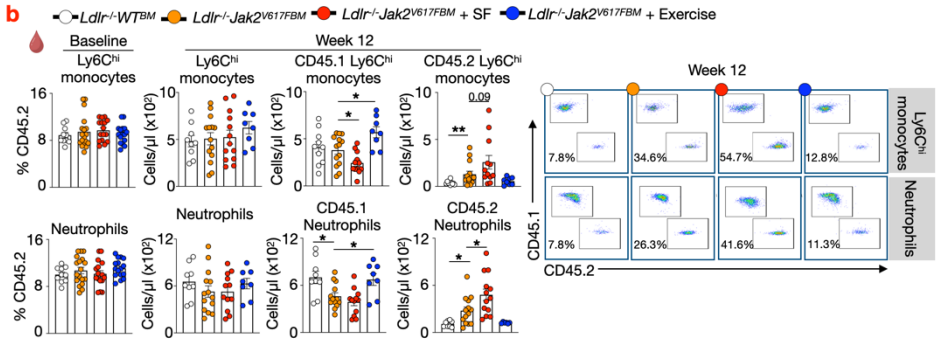


Figure 1f. Time course of plasma IL-1β levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM}Sedentary, SF, and Exercise (n=8,13,9,10; 4,4,4,4; 6,8,8,9 and 7,9,10,6 at baseline, 4,8 and 12 weeks respectively).

Moreover, we have also conducted an extensive analysis of total, CD45.1, and CD45.2 monocyte and neutrophil counts in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to SF or exercise. Intriguingly, we find that while lifestyle does not overtly impact the total number of blood monocytes or neutrophils, SF increases while exercise decreases the number of CD45.2 mutant cells while reciprocally altering the number of WT CD45.1 cells (**Extended Dat Fig 2 b**). These new findings affirm the notion that lifestyle has a selective and unique effect on mutant myeloid cells.



Extended Data Figure 2b. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice at baseline (n= 10 wt, 18 sed, 16 SF, 16 exercise) and total cell counts at the time of euthanasia (n= 8-10 wt, 13-14 sed, 12-13 SF, 6-8 exercise), with representative flow cytometry plots.

We also performed the same analyses in Ldlr-/- mice with Tet2, and p53 CH. In mice with Tet2 CH, we also found temporal kinetics of plasma IL-1β elicited by SF and exercise (**Figure 1 g**) and that the total number of blood monocytes and neutrophils were unchanged but rather, their relative abundances were impacted by lifestyles (**Extended Data Fig 2h**).

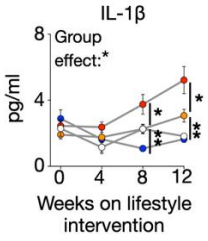
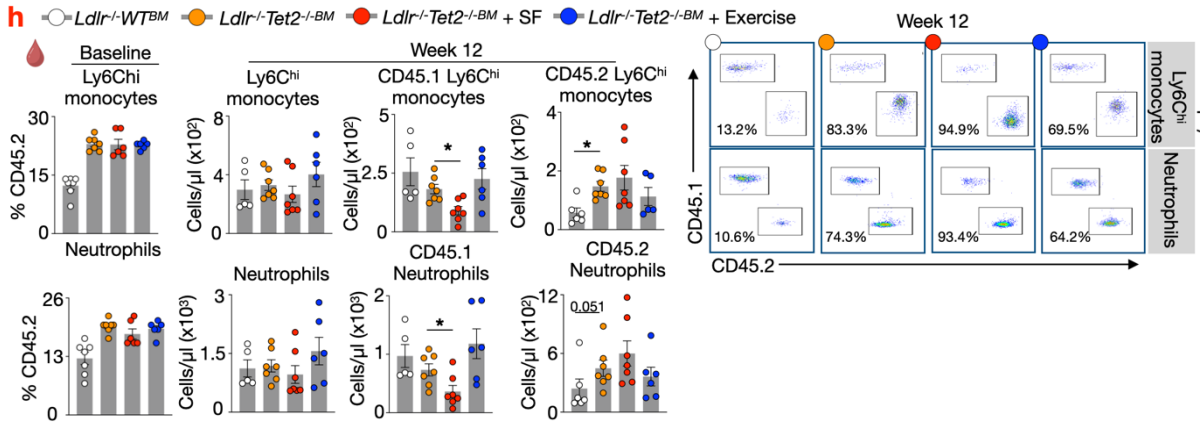


Figure 1g. Time course of plasma IL-1β levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Tet2*$^{-/-}$ Sedentary, SF, and Exercise (n=8,5,6,5; 4,5,5,5; 6,7,5,5, and 6,6,7,6 at baseline, 4,8 and 12 weeks respectively).



Extended Data Figure 2h. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$ mice at baseline (n= 7 wt, 7 sed, 6 SF, 6 exercise) and total cell counts at the time of euthanasia (n= 5-6 wt, 7 sed, 7 SF, 5-6 exercise), with representative flow cytometry plots.

In mice with p53 CH, we discovered that lifestyle does not impact plasma IL-1β over the course of the experiment (**Figure 1h**), nor monocyte or neutrophils numbers or the abundance of CD45.1 and CD45.2 cells (**Extended Data Figure 2m**). Together, these findings reveal nuanced and specific effects of lifestyle on myeloid cell abundance that depend on the presence of CH mutations and the gene mutated.

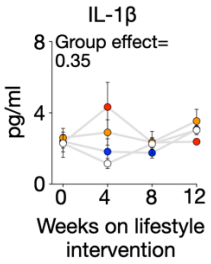
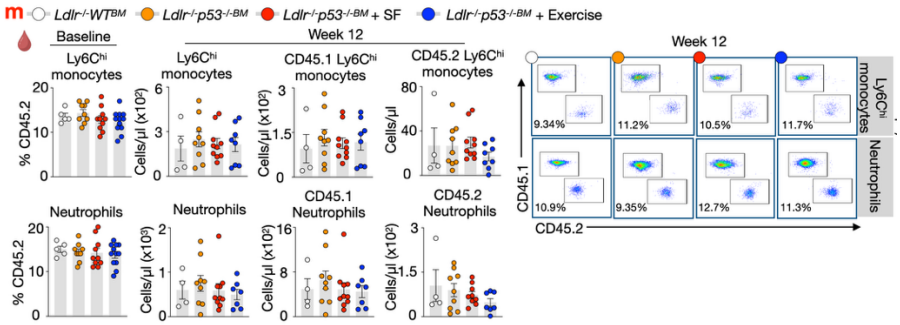


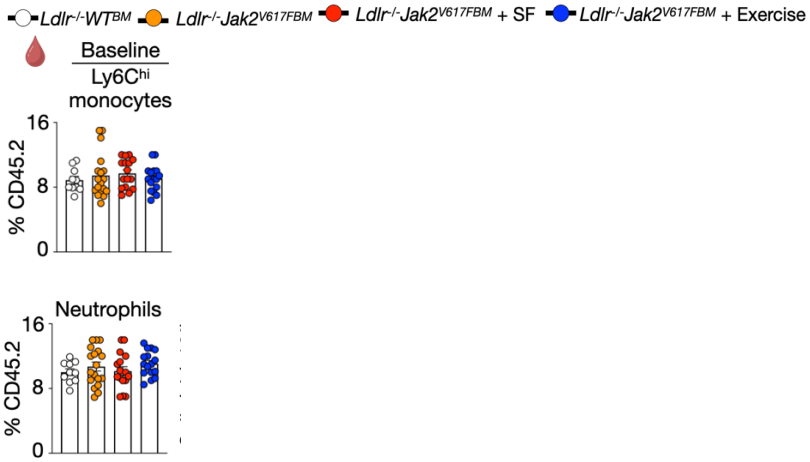
Figure 1h. Time course of plasma IL-1β levels (n=8,5,5,5; 4,5,5,5; 6,5,5,5 and 4,9,9,7 at baseline, 4,8 and 12 weeks in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*p53*$^{-/-}$ Sedentary, SF, and Exercise, respectively).



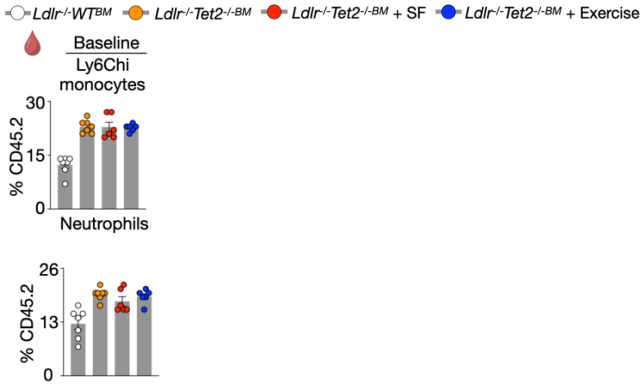
Extended Data Figure 2m. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$*p53*$^{-/-BM}$ mice at baseline (n= 5 wt, 9 sed, 10 SF, 12 exercise) and total cell counts at the time of euthanasia (n= 4 wt, 9 sed, 9-10 SF, 7-8 exercise) with representative flow cytometry plots.

What is meant by “CD45.2 fraction growth”? These numbers could be influenced by the starting mosaicism which should be shown for all mice. Absolute fractional growth of 2% is different if the starting mosaicism is 1% versus 10%.

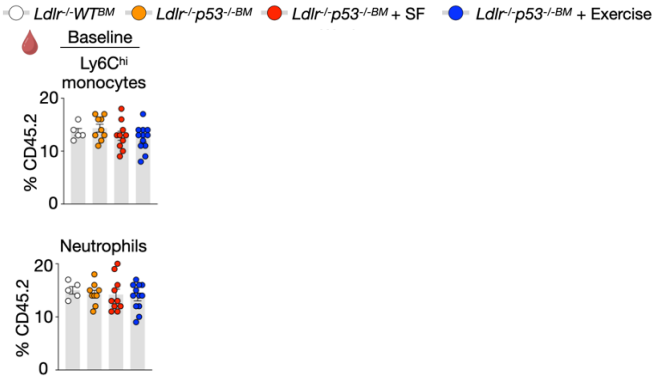
Thank you for raising this important point. CD45.2 fraction growth refers to the fold change in the percentage of CD45.2 cells over time relative to baseline. Baseline measures were assessed immediately prior to initiating the mutations with Poly(I:C) and lifestyle exposure. This has been clarified in the text. As suggested by the reviewer, in the revised manuscript we have now included all of the baseline %CD45.2 values across all mutations (**Extended Data Figure 2b, h, m, q**).



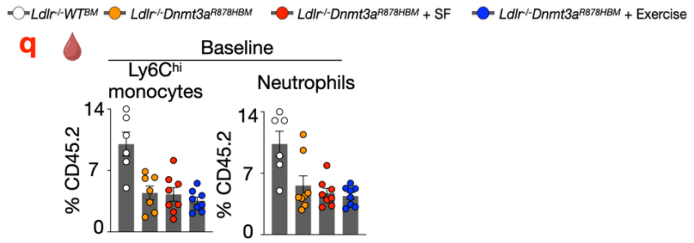
Extended Data Fig 2b. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$Jak2^{V617FBM}* mice at baseline.



Extended Data Fig 2h. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$Tet2$^{-/-BM}$* mice at baseline.



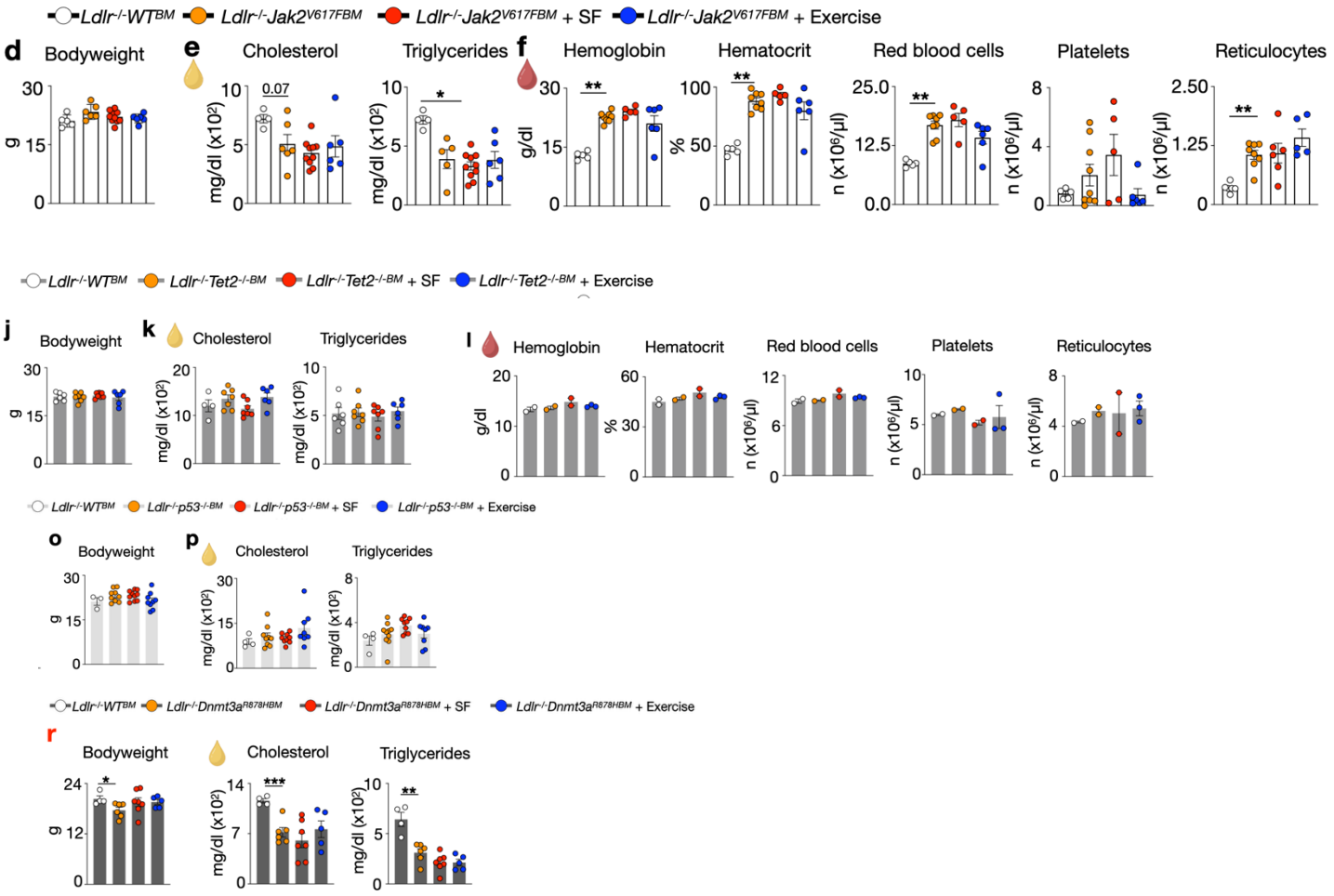
Extended Data Fig 2m. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$p53$^{-/-BM}$* mice at baseline.



