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## Physioxia improves the selectivity of hematopoietic stem cell expansion cultures

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### Abstract:

Hematopoietic stem cells (HSCs) are a rare hematopoietic cell type that can entirely reconstitute the blood and immune systems following transplantation. Allogeneic HSC transplantation (HSCT) is used clinically as a curative therapy for a range of hematolymphoid diseases, but remains a high-risk therapy due to potential side effects including poor graft function and graft-vs-host disease (GvHD). Ex vivo HSC expansion has been suggested as an approach to improve hematopoietic reconstitution from low-cell dose grafts. Here, we demonstrate that we can improve the selectivity of polyvinyl alcohol (PVA)-based mouse HSC cultures through the use of physioxic culture conditions. Single-cell transcriptomic analysis confirmed inhibition of lineage-committed progenitor cells in physioxic cultures. Long-term physioxic expansion also afforded culture-based ex vivo HSC selection from whole bone marrow, spleen, and embryonic tissues. Furthermore, we provide evidence that HSC-selective ex vivo cultures deplete GvHD-causing T cells and that this approach can be combined with genotoxic-free antibody-based conditioning HSCT approaches. Our results offer a simple approach to improve PVA-based HSC cultures and the underlying molecular phenotype, as well as highlight the potential translational implications of selective HSC expansion systems for allogeneic HSCT.

**Conflict of interest:** COI declared - see note

**COI notes:** HN is a co-founder and shareholder in Megakaryon, and Century Therapeutics. ACW is a consultant for Graphite Bio and ImmuneBridge. AC discloses financial interests in the following entities working in the rare genetic disease space: Beam Therapeutics, Decibel Therapeutics, Editas Medicines, Global Blood Therapeutics, GV, Lyrik Therapeutics, Magenta Therapeutics, and Spotlight Therapeutics. However, none of these companies had input into the design, execution, interpretation, or publication of the work in this manuscript.

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**Non-author contributions and disclosures:** No;

**Agreement to Share Publication-Related Data and Data Sharing Statement:** A single cell RNA-seq website resource is available here: <http://128.232.227.172/Igarashi2022/> (username: BGlabb; password: RHQoz89jc). Raw and processed data are available on GEO (GSE207743, GSE175400, GSE207740).

Clinical trial registration information (if any) :

1           **Physioxia improves the selectivity of hematopoietic stem cell expansion cultures**

2  
3   **SHORT TITLE:**

4   Selective blood stem cell cultures via physioxia

5  
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30

1 **ABSTRACT:**

2 Hematopoietic stem cells (HSCs) are a rare hematopoietic cell type that can entirely reconstitute  
3 the blood and immune systems following transplantation. Allogeneic HSC transplantation  
4 (HSCT) is used clinically as a curative therapy for a range of hematolymphoid diseases, but  
5 remains a high-risk therapy due to potential side effects including poor graft function and graft-  
6 vs-host disease (GvHD). Ex vivo HSC expansion has been suggested as an approach to improve  
7 hematopoietic reconstitution from low-cell dose grafts. Here, we demonstrate that we can  
8 improve the selectivity of polyvinyl alcohol (PVA)-based mouse HSC cultures through the use  
9 of physioxic culture conditions. Single-cell transcriptomic analysis confirmed inhibition of  
10 lineage-committed progenitor cells in physioxic cultures. Long-term physioxic expansion also  
11 afforded culture-based ex vivo HSC selection from whole bone marrow, spleen, and embryonic  
12 tissues. Furthermore, we provide evidence that HSC-selective ex vivo cultures deplete GvHD-  
13 causing T cells and that this approach can be combined with genotoxic-free antibody-based  
14 conditioning HSCT approaches. Our results offer a simple approach to improve PVA-based HSC  
15 cultures and the underlying molecular phenotype, as well as highlight the potential translational  
16 implications of selective HSC expansion systems for allogeneic HSCT.

17

18 **KEY POINTS:**

- 19 • Physioxia improves the selectivity of polyvinyl alcohol-based mouse hematopoietic stem  
20 cell cultures
- 21 • Selective hematopoietic stem cell cultures deplete graft-vs-host disease causing T cells

22

23

## 1 INTRODUCTION:

2 Self-renewing multipotent hematopoietic stem cells (HSCs) support the blood and immune  
3 systems throughout life<sup>1-3</sup>. HSCs first arise in the mouse embryo around E10.5 and initially  
4 localize to the fetal liver before moving to the bone marrow around the time of birth<sup>4</sup>. While the  
5 bone marrow microenvironment is the most studied HSC niche, HSCs have also been described  
6 in other organs such as the spleen<sup>5,6</sup>. HSCs are usually very rare, at an estimated frequency of  
7 ~1:30,000 cells within the adult bone marrow, and just ~1:300,000 in the adult spleen<sup>7</sup>. This has  
8 hindered efforts to characterize this biologically interesting stem cell population.

9  
10 The transplantation of healthy HSCs into a patient (termed HSCT) is a potentially curative  
11 therapy for a wide range of blood diseases<sup>8</sup>. However, although HSCT has been used for over 60  
12 years, this therapy often still remains a treatment of last resort due to several safety concerns<sup>9,10</sup>.  
13 First, allogeneic HSCT currently requires patients to undergo genotoxic pre-conditioning via  
14 radiation therapy and/or chemotherapy to enhance donor engraftment and prevent rejection.  
15 Second, donor and recipient must be sufficiently immune-matched to avoid graft-vs-host disease  
16 (GvHD), which is caused by allo-reactive T cells that contaminate the donor HSC graft. GvHD  
17 represents a serious potential side effect of HSCT and the donor-recipient immune-matching  
18 requirement to avoid it also limits the number of potential donors for a transplant. An ideal  
19 HSCT paradigm would involve availability of large numbers of HSCs so that toxic chemo-  
20 irradiative pre-conditioning would not be required and cell products that lack GvHD-inducing T  
21 cells to improve donor-recipient immune compatibility.

22  
23 The biological and clinical importance of HSCs has driven wide efforts to sustain HSCs ex  
24 vivo<sup>11,12</sup>. However, for a long time, stable expansion of HSCs has remained a major challenge for  
25 the field. We recently discovered that polymer-based media could support long-term ex vivo  
26 expansion of functional mouse HSCs<sup>13,14</sup>. While these cultures were initiated from FACS-  
27 purified CD150<sup>+</sup>CD34<sup>-/lo</sup>c-Kit<sup>+</sup>Sca1<sup>+</sup>Lineage<sup>-</sup> (CD150<sup>+</sup>CD34<sup>-/lo</sup>KSL) HSCs, a range of c-  
28 Kit<sup>+</sup>Sca1<sup>+</sup>Lineage<sup>-</sup> progenitor cells built up in these cultures over time, reducing HSC purity.  
29 Here, we describe that through optimizing O<sub>2</sub> concentrations for ex vivo HSC cultures, we could  
30 limit the buildup of progenitors and mature hematopoietic cells within long-term polyvinyl  
31 alcohol (PVA)-based cultures and thereby improve HSC culture selectivity. These optimized  
32 culture conditions