Extended Data Fig 2q. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$ Dnmt3a^{R878HBM}* mice at baseline.

What were the weights and cholesterol levels of mice on SF and exercise? BMI and cholesterol levels for all groups should be provided. What about differences in WT for weights and lipid levels? Is dyslipidemia necessary to observe the beneficial effects of exercise?

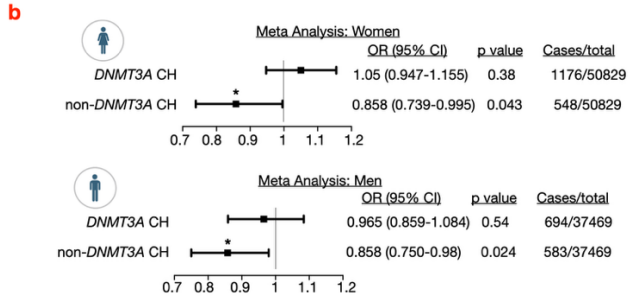
To address this point, we have measured body weight, plasma cholesterol and triglycerides across all of our models. We find that SF and exercise do not impact bodyweight or plasma lipid levels (**Extended Data Figure 2**). These data are expected because *Ldlr$^{-/-}$* mice do not gain weight when consuming a HCD.



Extended Data Figure 2. Body weight (**d**), plasma lipids (**e**), and blood parameters (**f**) in *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$Jak2^{V617FBM}* mice exposed to lifestyle at the time of euthanasia. Body weight (**j**), plasma lipids (**k**), and blood parameters (**l**) in *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$Tet2$^{-/-BM}$* mice exposed to lifestyle at the time of euthanasia. Body weight (**o**) and plasma lipids (**p**) in *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$p53$^{-/-BM}$* mice exposed to lifestyle at the time of euthanasia. **r** Body weight and plasma lipid levels in *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$ Dnmt3a^{R878HBM}* mice exposed to lifestyle at the time of euthanasia.

Only female mice were used in the study. This should be acknowledged as a limitation of the study and all conclusions should specify that these effects are true for female mice. Human studies should include subset analysis of women and men separately to determine if there is a sex-specific effect.

We fully agree with the reviewer and have indicated in the discussion that our use of female mice is a limitation. We used female mice for several reasons: they have larger atherosclerotic lesions than male mice thus shorter feeding times are needed, their lesion size is more consistent and predictable than males, and they do not succumb to as many comorbid issues like skin lesions. Based on the reviewer's suggestion we now provide new human data stratifying by sex. We find that moderate-to-vigorous physical activity reduces non-*DNMT3A* CH, but not *DNMT3A* CH, in both men and women (**Extended Data Figure 1b**).



Extended Data Figure 1b. Meta-analysis of the association of moderate-to-vigorous physical activity with the prevalence of *DNMT3A* CH and non-*DNMT3A CH*, split by sex.

The mouse experiments were only done once, which is not the scientific standard**.**

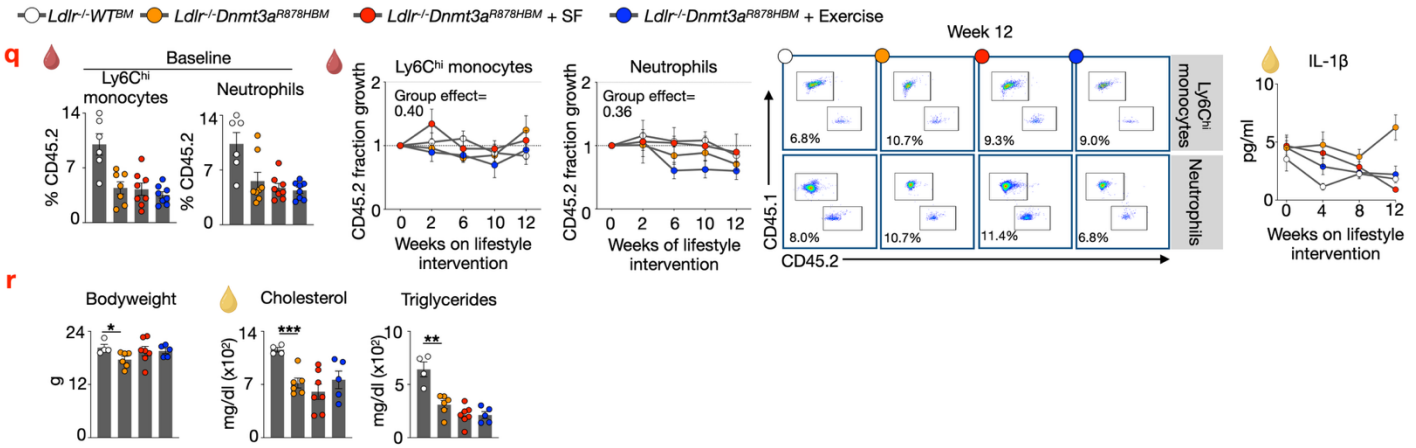
To clarify, all mouse experiments were repeated at least two to three times with large, predefined group sizes determined by power calculations for appropriate statistical inference. This ensures the robustness and reproducibility of our findings and enables rigorous statistical comparisons. This has been clarified in the methods. Please see the updated murine statistics section.

Uneven numbers of mice were used in the various groups, which biases the results to show differences in the larger groups.

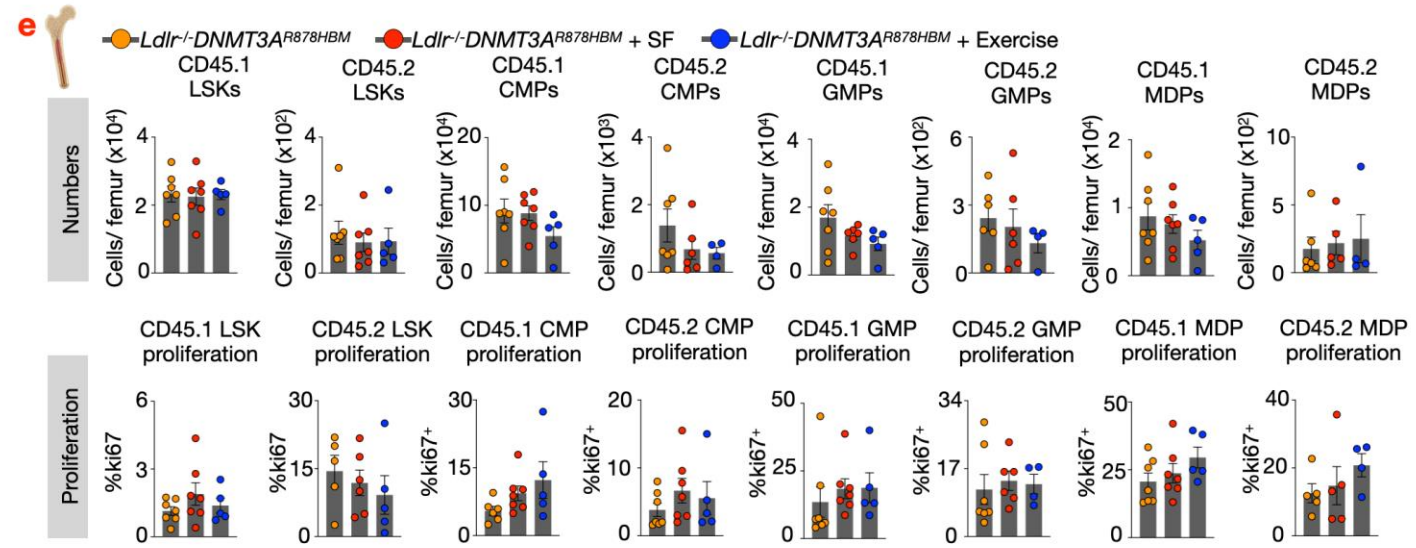
Groups contain uneven numbers of mice for several scientifically and experimentally justified reasons. First, most of our mice were derived from in-house breeding which generates uneven numbers of mice that are randomized to interventions. Second, as expected in BM transplant experiments, some mice do not survive the BMT procedure before randomization to lifestyle intervention. Finally, some experimental readouts necessitate larger group sizes (like atherosclerosis quantification) while other cellular and molecular analyses can be conducted with smaller group sizes. Importantly, unequal group sizes do not introduce systematic bias in estimating group differences when appropriate statistical methods are used. Accordingly, we employed statistical tests that are robust to unequal sample sizes and non-normal distributions, including Welch's *t*-tests, non-parametric tests (Mann–Whitney U and Kruskal–Wallis), and mixed-effects models where appropriate. This has all been clarified in the revised statistical section.

No rationale is provided for the choice of Tet2, Jak2, and p53 for the murine experiments. The paper might be more logical if the human epidemiologic data are presented first and then the mouse studies are presented next, as the human data can used as rationale for the choice of these mutations.

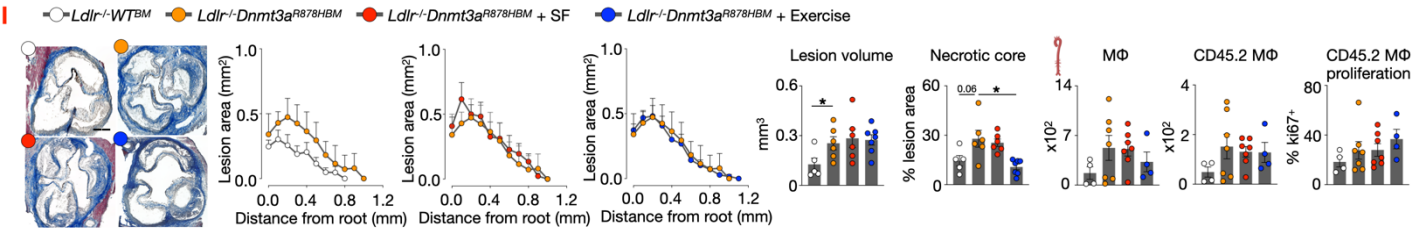
This is a fantastic suggestion that we have implemented. The human data now appear at the beginning of **Figure 1** with our mouse experimental data following. In our human data we find that physical activity does not influence the incidence of *Dnmt3a* CH but does reduce non-Dnmt3a CH. Thus, these human data justify our focus on CH driven by genes other than Dnmt3a and thus we focused on three common non-*Dnmt3a* mutations in *Jak2*, *Tet2*, and *p53*. Nevertheless, we performed additional new experiments analyzing the effects of exercise and SF on Dnmt3a CH in mice and these new data are included in the revised manuscript. In agreement with our human findings, we discovered that in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice, clone expansion and atherosclerosis are not impacted by SF or exercise. These new data are in **Extended Data Figures 2q-r, 4e, and 6l**.



Extended Data Figure 2. q. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$ Dnmt3a^{R878HBM}* mice at baseline (n= 6 wt, 7-8 sed, 8 SF, 8 exercise). Analysis of CD45.2 Ly6C^{hi} monocyte and CD45.2 neutrophil fraction growth in *Ldlr$^{-/-}$WT^{BM}* or *Ldlr$^{-/-}$ Dnmt3a^{R878HBM}* mice undergoing lifestyle intervention (n= 6 wt, 10 sed, 7 SF, 8 exercise) with representative flow cytometry plots. Time course of plasma IL-1β levels (n= 6 wt, 11 sed, 8 SF, 8 exercise). **r** Body weight and plasma lipid levels in *Ldlr$^{-/-}$WT^{BM}* and *Ldlr$^{-/-}$ Dnmt3a^{R878HBM}* mice exposed to lifestyle at the time of euthanasia (4, 4 wt; 6, 6 sed; 7, 7, SF and 5, 5 exercise).



Extended Data Figure 4e. Cell counts of CD45.2 mutant or CD45.1 wild-type LSKs, CMPs, GMPs, and MDPs in *Ldlr$^{-/-}$Dnmt3A^{R878HBM}* (**e**) mice. Proliferation of cell populations, quantified by the proportion of ki67-positive cells using flow cytometry.



Extended Data Figure 6. l. Lesion size, volume and necrotic core area (n= 5 wt, 6 sed, 6 SF, 7 exercise) with representative images of aortic roots stained with Masson's Trichrome stain, quantification of total and CD45.2 aortic macrophage number, and CD45.2 macrophage proliferation in *Ldlr$^{-/-}$Dnmt3a^{R878HBM}* and *Ldlr$^{-/-}$WT^{BM}* control mice at the time of euthanasia (n = 4 wt, 7 sed, 7 SF, 4 exercise); scale bar=200 µm.

Gene specific effects of environment on CH have been studied in other contexts (reviewed in Florez Cell Stem Cell) and should be cited.

Thank you, we have cited this paper.

The authors claim that: “Consistent with these dynamics, BM IL-1β and IL-6 were elevated in Ldlr-/-Jak2V617FBM mice exposed to SF and tended to be lowered by exercise (Fig 2c).” However the data are not significant and therefore the data do not support this conclusion. This again points to the question of whether other cytokines or other factors are being affected.

Thank you for raising this important point. We agree that the wording in the original text could be interpreted as implying a statistically significant effect of exercise on bone marrow IL-1β and IL-6 levels, which is not supported by the data. We have revised the text to clarify that, in *Jak2* CH mice, sleep fragmentation induces a statistically significant increase in bone marrow IL-1β and IL-6, whereas the reduction observed with exercise does not reach statistical significance and is therefore described as a trend. However, these cytokines are powerful and even minimal changes can exert profound biological influence.

Given the modest magnitude of the cytokine changes, we agree that alternative mediators could also contribute. To remain focused and avoid overinterpretation, we have moved the IL-6 data to the Extended Data Figures and centered our mechanistic analysis on IL-1β. Supporting this focus, we now show that mutant progenitors are selectively sensitized to IL-1β signaling, with IL-1β response scores increased in CD45.2 CMP/GMP populations under sleep fragmentation and reduced by exercise (**Fig. 2n**). Moreover, functional relevance is demonstrated by the finding that IL-1β neutralization completely abrogates sleep-fragmentation–induced clonal expansion (**Fig. 2r–t**). Together, these data indicate that IL-1β signaling is biologically important in this context.

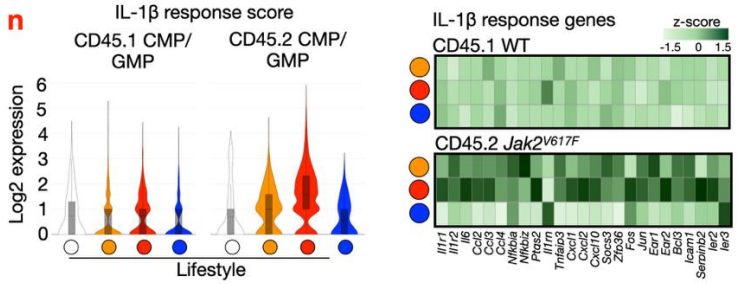


Figure 2. n. IL-1β response scores for CD45.1 and CD45.2 CMP/GMPs. Response scores represent composite Log2 expression of *Il1r1, Il1r2, Il6, Tnf, Ccl2, Ccl3, Ccl4, Nfkbia, Nfkbiz, Ptgs2,* and *Il1rn.* Heatmap of *IL1r1*, *IL1r2* and select IL-1β response genes in CD45.1 WT and *JAK2*^{V617F} mutant myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition.

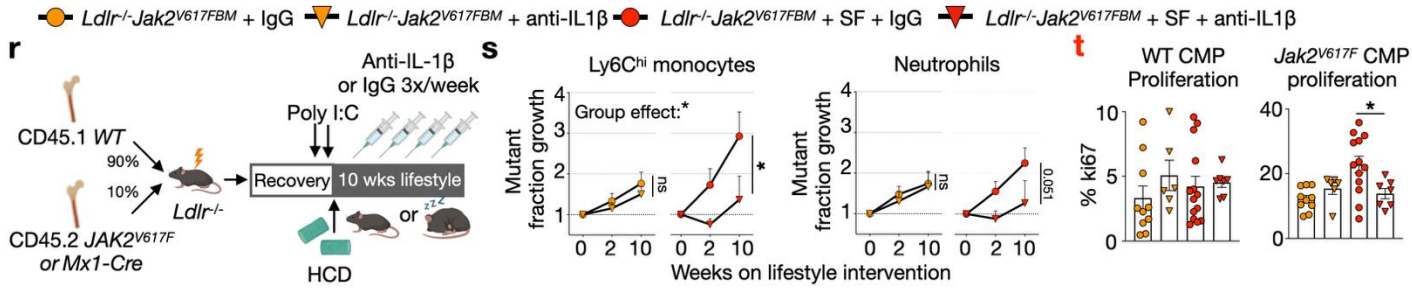


Figure 2. r. Schematic of experimental design. **s.** Expansion of blood *Jak2*^{V617F} Ly6C^{hi}monocytes (n=19,6,16,10 for Sedentary+IgG, Sedentary+anti-IL-1β, SF+IgG and SF+anti-IL-1β, respectively) and neutrophils (n=19,7,16,9; same groups) relative to baseline. **t.** Proliferation of WT CMPs (n=10,6,14,7; groups as in (**s**)) and *Jak2*^{V617F} CMPs (n=10,6,13,7; groups as in (**s**)).

For the human data, the authors should do a subset analysis of the association between exercise and CH by mutation type. Or an association of the combined people with Tet2 and Jak2 CH and exercise.

We agree that stratifying human analyses by mutation type would conceptually align with our murine models. However, in both the UK Biobank and All of Us cohorts, the number of participants carrying *TET2*, *JAK2*, or *TP53* mutations with available MVPA data was very small (see Table below), precluding adequately

powered analyses for any specific genotype. To address the reviewer's point, we did conduct exploratory mutation-specific analyses. Point estimates for *TET2*, *TP53*, and *JAK2* carriers were all below 1, suggesting a similar inverse association between physical activity and CH prevalence as observed for the Non-*DNMT3A* group, but with wide confidence intervals due to limited power. We therefore retained the *DNMT3A* vs. non-*DNMT3A* stratification for the main analysis, which maximizes statistical power and is consistent with prior large-scale CHIP studies (PMID:38454125).

Table.

CH mutation	UKB	AoU
Any	2638	302
DNMT3A	1717	153
TET2	402	49
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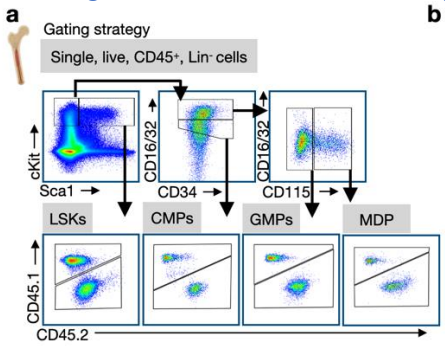
BCORL1	3	<21
CREBBP	12	<21
SRSF2	41	<21
ASXL1	253	<21
PPM1D	58	<21
GNAS	13	<21

How were CMPs and GMPs defined? How did the authors account for Sca shifts induced by inflammation that influence the definition of these populations?

Our BM gating strategy is provided in **Extended Data Figure 4a**. While we can not preclude shifts in Sca, this effect was not observed in our mouse models. For example, Sca MFI in the progenitor populations was unchanged in all of our models, only the abundance of these cells was impacted.



Extended Data Figure 4a. Flow cytometry gating strategy to identify CD45.2 mutant or control and CD45.1 wild-type progenitor populations in bone marrow.

The differences in IL1 levels with exercise or SF were 1-3 ng/mL --- how were these levels measured? Most ELISA and bead array methods do not measure cytokines at these levels at a sufficient level of precision to detect such small differences. Such small differences are unlikely to be physiologically relevant. Can the authors confirm IL1b or IL6 regulated genes were different between the groups?

Thank you for this important point. We used the MHSLB00 kit from R&D Systems which has a detection range of 0.8-50pg/ml and a sensitivity of 0.312 pg/ml. All BM fluid samples were diluted approximately 100 fold so that values fall within this range. The differences in IL-1β levels are physiologically relevant. This is demonstrated by our anti-IL1β experiments. IL-1β blockade, completely abrogates the SF induced acceleration of *Jak2*^{V617F} clone expansion and atherogenesis in the *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice. These findings demonstrate the importance of the changes in this powerful and impactful cytokine. Further, we conducted additional mechanistic analyses and in the updated manuscript reveal a new signaling mechanism for IL-1β in the BM: We evaluated the signals that mediate lifestyle-regulated *Jak2*^{V617F} progenitor proliferation and expansion. IL-1β response genes, including *Il1r1* and *IL1r2*, were elevated by SF and repressed by exercise in *Jak2*^{V617F}

CMPs and GMPs but not in their WT counterparts (**Fig 2n and Extended Data Fig 5e**). To identify the source of IL-1β in the BM, we measured pro-IL-1β among all BM hematopoietic cells, and found that BM macrophages were the highest producers (**Fig 2o and Extended Data Fig 5f**). *Jak2^{V617F}* BM macrophages produced more pro-IL-1β relative to WT cells (**Fig 2p**) and SF increased while exercise decreased pro-IL1β producing *Jak2^{V617F}* BM macrophages (**Fig 2q**). To test if IL-1β preferentially promotes the proliferation of *Jak2^{V617F}* progenitors, we injected IL-1β protein into WT mice that had been irradiated and reconstituted with 10% CD45.2 *Jak2^{V617F}* BM and 90% CD45.1 WT BM. While IL-1β induced WT BM CMP proliferation, the proliferative increase among *Jak2^{V617F}* CMPs was greater (**Extended Data Fig 5g**), demonstrating enhanced responsiveness. Finally, to directly test if SF expedites *Jak2^{V617F}* clone expansion via IL-1β, we exposed *Ldlr$^{-/-}$ Jak2^{V617FBM}* mice to either antibody-mediated IL-1β blockade or IgG control throughout sedentary and SF lifestyles (**Fig 2r**). Anti-IL-1β treatment did not influence body weight or plasma lipid levels (**Extended Data Fig 5h**). We quantified clone expansion in the blood and discovered that in sedentary *Ldlr$^{-/-}$Jak2^{V617FBM}* mice, IL-1β blockade did not alter the expansion of *Jak2^{V617F}* monocytes and neutrophils (**Fig 2s**). In *Ldlr$^{-/-}$Jak2^{V617FBM}* mice exposed to SF, meanwhile, IL-1β blockade strongly diminished SF-induced expedited expansion of *Jak2^{V617F}* clones (**Fig 2s**). In the BM, IL-1β blockade did not alter WT or *Jak2^{V617F}* CMP proliferation in sedentary mice, but it repressed the proliferation of *Jak2^{V617F}*, but not WT, CMPs in SF animals (**Fig 2t and Extended Data Fig 5i**). Altogether, these findings show that sleep and exercise selectively reprogram *Jak2^{V617F}* myeloid progenitor cells towards proliferative and metabolically active phenotypes by modulating the abundance of IL-1β-producing *Jak2^{V617F}* BM macrophages and the IL-1β sensitivity of *Jak2^{V617F}* progenitors.

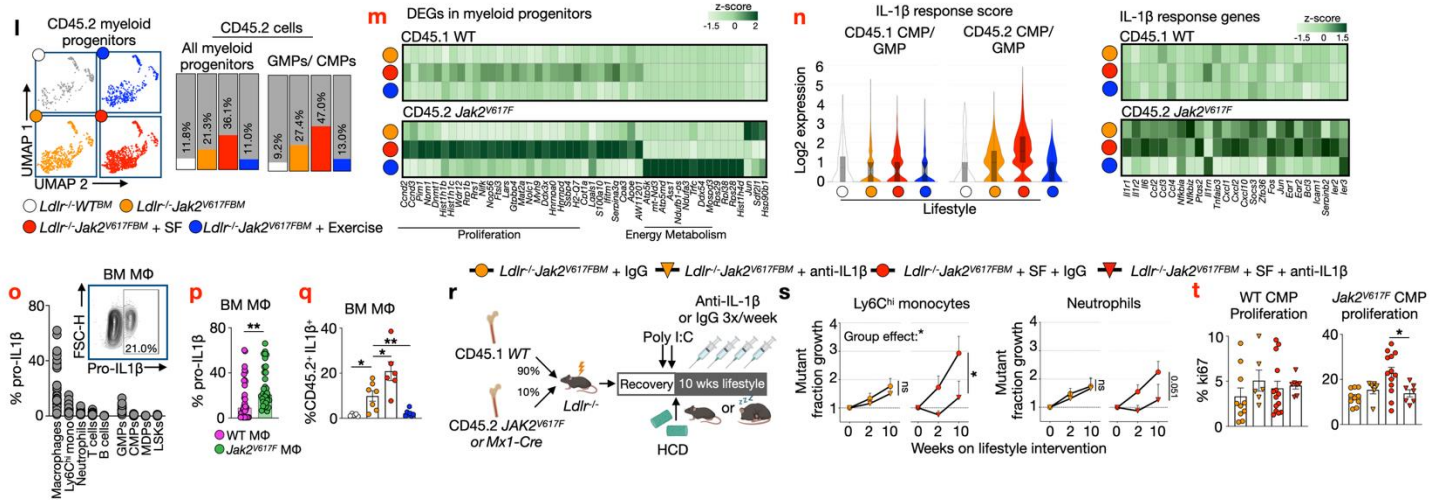
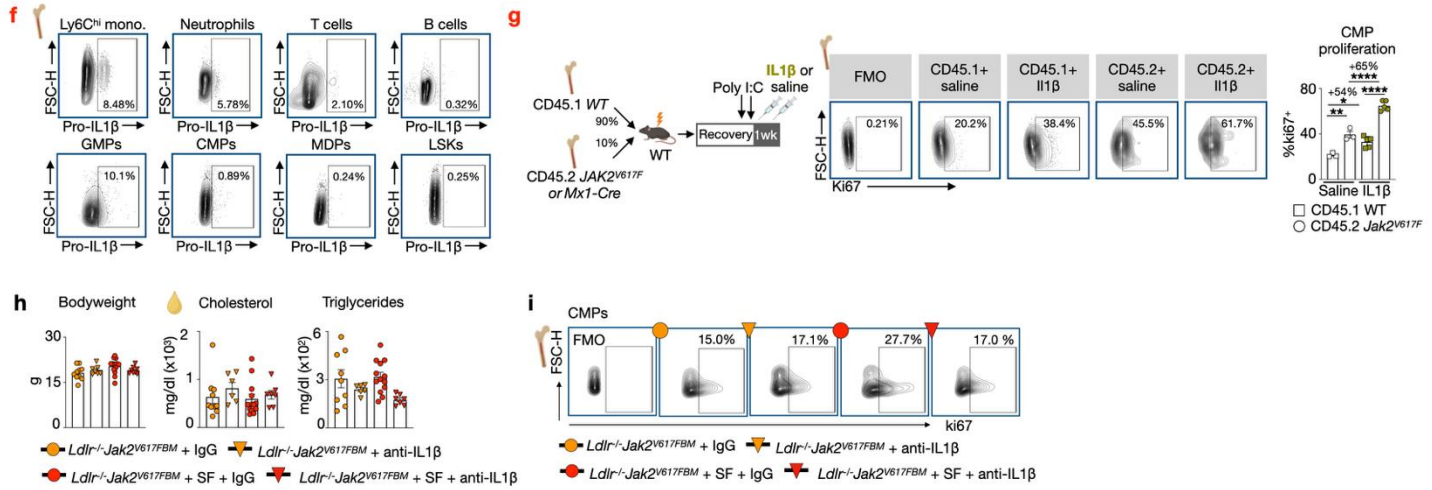


Figure 2. l. UMAP of CD45.2 myeloid progenitors split by experimental group and proportion of CD45.2^{+} cells within the indicated populations. **m**. Heatmap of all genes significantly upregulated in *JAK2^{V617F}* myeloid progenitors for Sed, SF and exercise across CD45.1 WT and *JAK2^{V617F}* myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition. **n**. IL-1β response scores for CD45.1 and CD45.2 CMP/GMPs. Response scores represent composite Log2 expression of *Il1r1, Il1r2, Il6, Tnf, Ccl2, Ccl3, Ccl4, Nfkbia, Nfkbiz, Ptgs2,* and *Il1rn.* Heatmap of *IL1r1*, *IL1r2* and select IL-1β response genes in CD45.1 WT and *JAK2^{V617F}* mutant myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition. **o.** pro-IL-1β expression measured by flow cytometry across different hematopoietic cell types with representative flow plot in *Ldlr$^{-/-}$Jak2^{V617FBM}* chimeras (n=36 mature cell types, n=20 progenitors). **p.** pro-IL-1β expression in CD45.1 WT and CD45.2 *Ldlr$^{-/-}$Jak2^{V617FBM}* BM-macrophages (n=35)**. q.** pro-IL-1β expression in CD45.2 BM-macrophages by lifestyle group (n=5,7,6,7; groups as in (**a**)). **r.** Schematic of experimental design. **s.** Expansion of blood *Jak2^{V617F}* Ly6C^{hi}monocytes (n=19,6,16,10 for Sedentary+IgG, Sedentary+anti-IL-1β, SF+IgG and SF+anti-IL-1β, respectively) and neutrophils (n=19,7,16,9; same groups) relative to baseline. **t.** Proliferation of WT CMPs (n=10,6,14,7; groups as in (**s**)) and *Jak2^{V617F}* CMPs (n=10,6,13,7; groups as in (**s**)).



Extended Data Figure 5. f. Representative flow cytometry plots showing pro-IL-1β expression in various cell types from the BM of *Ldlr*$^{-/-}$ *Jak2*^{V617FBM} mice. **g.** Experimental setup, and representative flow cytometry plots showing ki67 expression in bone marrow CMPs in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} with and without SF, with IgG or anti-IL-1β as well as quantification of %ki67 positivity in CMPs. **h.** Bodyweight, plasma cholesterol, and plasma triglyceride levels in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β at the time of euthanasia. n= 10, 10, 9 no SF + IgG; 7, 6, 6 no SF + anti-IL-1β; 13, 13, 13 SF + IgG and 8, 7, 7 SF + anti-IL-1β for bodyweight, cholesterol and triglycerides, respectively. **i.** Flow cytometry plots for ki67 in CMPs from *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β.

Does KO or inhibition of CLEC4A rescue the expansion of Jak2 or Tet2 mutant clones in the setting of SF? This is an astute question, and we conducted the experiment to answer it. To directly test if CLEC4E-IL-1β signaling is a causal mechanism by which SF accelerates *Jak2*^{V617F}-driven atherosclerosis, we exposed sedentary and SF *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice to either antibody-mediated CLEC4E blockade or IgG control throughout atherogenesis (**Fig 4i**). Anti-CLEC4E treatment did not influence body weight or plasma lipid levels (**Extended Data Fig 9e**) nor alter *Jak2*^{V617F} clone expansion in the blood or CMP proliferation in the BM of sedentary and SF *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice (**Fig 4j**). In the aortic root, however, anti-CLEC4E completely abrogated accelerated atherogenesis and necrotic core formation in SF mice, but did not influence lesion size in sedentary mice (**Fig 4k-l**), demonstrating atherogenic regulation independent of peripheral clonal dynamics. We then evaluated genotype-dependent IL-1β generation by aortic macrophages and found that CLEC4E blockade did not alter pro-IL1β in WT macrophages but repressed the cytokine in *Jak2*^{V617F} macrophages of SF but not sedentary mice (**Fig 4m**). Immunostaining of aortic root cross sections revealed that CLEC4E blockade diminished IL-1β, NLRP3, and AIM2 most strongly or solely in the CD45.2 *Jak2*^{V617F} lesional macrophages of SF mice (**Fig 4n-p**).

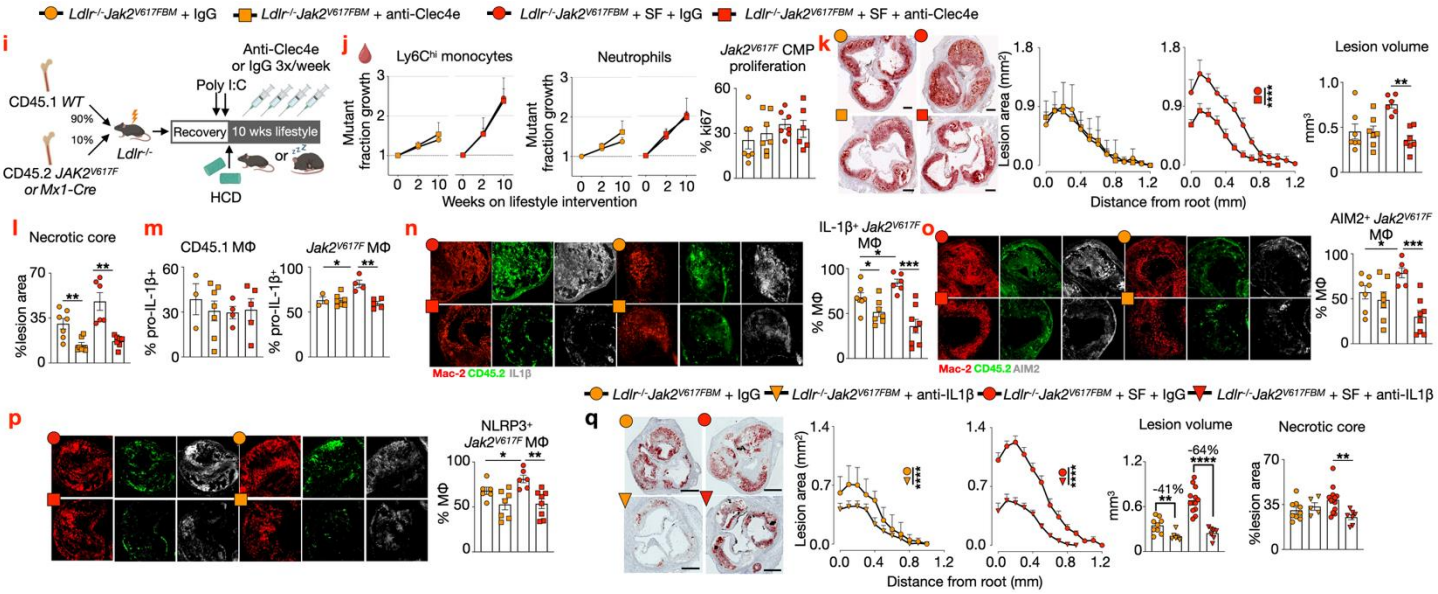


Figure 4. i. Experimental outline. **j**. Expansion of *Jak2*^{V617F} blood Ly6C^{hi} monocytes (n=6,7,5,8 for Sedentary+IgG, Sedentary+anti-Clec4e, SF+IgG, SF+anti-Clec4e) and neutrophils (n= 6,7,5,7) relative to baseline. Proliferation of *Jak2*^{V617F} CMPs (n=7,7,7,6; same groups) **k**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size and volume (both n=7,7,6,7; groups as in (**j**)). Scale bars are 200μm. **l.** Proportion of the necrotic core relative to plaque area (n= 7,7,6,7; groups as in (**j**)). **m.** pro-IL1β expression in CD45.1 WT and *Jak2*^{V617F} macrophages (both n=3,7,4,5; groups as in (**j**)) by flow cytometry. **n.** MAC-2, CD45.2, and IL-1β immunofluorescence and quantification of CD45.2, and IL-1β pixel overlap (n=7, 7,6,7, groups as in (**j**)). Scale bar=100 μm **o.** MAC-2, CD45.2, and AIM2 immunofluorescence and quantification of CD45.2 and AIM2 pixel overlap (n=7,7,6,7; groups as in (**j**)). Scale bar=100 μm. **p.** MAC-2, CD45.2, and NLRP3 immunofluorescence and quantification of CD45.2 and NLRP3 pixel overlap (n=6,7,6,7; groups as in (**j**)). Scale bar=100 μm**. q**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size (n=10, 6, 13, 7) and volume (n=9, 6, 13, 7) in sequential sections and proportion of the necrotic core (n=9, 5, 13, 7) relative to plaque area; groups as in (**j**).

The authors did not fully address the question of whether expansion of macrophages versus reprogramming of macrophages is critical for CH expansion and progression of atherosclerosis. They imply that reversing the effect by reversing the reprogramming works; however what happens if mutant macrophage cell numbers are controlled – is this sufficient to abrogate atherosclerosis exacerbation?

This is a critical point. We have conducted several new analyses and experiments to answer this question. In two major experiments (anti-Clec4e and ADRB2 antagonism), we demonstrate that distinct pharmacological interventions do not alter peripheral clonal dynamics including the modulated rate of Jak2^{V617F} clone expansion or BM mutant progenitor proliferation, but reprogram the inflammatory status of mutant aortic macrophages and alter atherosclerosis. Thus, we demonstrate atherosclerosis modulation WITHOUT impacting peripheral clone dynamics in the blood or BM, pointing to a critical role for lifestyle in influencing local macrophage programming in atherosclerotic lesions.

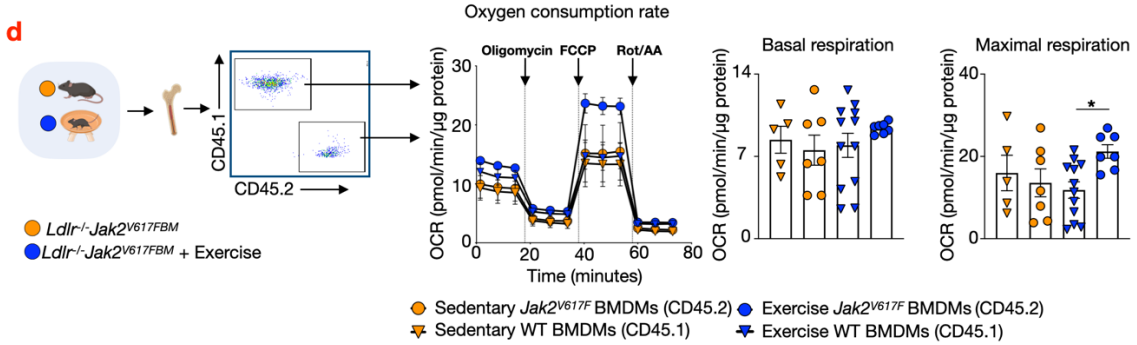
First, we blocked CLEC4E in Jak2 CH mice and discovered that this intervention did not alter Jak2V617F clone expansion or mutant progenitor proliferation in the BM in sedentary and SF mice. Anti-CLEC4E therapy, however, abrogated CD45.2 mutant macrophage IL-1B, NLRP3, and AIM2 and reverted the SF-induced accelerated atherosclerosis in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice. This new experiment, detailed in **Figure 4 (**and in the above comment**)**, allowed us to dissociate peripheral clonal dynamics in the BM and blood from SF-induced macrophage reprogramming locally in the lesions, demonstrating that the latter is a critical and sufficient factor to modify atherosclerosis in CH.

Likewise, in *Ldlr*$^{-/-}$*Jak2V*^{617FBM} mice exposed to exercise, ADRB2 antagonism did not influence peripheral clonal dynamics and mutant cell abundance and progenitor proliferation in the BM were unaffected by ADRB2 antagonism. However, ADRB2 antagonism completely reverted the aorta macrophage anti-inflammatory and anti-atherogenic effects of exercise in *Ldlr*$^{-/-}$*Jak2V*^{617FBM} mice. Again, these findings in **Figure 5** demonstrate our ability to disassociate peripheral clonal expansion trajectories from local macrophage programming in the lesion. Indeed, lifestyle-induced mutant aortic macrophage reprogramming is sufficient to alter atherosclerosis in CH while preserving peripheral clone dynamics.

Salmeterol appeared to be sufficient to replicate the b2-agonist effects of exercise. Do patients with asthma and frequent use of salmeterol or albuterol experience protective effects against CH-associated atherosclerosis?
This is a fantastic question and worth answering in a follow-up study. We reviewed the FDA manual for Salmeterol(https://www.accessdata.fda.gov/drugsatfda_docs/label/2003/20236slr028_serevent_lbl.pdf) which notes that “Because of the small therapeutic dose, systemic levels of salmeterol are low or undetectable after inhalation of recommended doses (42 mcg of salmeterol inhalation aerosol twice daily). Following chronic administration of an inhaled dose of 42 mcg twice daily, salmeterol was detected in plasma within 5 to 10 minutes in 6 asthmatic patients; plasma concentrations were very low, with peak concentrations of 150 pg/mL and no accumulation with repeated dose.” Therefore, it is uncertain if Salmeterol will be sufficient to modify CH-associated atherosclerosis, but this idea is worth testing in future studies.

Minor comments:
- In the methods, the killing of mice should be described as “euthanasia” not “sacrifice”
Thank you, this has been changed.

- Seahorse data provided in Extended Data Figure 4g should be quantified.
Thank you, we have now provided the quantification of these data in **Extended Data Figure 5d**.



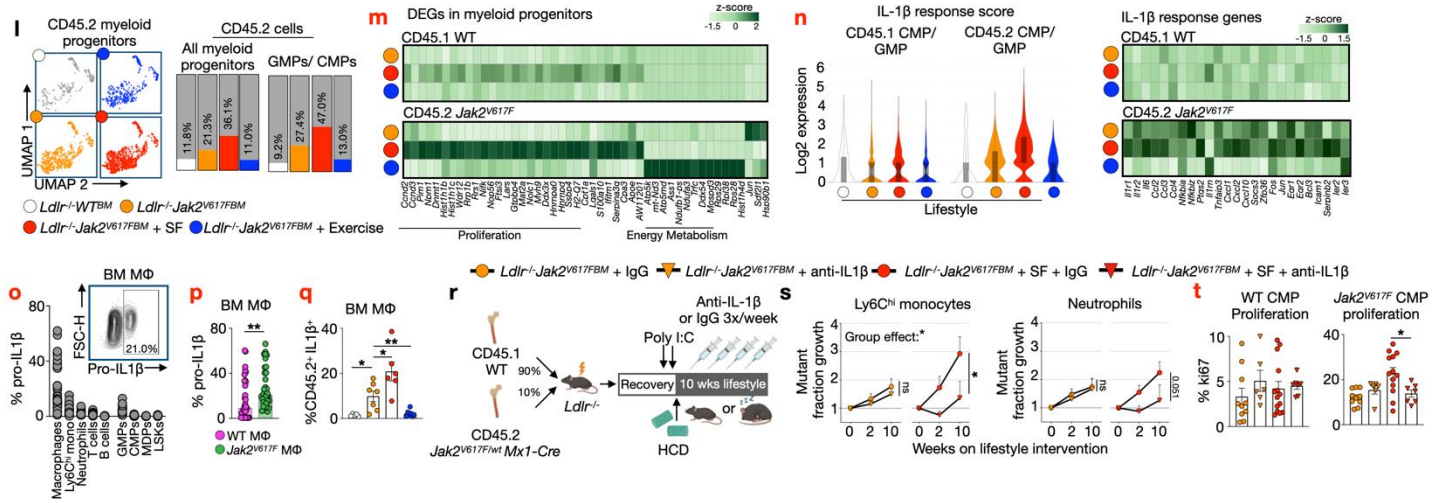
Extended Data Figure 5d. Experimental setup and mitochondrial respiration marked by OCR in BMDMs isolated from *Ldlr*$^{-/-}$*Jak2*^{V617FBM} *mice* and sorted into mutant CD45.2 and non-mutant CD45.1 groups; n = 8 and 6 technical replicates for the sedentary CD45.1 and CD45.2 groups; n = 12 and 8 technical replicates for the CD45.1 and CD45.2 exercise groups.

Referee #4

Gerhardt et al. investigated the mutation-dependent effects of the lifestyle interventions sleep and exercise on clonal hematopoiesis and further examined how these interventions influence atherosclerosis and its underlying mechanisms. Through a combination of bone marrow transplantation experiments, single-cell RNA sequencing, in vivo chemical delivery, and physiological interventions—including sleep fragmentation and exercise—the authors demonstrated that clonal hematopoiesis can be exacerbated or mitigated in vivo depending on lifestyle intervention. They also leveraged large human cohort datasets, including the UK Biobank and All of Us Research Program, to propose that physical activity may attenuate non-DNMT3A-associated clonal hematopoiesis. The study addresses an important area of biological research, is thorough, and an impressive technical tour de force. The findings showing that exercise modulates clonal hematopoiesis (CH) and macrophage function via norepinephrine-mediated signaling is intriguing and a major scientific advance for the field. The role of sleep in promoting CH and influencing atherosclerosis had already been established by this same group in prior work (McAlpine et al., Nature, 2019; Yates et al., Nature Communications, 2025). Moreover, considering the literature that has identified IL-1β and inflammasome activation as central drivers of atherosclerosis, the current study's contribution to advancing our mechanistic understanding of the link between sleep and atherosclerosis may be somewhat incremental and needs to be better defined by the authors.
Thank you for the insightful and helpful comments. We have addressed each comment individually.

Overall, this study demonstrates that sleep fragmentation and exercise do not affect wild-type hematopoiesis but selectively modulate hematopoiesis derived from mutant myeloid progenitors, including Jak2 and Tet2 mutants. These are important finds but the mechanisms by which sleep and exercise exert mutation-specific regulation of clonal hematopoiesis is not fully explored at this stage.

Thank you for raising this important point. We have performed numerous new experiments to delineate the specific mechanisms by which lifestyle regulates CH. Our data reveal a new and novel BM macrophage-HSC signaling mechanism. First, we performed a deeper analysis of Jak2V617F and WT BM HSC transcriptional programming. SF- and exercise-induced transcriptional reprogramming was specific to *Jak2*^{V617F} mutant cells because neighboring WT CD45.1 myeloid progenitor cells, existing in the same BM, were largely unaffected by lifestyle as their proliferative and metabolic programming were unchanged (**Fig 2m and Extended Data Fig 5b**). We then evaluated the signals that mediate lifestyle-regulated *Jak2*^{V617F} progenitor proliferation and expansion. IL-1β response genes, including *Il1r1* and *IL1r2*, were elevated by SF and repressed by exercise in *Jak2*^{V617F} CMPs and GMPs but not in their WT counterparts (**Fig 2n and Extended Data Fig 5e**). To identify the source of IL-1β in the BM, we measured pro-IL-1β among all BM hematopoietic cells, and found that BM macrophages were the highest producers (**Fig 2o and Extended Data Fig 5f**). *Jak2*^{V617F} BM macrophages produced more pro-IL-1β relative to WT cells (**Fig 2p**) and SF increased while exercise decreased pro-IL1β producing *Jak2*^{V617F} BM macrophages (**Fig 2q**). To test if IL-1β preferentially promotes the proliferation of *Jak2*^{V617F} progenitors, we injected IL-1β protein into WT mice that had been irradiated and reconstituted with 10% CD45.2 *Jak2*^{V617F} BM and 90% CD45.1 WT BM. While IL-1β induced WT BM CMP proliferation, the proliferative increase among *Jak2*^{V617F} CMPs was greater (**Extended Data Fig 5g**). Finally, to directly test if SF expedites *Jak2*^{V617F} clone expansion via IL-1β, we exposed *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice to either antibody-mediated IL-1β blockade or IgG control throughout sedentary and SF lifestyles (**Fig 2r**). Anti-IL-1β treatment did not influence body weight or plasma lipid levels (**Extended Data Fig 5h**). We quantified clone expansion in the blood and discovered that in sedentary *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice, IL-1β blockade did not alter the expansion of *Jak2*^{V617F} monocytes and neutrophils (**Fig 2r**). In *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to SF, meanwhile, IL-1β blockade strongly diminished SF-induced expedited expansion of *Jak2*^{V617F} clones (**Fig 2r**). In the BM, IL-1β blockade did not alter WT or *Jak2*^{V617F} CMP proliferation in sedentary mice, but it repressed the proliferation of *Jak2*^{V617F}, but not WT, CMPs in SF animals (**Fig 2s and Extended Data Fig 5i**). Altogether, these findings show that sleep and exercise selectively reprogram *Jak2*^{V617F} myeloid progenitor cells towards proliferative and metabolically active phenotypes by modulating the abundance of IL-1β-producing *Jak2*^{V617F} BM macrophages and the IL-1β sensitivity of *Jak2*^{V617F} progenitors.



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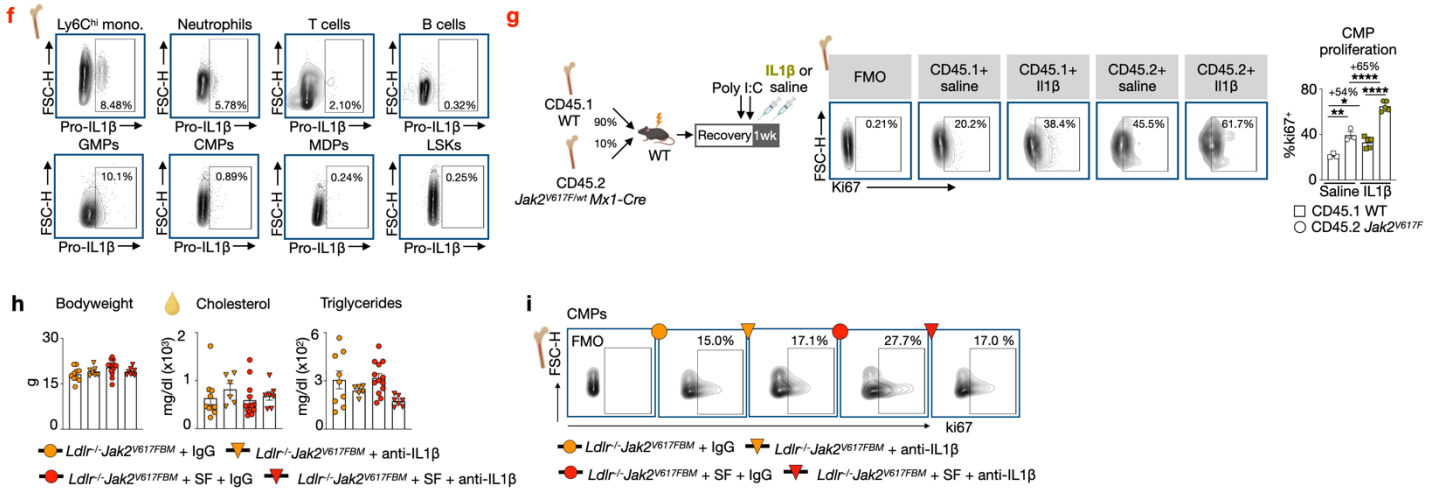
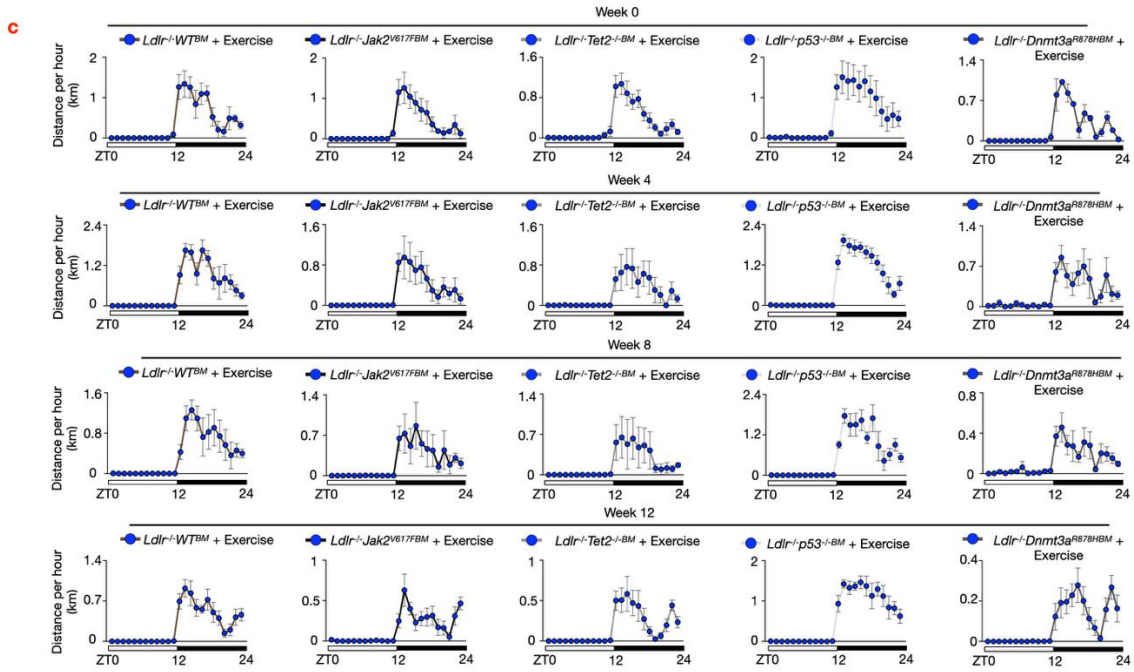
pro-IL-1β expression in CD45.2 BM-macrophages by lifestyle group (n=5,7,6,7; groups as in (**a**)). **r.** Schematic of experimental design. **s.** Expansion of blood *Jak2*^{V617F} Ly6C^{hi}monocytes (n=19,6,16,10 for Sedentary+IgG, Sedentary+anti-IL-1β, SF+IgG and SF+anti-IL-1β, respectively) and neutrophils (n=19,7,16,9; same groups) relative to baseline. **t.** Proliferation of WT CMPs (n=10,6,14,7; groups as in (**s**)) and *Jak2*^{V617F} CMPs (n=10,6,13,7; groups as in (**s**)).



Extended Data Figure 5. f. Representative flow cytometry plots showing pro-IL-1β expression in various cell types from the BM of *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice. **g.** Experimental setup, and representative flow cytometry plots showing ki67 expression in bone marrow CMPs in WT*Jak2*^{V617FBM} mice injected with saline or IL-1β, as well as quantification of %ki67 positivity in CMPs. **h.** Bodyweight, plasma cholesterol, and plasma triglyceride levels in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β at the time of euthanasia. n= 10, 10, 9 no SF + IgG; 7, 6, 6 no SF + anti-IL-1β; 13, 13, 13 SF + IgG and 8, 7, 7 SF + anti-IL-1β for bodyweight, cholesterol and triglycerides, respectively. **i.** Flow cytometry plots for ki67 in CMPs from *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice with and without SF, with IgG or anti-IL-1β.

The total amount of exercise performed by each mouse line/group over the 12- or 16- week training periods must be presented. The different strains and lines may have performed vastly different quantities of exercise, which could be a mechanism for differences between groups and treatments**.**

Thank you for raising this important point. We have performed the analysis suggested and quantified the amount of running by every mouse line throughout the duration of the 12 week experiment (**Extended Data Figure 1c**). We quantified voluntary wheel running activity at baseline (following transplantation, recovery, and mutation induction after one week of habituation) and after 4, 8, and 12 weeks. Mice ran almost exclusively during the dark cycle, with minimal activity during the light cycle. Importantly, all exercise groups, independent of genotype, maintained robust running activity throughout the study, confirming effective engagement in the exercise paradigm. Further, all analyses were performed as within-genotype comparisons (e.g. exercise vs. sedentary for each CH mouse model), ensuring that the observed effects reflect biological responses to exercise rather than any possible differences in total running distance between genotypes.



Extended Data Figure 1c. c. Quantification of distance run per hour over 24 hours by *Ldlr*$^{-/-}$*WT*^{BM}, *Ldlr*$^{-/-}$*Jak2*^{V617FBM}, *Ldlr*$^{-/-}$*Tet2*$^{-/-BM}$, *Ldlr*$^{-/-}$*p53*$^{-/-BM}$, and *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice undergoing exercise at baseline, 4, 8, and 12 weeks (n= 4, 4, 4, 4; 3, 4, 4, 4; 5, 5, 5, 5; 5, 5, 4, 5 and 2, 5, 7, 4 at baseline, 4, 8 and 12 weeks, respectively).

In Figure 1, the use of UK Biobank and All of Us datasets to examine associations between physical activity and DNMT3A vs. non-DNMT3A clonal hematopoiesis is intriguing. However, since the mutant models employed in this study are Tet2, Jak2, and p53, it would be more appropriate and aligned with the paper's main focus to stratify the human cohort analyses based on these specific mutations.

We agree and have performed several new analyses and experiments to address this point. Unfortunately, in both the UK Biobank and All of Us cohorts, the number of participants carrying *TET2*, *JAK2*, or *TP53* mutations with available MVPA data was very small (see table below), precluding adequately powered analyses for any specific genotype. To address the reviewer's point, we did conduct exploratory mutation-specific analyses. Point estimates for *TET2*, *TP53*, and *JAK2* carriers were all below 1, suggesting a similar inverse association between physical activity and CHIP prevalence as observed for the non-*DNMT3A* group, but with wide confidence intervals due to limited power. We therefore retained the *DNMT3A* vs. non-*DNMT3A* stratification for the main analysis, which maximizes statistical power and is consistent with prior large-scale CHIP studies (PMID: 38454125).

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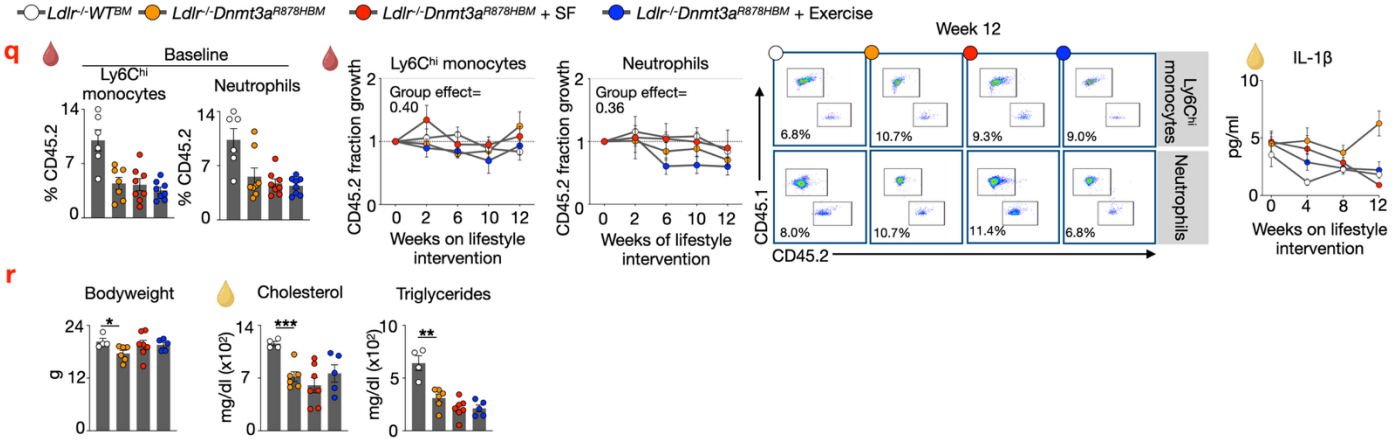
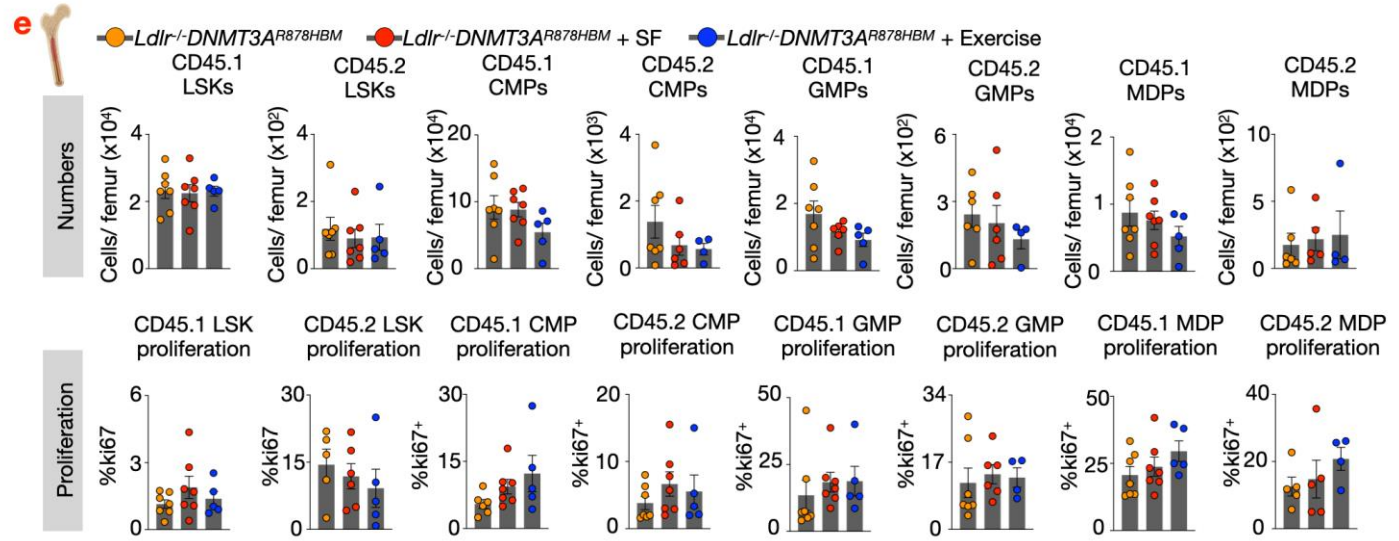
CREBBP	12	<21
SRSF2	41	<21
ASXL1	253	<21
PPM1D	58	<21
GNAS	13	<21

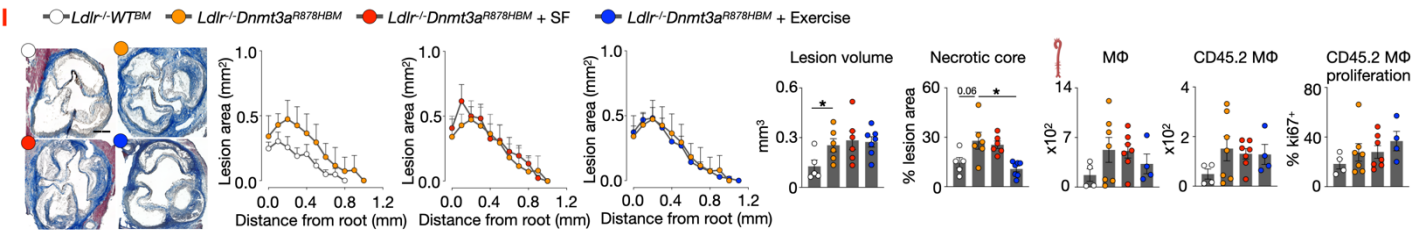
Second, our human analysis (now also stratified by sex) has been moved to the beginning of Figure 1 with the murine data following. Finally, to directly address *Dnmt3a* clonal hematopoiesis in our murine models, we generated in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice and subjected them to sedentary, SF, or exercise lifestyles. In agreement with our human data, we discovered that in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} mice, clone expansion and atherosclerosis are not impacted by SF or exercise. These new data are in **Extended Data Figures 2q-r, 4e, and 6l**.



Extended Data Figure 2 q. Engraftment frequencies of CD45.2 and CD45.1 Ly6C^{hi} monocytes and neutrophils in the blood of *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice at baseline (n= 6 wt, 7-8 sed, 8 SF, 8 exercise). Analysis of CD45.2 Ly6C^{hi} monocyte and CD45.2 neutrophil fraction growth in *Ldlr*$^{-/-}$*WT*^{BM} or *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice undergoing lifestyle intervention (n= 6 wt, 10 sed, 7 SF, 8 exercise) with representative flow cytometry plots. Time course of plasma IL-1β levels (n= 6 wt, 11 sed, 8 SF, 8 exercise). **r** Body weight and plasma lipid levels in *Ldlr*$^{-/-}$*WT*^{BM} and *Ldlr*$^{-/-}$ *Dnmt3a*^{R878HBM} mice exposed to lifestyle at the time of euthanasia (4, 4 wt; 6, 6 sed; 7, 7, SF and 5, 5 exercise).

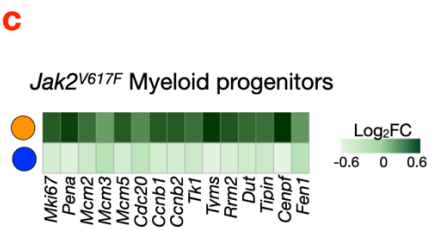


Extended Data Figure 4e. Cell counts of CD45.2 mutant or CD45.1 wild-type LSKs, CMPs, GMPs, and MDPs in *Ldlr*$^{-/-}$*Dnmt3A*^{R878HBM} (**e**) mice. Proliferation of cell populations, quantified by the proportion of ki67-positive cells using flow cytometry.



Extended Data Figure 6. l. Lesion size, volume and necrotic core area (n= 5 wt, 6 sed, 6 SF, 7 exercise) with representative images of aortic roots stained with Masson's Trichrome stain, quantification of total and CD45.2 aortic macrophage number, and CD45.2 macrophage proliferation in *Ldlr*$^{-/-}$*Dnmt3a*^{R878HBM} and *Ldlr*$^{-/-}$*WT*^{BM} control mice at the time of euthanasia (n = 4 wt, 7 sed, 7 SF, 4 exercise); scale bar=200 µm.

In Figure 2a, exercise reduced the proliferation of CD45.2-positive common myeloid progenitors (CMPs) and granulocyte macrophage progenitors (GMPs) in Ldlr−/− Jak2V617F bone marrow. However, in Figure 2m, no notable differences in proliferation markers were observed between the Ldlr−/−Jak2V617F and Ldlr−/−Jak2V617F+Exercise groups.
Does this suggest that exercise reduces proliferation in CMPs and GMPs without altering proliferation-related gene expression?
This is an important point. The heat map shown in **Fig. 2m** represents a selected subset of genes that were significantly upregulated in sleep-fragmentation relative to both sedentary and exercise groups, rather than a comprehensive panel of proliferation-associated genes. As such, the absence of a difference between the *Ldlr*$^{-/-}$*Jak2*^{V617FBM} and *Ldlr*$^{-/-}$*Jak2*^{V617FBM} + Exercise groups in **Fig. 2m** reflects the gene-selection strategy for that panel and does not imply a lack of transcriptional modulation of proliferation pathways by exercise. To clarify and directly address this, we conducted an expanded analysis of proliferation genes in the CD45.2-positive myeloid progenitors in *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice exposed to sedentary or exercise. This analysis revealed the downregulation of several proliferation genes in the mutant myeloid progenitors of exercised mice. These new data are in **Extended Data Figure 5c**. Together with the reduced proliferative activity observed at the cellular level (**Fig. 2a**), these findings suggest that exercise attenuates proliferation of mutant progenitor cells, although only a subset of proliferation-associated genes is transcriptionally regulated under these conditions.



Extended Data Figure 5c. Heat map of select proliferation-associated gene expression in *Jak2*^{V617F} myeloid progenitor cells from sedentary and exercised mice.

Furthermore, in CD45.1-derived cells, are the proliferation- and energy metabolism-related transcriptional changes observed in CD45.2 cells under sleep fragmentation or exercise conditions? A more in-depth scRNA-seq comparison between CD45.1 and CD45.2 populations would provide valuable insights into the mutation-dependent effects of sleep fragmentation and exercise.
Thank you for raising this. We have performed a more in-depth scRNAseq comparison between CD45.1 and CD45.2 cells in the BM of sedentary, SF, and exercised Jak2 CH mice. Strikingly, this new analysis reveals that the SF-induced proliferative programming and the exercise-induced metabolic programming is specific to CD45.2 mutant cells, whereas neighboring WT CD45.1 cells, residing in the same BM, have minimal responses. These new data are in **Figure 2m** and affirm the idea that mutant cells are selectively sensitive to lifestyle manipulations.

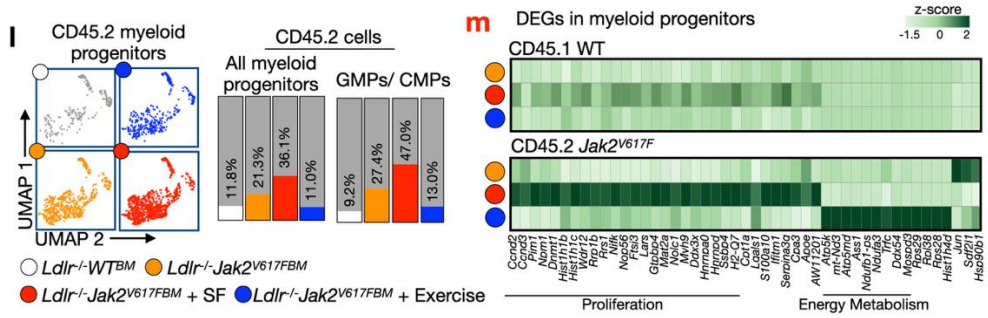


Figure 2. l. UMAP of CD45.2 myeloid progenitors split by experimental group and proportion of CD45.2^{+} cells within the indicated populations. **m**. Heatmap of all genes significantly upregulated in *JAK2*^{V617F} myeloid progenitors for Sed, SF and exercise across

CD45.1 WT and *JAK2*^{V617F} myeloid progenitors. Columns represent individual genes, expression levels were normalized by z-score and scaled by column. Rows correspond to lifestyle condition.

In Figure 4, the authors suggest that sleep fragmentation induces CLEC4E-dependent inflammasome activation, thereby promoting atherosclerosis, as predicted by single-cell RNA-seq and supported by in vitro treatment of BMDMs with a CLEC4E agonist, which increased Il-1β, Nlrp3, and Aim2 expression. However, agonist stimulation of BMDMs alone is insufficient to establish that CLEC4E mediates atherosclerosis in the sleep fragmentation context. It would be more convincing to treat Ldlr−/− Jak2V617F mice with the CLEC4E agonist and evaluate its impact on atherosclerosis progression in vivo.

Thank you, we fully agree and therefore performed the experiment suggested by the reviewer. To directly test if CLEC4E-IL-1β signaling is a causal mechanism by which SF accelerates *Jak2*^{V617F}-driven atherosclerosis, we exposed sedentary and SF *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice to either antibody-mediated CLEC4E blockade or IgG control throughout atherogenesis (**Fig 4i**). Anti- CLEC4E treatment did not influence body weight or plasma lipid levels (**Extended Data Fig 9e**) nor alter *Jak2*^{V617F} clone expansion in the blood and CMP proliferation in the BM of sedentary and SF *Ldlr*$^{-/-}$*Jak2*^{V617FBM} mice (**Fig 4j**). In the aortic root, however, anti- CLEC4E completely abrogated SF-induced accelerated atherogenesis and necrotic core formation but did not influence lesion size in sedentary mice (**Fig 4k-l**), demonstrating atherogenic regulation independent of peripheral clonal dynamics. We then evaluated genotype-dependent IL-1β generation by aortic macrophages and found that CLEC4E blockade did not alter pro-IL1β in WT macrophages but repressed the cytokine in *Jak2*^{V617F} macrophages of SF but not sedentary mice (**Fig 4m**). Immunostaining of aortic root cross sections revealed that CLEC4E blockade diminished IL-1β, NLRP3, and AIM2 in the CD45.2 *Jak2*^{V617F} lesional macrophages of SF mice (**Fig 4n-p**). Together with the in vitro data, these new in vivo data demonstrate the role of CLEC4E signaling in SF-mediated atherosclerosis in Jak2 CH.

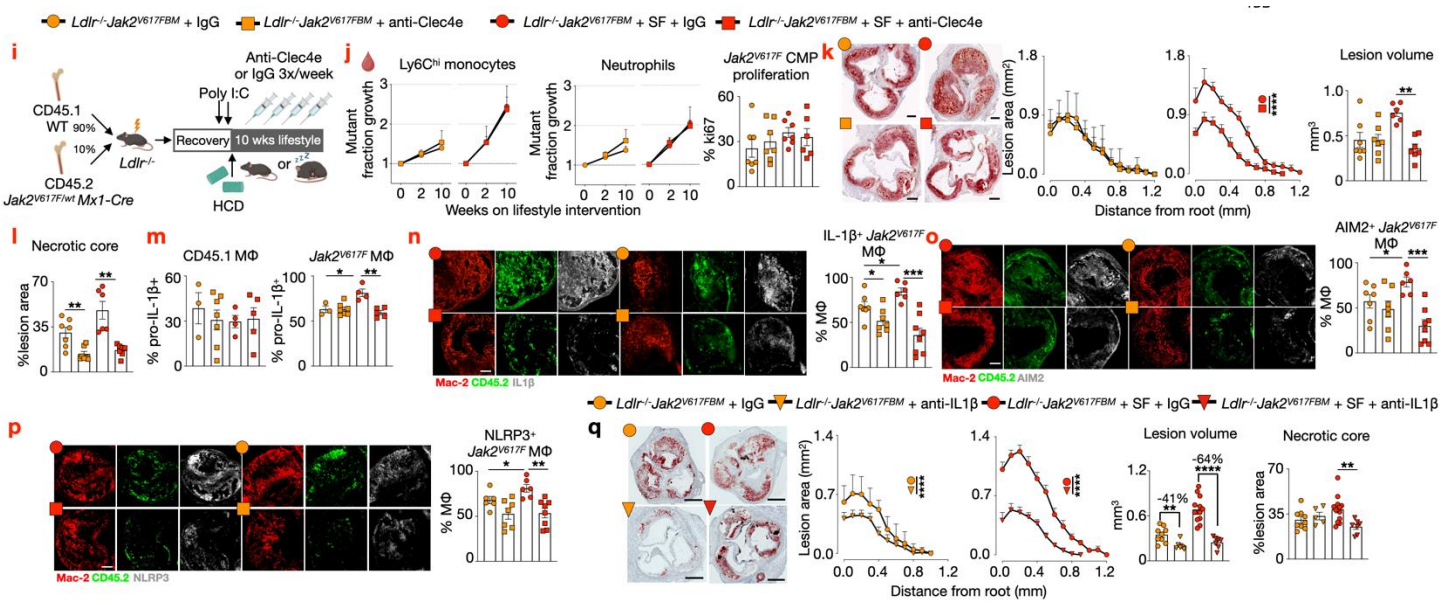


Figure 4. i. Experimental outline. **j**. Expansion of *Jak2*^{V617F} blood Ly6C^{hi} monocytes (n=6,7,5,8 for Sedentary+IgG, Sedentary+anti-Clec4e, SF+IgG, SF+anti-Clec4e) and neutrophils (n= 6,7,5,7) relative to baseline. Proliferation of *Jak2*^{V617F} CMPs (n=7,7,7,6; same groups) **k**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size and volume (both n=7,7,6,7; groups as in (**j**)). Scale bars are 200μm. **l.** Proportion of the necrotic core relative to plaque area (n= 7,7,6,7; groups as in (**j**)). **m.** pro-IL1β expression in CD45.1 WT and *Jak2*^{V617F} macrophages (both n=3,7,4,5; groups as in (**j**)) by flow cytometry. **n.** MAC-2, CD45.2, and IL-1β immunofluorescence and quantification of CD45.2, and IL-1β pixel overlap (n=7, 7,6,7, groups as in (**j**)). Scale bar=100 μm **o.** MAC-2, CD45.2, and AIM2 immunofluorescence and quantification of CD45.2 and AIM2 pixel overlap (n=7,7,6,7; groups as in (**j**)). Scale bar=100 μm. **p.** MAC-2, CD45.2, and NLRP3 immunofluorescence and quantification of CD45.2 and NLRP3 pixel overlap (n=6,7,6,7; groups as in (**j**)). Scale bar=100 μm. **q**. Representative aortic root cross-sections stained with Oil-Red O, quantification of lesion size (n=10, 6, 13, 7) and volume (n=9, 6, 13, 7) in sequential sections and proportion of the necrotic core (n=9, 5, 13, 7) relative to plaque area; groups as in (**j**).

In Figure 5, the authors propose that exercise suppresses atherosclerosis via norepinephrine-mediated signaling. However, as shown in Figure 5l, Adrb2 antagonism failed to alter CMP and GMP proliferation. This

result would suggest that the observed effect of exercise may be more directly related to atherosclerosis modulation, rather than the regulation of clonal hematopoiesis per se. Thus, the mechanistic link between norepinephrine signaling and mutant clone suppression remains speculative and warrants further clarification.
We thank the reviewer for this thoughtful comment. Indeed, Adrb2 blockade did not alter CMP or GMP WT or mutant proliferation in the bone marrow or mutant cell expansion in the blood, indicating that β_2-adrenergic signaling does not directly regulate mutant cell number or clonal expansion. This provides us with a tangible model to dissociate peripheral clonal dynamics from local macrophage programming in the aorta which has a consequence on atherogenesis. Instead, exercise-induced norepinephrine appears to reduce CH-driven atherosclerosis by reducing the inflammatory activity of mutant aortic macrophages, where Adrb2 is selectively overexpressed. In line with this interpretation, we now include immunofluorescence staining of aortic roots showing that ADRβ_2-blockade increases IL-1β expression in CD45.2$^+$ macrophages specifically under exercise conditions (**Figure 5o**). These data demonstrate that β_2-adrenergic signaling restrains inflammasome activation in mutant macrophages during exercise, thereby reducing plaque inflammation and atherosclerosis without directly affecting bone marrow proliferation or peripheral clonal dynamics.

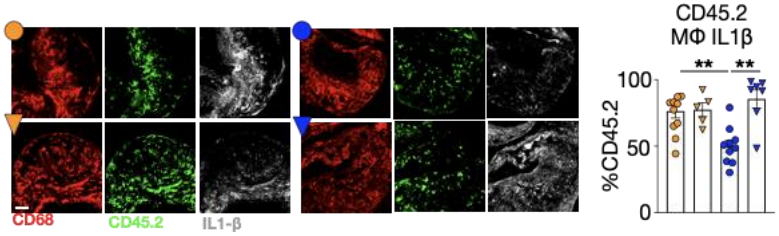
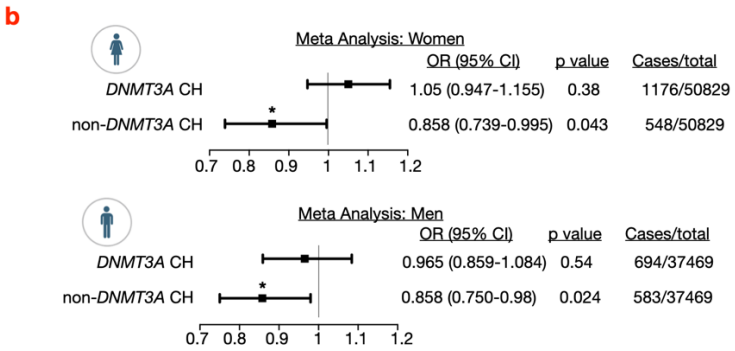


Figure 5. o. IL-1β and CD45.2 immunofluorescence and quantification of pixel overlap in the aortic root (n=10,5,10,7). Groups as in (**I**). Scale bar=100μm.

Together with the Clec4e blocking experiments described above, which also did not alter peripheral clonal dynamics but repressed atherosclerosis and aortic mutant macrophage inflammation, these new findings show that lifestyle modulates CH-driven atherosclerosis through independent hematopoietic (cell number–dependent) and mutant macrophage-specific (local macrophage phenotype–dependent) pathways.

The mouse studies are only performed in female mice. No rationale is stated for the choice of female mice, or why both sexes were not studied. This is a significant limitation of the work. Given that the literature shows marked sexual dimorphism in exercise training responses in terms of transcriptional, proteomic, metabolomic, and phenotypic responses adaptations, the current results are only applicable to females. Thus, the summary, results and conclusions should acknowledge this fact, and the term female should be included where appropriate.
We agree with the reviewer that sex differences can significantly influence exercise-induced transcriptional, metabolic, and inflammatory adaptations. All mouse experiments in this study were therefore performed in female donor–recipient pairs, consistent with prior mechanistic work on *Jak2*^{V617F} (PMID:33731931)- and *Tet2*$^{-/-}$ - (PMID:28104796) driven CH in the context of murine atherosclerosis which used only female mice. We used female mice because female *Ldlr*$^{-/-}$ mice exhibit more stable lipid profiles and a higher, less variable baseline lesion burden than males (PMID: 29430260), reducing biological noise in atherosclerosis experiments. Including both sexes in bone marrow chimeras would require all four sex-matched and -mismatched donor–recipient combinations, substantially increasing complexity and animal use. We have amended the text and now explicitly state this design choice in the Methods, and have revised the text, figure legends, and conclusions to indicate that the results apply specifically to female mice. To specifically address this point and address potential sex differences, we performed sex-stratified analyses in our human datasets, which show consistency and demonstrate that MVPA reduced non-*DNMT3A* CH, but not *DNMT3A* CH, in both men and women (**Extended Data Figure 1b**).



Extended Data Figure 1b. Meta-analysis of the effect of moderate-to-vigorous physical activity on the prevalence of DNMT3A CH and non-DNMT3A CH, split by sex.

Referee #5

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.
Thank you!

Referee #6

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.
Thank you!

We thank the referees and editors for their thoughtful and constructive comments which have significantly strengthened our manuscript. Below is a point-by-point reply. New text and data are marked in red.

Referee #1 (Remarks to the Author):

I carefully evaluated the revised manuscript submitted by Gerhardt et al.

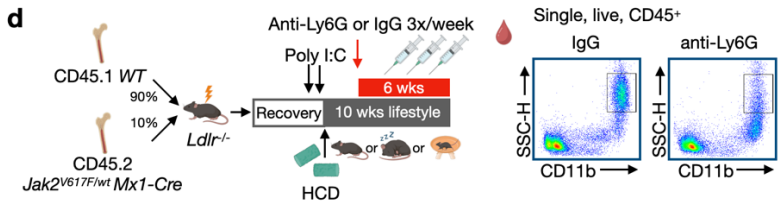
As previously stated, the overall novelty of the study remains limited. Indeed, the pro-atherogenic effects of sleep fragmentation have already been reported by the same group (MacAlpine et al., Nature, 2019), and the association between clonal hematopoiesis and atherosclerosis is now well established (e.g., Oren et al., JCI, 2024).

That said, the mechanistic investigations have been clearly improved. The authors performed a large number of additional and convincing experiments that substantially clarify the mechanisms by which lifestyle factors modulate atherosclerosis in the context of clonal hematopoiesis. The identification of increased IL-1β production by mutant bone-marrow–derived macrophages, together with the specific activation of IL-1β–related pathways in mutant progenitors, is particularly interesting. In addition, the authors performed a more in-depth analysis of macrophage subsets within atherosclerotic lesions.

One methodological issue should nevertheless be addressed :

Extended Data Figure 8a. To evaluate the contribution of neutrophils to the vascular phenotype, the authors used an anti-Ly6G depleting antibody during the last six weeks of the protocol. While this approach is appropriate, neutrophil depletion was assessed by flow cytometry using a gating strategy that includes the Ly6G marker. This represents a major confounding factor, as it is not appropriate to use the same target for both depletion and flow-cytometric identification, since epitope masking by the depleting monoclonal antibody may lead to inaccurate assessment of neutrophil numbers.

Thank you for pointing this out. We have adjusted our gating strategy and provide new flow cytometry plots that do not rely on Ly6G gating but nonetheless demonstrate successful neutrophil depletion (**Extended Data Fig 8d**).



Extended Data Fig 8d.

Referee #2 (Remarks to the Author):

In the revised manuscript, the authors have addressed many of the concerns raised in the initial review. Several issues that were unclear in the first round, particularly regarding experimental design and animal sex, have now been clarified, and overall transparency has improved.

That said, I still have some reservations about aspects of the statistical approach, which, while acceptable, do not fully incorporate the most rigorous hierarchical or cohort-level modeling strategies that might strengthen confidence in certain conclusions. In addition, although the authors now clearly state that all murine experiments were conducted in female mice and have acknowledged this as a limitation, the lack of male data in experimental groups continues to constrain the generalizability of the findings.

These remaining issues temper the strength of the conclusions but do not fundamentally undermine the study's core findings. I am not certain that further rounds of revision would substantially resolve these issues.

Thank you for your helpful and constructive suggestions. We have added two points to the discuss that address sex and statistical analyses. First, we have noted that our data derive from female mice and that further analyses in males is warranted to better assess generalizability and translation. Second, we have added the following sentence to the discussion regarding our statistical approach:

"Lastly, our analyses were conducted at the level of the individual mouse; however, potential hierarchical clustering effects were not explicitly modeled and may contribute to residual variability."

Referee #3 (Remarks to the Author):

Gerhardt et al have revised the manuscript in response to prior reviews. Most of our prior comments have been addressed but we do have some remaining questions:

1. Is the metric of moderate-to-vigorous activity comparable between the UK biobank and All of Us cohorts to perform a meta-analysis?

Yes, both cohorts used actigraphy-based quantification of physical activity. In each cohort, MVPA was defined in the same way as 150 minutes per week.

2. The authors write that" "Whether the speed and extent of clonal expansion is strictly inscribed by mutation-directed intracellular signaling or if external cues also play a role remains unclear. " This statement from the text is not completely true – there have been many publications that you have cited in your manuscript that have shown that external cues have a significant role in promoting the expansion of specific CH clones.

We agree with the reviewer. Indeed, emerging evidence suggests external factors modify clone expansion. However, this idea is not universally accepted as there are studies suggesting cardiovascular risk factors and disease do *not* alter clone expansion and promote the idea that the behavior of mutant cells is not impacted by external cues (References 22 and 23). Thus, uncertainty remains. Further, the impact of sleep and exercise have not been tested and the underlying mechanisms remain unclear. Our study significantly advances this concept and identifies new therapeutically relevant mechanisms and biology. Moreover, it has remained unclear whether mutant cells and WT cells, existing in the same context, are similarly responsive to external cues. Our findings suggest that mutant cells are selectively influenced by sleep and exercise in unique ways.

3. All of the mosaic mice were treated with PIPC after transplant to induce the mutation; so the model was entirely PIPC exposed. Also all had a high cholesterol diet – these confounders should be discussed as potential limitations of the applicability of the model to other conditions.

Thank you for raising this limitation, we have added this limitation to the discussion section. We have updated the discussion to reflect the need to better understand the influence of lifestyle on CH in alternate models and different diets.

"Although our data suggest that even in the absence of HCD and atherosclerosis lifestyle modulates clone expansion, most of our experiments were performed in the setting of HCD with Poly:IC to initiate mutations. The impact of lipids, alternate diets and models on CH mutant cell behavior and lifestyle interactions can be further explored."

4. The reported change in VAF is described as fold change from baseline and would be more clearly labeled as such instead of "CD45.2 fraction growth".

We want to ensure that the data represent the growth of the CD45.2 cell fraction and avoid confusion with overall cell count or the CD45.1 fraction. In these graphs, the white data points represent WT CD45.2 cells where are all the other groups (orange, red, blue) are mutant CD45.2 cells. Therefore, we want to make it clear that these graphs show CD45.2 cell fraction growth over times, whether the CD45.2 cells are WT or mutant.

5. For the serum IL-1b time course data (figure 1f-h), are the same mice being serially sampled? If not, this graph is misleading by connecting the lines. It would be better suited to show as a bar graph or box-and-whisker over time.

The serum IL-1β measurements at each time point were obtained from independent groups of mice rather than serial sampling of the same animals. Statistical comparisons were therefore performed separately at each time point comparing groups of mice and not assessing time or multiple samplings, as indicated in the figure and legend. The connecting lines are intended only to aid visualization of overall temporal trends rather than to imply longitudinal tracking or statistical analysis of individual mice. To avoid any potential misunderstanding, we have clarified this in the statistical explanation in the figure legend.

6. We had previously asked if other cytokines besides just these two were measured and this question was not answered by the authors. Either way, it is an important caveat to note that other cytokines were not broadly surveyed; this should be mentioned as a limitation since people have tended to focus on these two without specific evidence that other cytokines might not be involved.

Thank you for this suggestion, we fully agree. While our data point to specific signaling mechanisms and contribution of IL-1β, other cytokines are likely to be involved and modified by lifestyle. Such investigations will certainly be the subject of future manuscripts. Identifying if sleep or exercise modify the level of other cytokines in CH will be valuable data, demonstrating that such changes causally and mechanistically influence clone expansion or atherosclerosis will also be next steps. In this study, our focus is on cytokines previously implicated in and undergoing active therapeutic targeting trials to mitigate CH and CVD, i.e. IL-1b and IL-6. This choice was intentional, allowing us to assess how lifestyle factors modify established CH-associated inflammatory pathways rather than broadly surveying cytokines without a specific mechanistic framework. It also adds translational value and clinical application to our findings.

7. The authors claim that exercise influences atherosclerotic lesion in LdlrKO mice but extended figure 3d shows that exercise has no effect on lesion area or volume.

That is correct. In our hands, voluntary exercise has the most profound effect on atherosclerosis in the setting of CH. There can be several reasons for this. This may be due to different forms of exercise (voluntary v.s forced), timing and length of exercise exposure, or the sex of the mice (our studies were performed in females while prior work has been done in males e.g. reference 33). Alternatively, a “two-hit” model could be envisioned where exercise has the most profound effect on atherosclerosis in the setting of an already highly inflammatory and atherogenic context. Our data support this concept.

8. The authors note shifts in the types of Macrophages among the Jak2 mutant cells with SF v exercise. Are there any changes in the phenotype of the WT macrophages with SF v exercise?

Yes, this is described and demonstrated in **Extended Figure 7g**. WT CD45.1 macrophages are impacted by SF which augments *Vcan*, *Thbs1*, and *Ptgs2* among other genes, while exercise represses *Folr2*, *Ccl8*, and *Cbr2* among other genes.

The use of the word “reprogramming” suggests a durable change, whereas the changes noted here are directly after acute exposure. The word “redirect” or “promote” would be more appropriate. Or “shift the transcriptomic landscape” as the authors describe.

The transcriptional changes we observe suggest molecular reprogramming. We do not use this term to infer or suggest permanent effects. Indeed, cellular reprogramming is plastic. At the timepoints we evaluate, our data demonstrate that cells are functionally and transcriptionally restructured and altered as several signaling pathways are either promoted or repressed. Further studies will have to evaluate the durability of these changes, perhaps by assessing the epigenome in experiments where the lifestyle stimulus is ceased.

9. “In the aortic root, however, anti-CLEC4E completely abrogated SF-induced accelerated atherogenesis and

necrotic core formation, but did not influence lesion size in sedentary mice (Fig 4k-l), revealing atherogenic regulation in the setting of SF independent of peripheral clone dynamics."
a. How do we know that the CLEC4E pathway is unique to Jak2 mutations? It could also be for relevant for Tet2 where there is an increase in IL-1b signaling. Is this receptor activated by the increased bacterial translocation known to occur with Tet2? Have the authors measured bacterial DNA in blood of SF v non SF mice?

Thank you for these fantastic suggestions. CLEC4E signaling may certainly be involved in Tet2-mediated inflammasome activation and signaling. In order to remain focused, we examine CLEC4E signaling in the context of Jak2. We have not assessed bacterial translocation in our Tet2 mice. We have amended to discussion to raise the point that further work assessing both CLEC4E and b-adrenergic signaling in the context of other CH mutation is needed.

"Deeper mechanistic investigations are warranted, including specific macrophage response to lesion environment, LC circuit tracing, and specific signaling mechanisms by which lifestyle modifies the molecular programming of leukocytes harboring CH mutations beyond *Jak2*^{V617F}."

10. The authors advance the hypothesis that exercise induces b-adrenergic signaling that protects against atherosclerosis by inducing an anti-inflammatory phenotype in macrophages. This hypothesis is opposite of a recent publication that shows that b adrenergic signaling amplified macrophage inflammatory responses: https://pubmed.ncbi.nlm.nih.gov/40466637/
Also, b adrenergic signaling promotes atherosclerosis https://pubmed.ncbi.nlm.nih.gov/39082138/
How do the authors address these contradictions?

This is an exciting and interesting point raised by the reviewer and something we have deeply considered. ADRB2 signaling appears to have both inflammatory and anti-inflammatory effects depending on context, tissue, cell, and disease state. Indeed, our own prior work (PMID 39478215) and the studies listed above by the reviewer demonstrate that ADRB2 signaling can have inflammatory consequences in certain settings. However, ADRB2 can also elicit strong anti-inflammatory responses (PMID 32045472, 30195028, 33875568, 22318967, 41107955) and our prior work has also shown how sympathetic signaling can repress multi-organ myeloid inflammation (PMID:36827982, 35636458). While more work is clearly needed to define ADRB2 signaling, these studies suggest that its influence on inflammation is context-, model-, and disease-dependent. One hypothesis is that at the steady state (low inflammation), ADRB2 stimulation leads to an increase in inflammatory responses. In settings of excessive inflammation such as CH and atherosclerosis, ADRB2 stimulation leads to anti-inflammatory responses. Our findings in this manuscript support this idea. This dichotomy will be the subject of future studies.

Minor comments:
1. "In line with these clonal dynamics, SF raised while exercise lowered plasma IL-1β and IL-6 in Ldlr-/-Jak2V617FBM mice (Fig 1g and Extended Data 2c)." in this part of the manuscript, I believe the figure being referenced is Fig 1F not G for Jak2 IL-1b levels.

Thank you, this has been corrected.

2. Is the endpoint for extended figure 3a/b different than figure 1e? Were the plasma serum levels taken at different times?

In Fig 3a/b mice were subjected to 16 weeks of lifestyle intervention whereas in Fig 1 they were subjected to 12 weeks. The reason for this is that in Fig 1 mice undergo a BMT whereas they do not in Fig 3. BMT on its own accelerates lesion growth and enlarges atherosclerotic lesion size, therefore a shorter timeframe was chosen.

3. "In the blood, the presence of Jak2V617F cells raised hemoglobin, hematocrit, red blood cells, and reticulocytes, but lifestyle did not influence these parameters (Extended Data Fig 1f). Importantly, the influence of sleep and exercise on Jak2V617F CH evolution was not restricted to the setting of atherosclerosis, as SF expanded and exercise diminished Jak2V617F cell expansion in non-atherogenic chow-fed WT (Ldlr+/+) mice as well (Extended Data Fig 2g)." ◊ I think you mean Extended data fig 2F as there is no extended figure 1F…

Thank you, this has been corrected.

4. “Exercise, meanwhile, significantly repressed clone expansion in Ldlr-/-Tet2-/-BM mice (Fig 1h and Extended Data Fig. 2h).” ◊ I think you mean Figure 1g as figure 1h refers to p53.

Thank you, this has been corrected.

5. In the plasma of Ldlr-/-Tet2-/-BM mice, SF raised while exercise lowered IL-1β and IL-6 (Fig. 1g and Extended Data Fig 2i). ◊ it is misleading to claim that exercise lowered IL-6 as the comparison is between sedentary v exercise and there is no statistical significance (per extended fig2i). Relevant non-significance bars should be noted.

Thank you, this has been corrected.

6. “Consistent with these dynamics, BM IL-1β and IL-6 were elevated in Ldlr-/-Jak2V617FBM mice exposed to SF and tended to be lowered by exercise (Fig 2c and Extended Data Fig 4b).” ◊ this is misleading as IL-1b and IL6 are not lower with exercise compared to sedentary Jak2. In fact, IL-6 is the same between SF and exercise!!

Thank you, this has been corrected. We have removed the sentence referring to exercise.

7. “In p53 CH, sedentary Ldlr-/-p53BM mice had accelerated atherosclerosis, but despite sleep not influencing clone expansion in the blood or BM (Fig 1), SF enhanced lesion area and volume, as well as p53-/-, but not WT, macrophage abundance in the aorta of Ldlr-/-p53BM mice (Fig 3d and Extended Data Fig 6i).” ◊ What is Fig 1 referring to? Figure 1h?

Yes, it is referring to Fig 1h. Thank you this has been corrected.

Referee #4 (Remarks to the Author):

The authors have adequately addressed my comments. The summary needs to state that the mouse studies The authors have done an excellent job addressing my comments. However, the summary needs to state that the mouse studies were done in females. This is an impressive study.

Thank you!

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Thank you!

Referee #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Thank you!

We thank the reviewers and editors for their constructive comments and suggestions. We have addressed each comment.

Referees' comments:

Referee #1 (Remarks to the Author):

No more comment

Thank you

Referee #2 (Remarks to the Author):

The authors have adequately addressed my comments.

Thank you

Referee #3 (Remarks to the Author):

Gerhardt et al have revised the manuscript in response to prior reviews. They have addressed most comments. In other cases, they agreed with our criticism but did not change the paper to reflect our suggestions. The items that still need to be revised are listed below:

1. The authors write that" "Whether the speed and extent of clonal expansion is strictly inscribed by mutation-directed intracellular signaling or if external cues also play a role remains unclear. " This statement from the text is not completely true – there have been many publications that you have cited in your manuscript that have shown that external cues have a significant role in promoting the expansion of specific CH clones.

We agree with the reviewer. Indeed, emerging evidence suggests external factors modify clone expansion. However, this idea is not universally accepted as there are studies suggesting cardiovascular risk factors and disease do not alter clone expansion and promote the idea that the behavior of mutant cells is not impacted by external cues (References 22 and 23). Thus, uncertainty remains. Further, the impact of sleep and exercise have not been tested and the underlying mechanisms remain unclear. Our study significantly advances this concept and identifies new therapeutically relevant mechanisms and biology. Moreover, it has remained unclear whether mutant cells and WT cells, existing in the same context, are similarly responsive to external cues. Our findings suggest that mutant cells are selectively influenced by sleep and exercise in unique ways.

Since the authors agree, they should modify their statement to acknowledge that while studies exist to demonstrate the importance of external cues, the impact of sleep and exercise have not been studied.

Thank you, we have modified our statement accordingly in the text, with appropriate references.

2. The reported change in VAF is described as fold change from baseline and would be more clearly labeled as such instead of "CD45.2 fraction growth.
We want to ensure that the data represent the growth of the CD45.2 cell fraction and avoid confusion with overall cell count or the CD45.1 fraction. In these graphs, the white data points represent WT CD45.2 cells where are all the other groups (orange, red, blue) are mutant CD45.2 cells. Therefore, we want to make it clear that these graphs show CD45.2 cell fraction growth over times, whether the CD45.2 cells are WT or mutant. To achieve their stated goals, the authors should then label the Y axis as CD45.2 fold change. "Fraction growth" is arithmetic whereas "fold change" is geometric.
Thank you, we have now included fold change in the graph labels.

3. For the serum IL-1b time course data (figure 1f-h), are the same mice being serially sampled? If not, this graph is misleading by connecting the lines. It would be better suited to show as a bar graph or box-and-whisker over time.

The serum IL-1β measurements at each time point were obtained from independent groups of mice rather than serial sampling of the same animals. Statistical comparisons were therefore performed separately at each time point comparing groups of mice and not assessing time or multiple samplings, as indicated in the figure and legend. The connecting lines are intended only to aid visualization of overall temporal trends rather than to imply longitudinal tracking or statistical analysis of individual mice. To avoid any potential misunderstanding, we have clarified this in the statistical explanation in the figure legend.

4. We had previously asked if other cytokines besides just these two were measured and this question was not answered by the authors. Either way, it is an important caveat to note that other cytokines were not broadly surveyed; this should be mentioned as a limitation since people have tended to focus on these two without specific evidence that other cytokines might not be involved.

Thank you for this suggestion, we fully agree. While our data point to specific signaling mechanisms and contribution of IL-1β, other cytokines are likely to be involved and modified by lifestyle. Such investigations will certainly be the subject of future manuscripts. Identifying if sleep or exercise modify the level of other cytokines in CH will be valuable data, demonstrating that such changes causally and mechanistically influence clone expansion or atherosclerosis will also be next steps. In this study, our focus is on cytokines previously implicated in and undergoing active therapeutic targeting trials to mitigate CH and CVD, i.e. IL-1b and IL-6. This choice was intentional, allowing us to assess how lifestyle factors modify established CH-associated inflammatory pathways rather than broadly surveying cytokines without a specific mechanistic framework. It also adds translational value and clinical application to our findings.

Given that they agree, the authors should add this as a limitation to the discussion.
Thank you, we have added this to the discussion.

5. The authors claim that exercise influences atherosclerotic lesion in LdlrKO mice but extended figure 3d shows that exercise has no effect on lesion area or volume.

That is correct. In our hands, voluntary exercise has the most profound effect on atherosclerosis in the setting of CH. There can be several reasons for this. This may be due to different forms of exercise (voluntary v.s forced), timing and length of exercise exposure, or the sex of the mice (our studies were performed in females while prior work has been done in males e.g. reference 33). Alternatively, a “two-hit” model could be envisioned where exercise has the most profound effect on atherosclerosis in the setting of an already highly inflammatory and atherogenic context. Our data support this concept.

If there is no effect of exercise on lesion area or volume, then none should be claimed.
We do not claim changes in lesions size in the exercising non-CH mice.

6. The authors note shifts in the types of Macrophages among the Jak2 mutant cells with SF v exercise. Are there any changes in the phenotype of the WT macrophages with SF v exercise?
The use of the word “reprogramming” suggests a durable change, whereas the changes noted here are directly after acute exposure. The word “redirect” or “promote” would be more appropriate. Or “shift the transcriptomic landscape” as the authors describe.

The transcriptional changes we observe suggest molecular reprogramming. We do not use this term to infer or suggest permanent effects. Indeed, cellular reprogramming is plastic. At the timepoints we evaluate, our data demonstrate that cells are functionally and transcriptionally restructured and altered as several signaling pathways are either promoted or repressed. Further studies will have to evaluate the durability of these changes, perhaps by assessing the epigenome in experiments where the lifestyle stimulus is ceased.

According to many definitions, cellular reprogramming involves not only transcriptional changes but also metabolic, epigenetic, and translational changes: PMID: 40140235. The authors should not use the word reprogramming when they are simply referring to changes in transcriptional states.
Thank you for this suggestion, we have clarified that we are specifically talking about transcriptomic

programming. Additional programming feature will need to be evaluated in future studies.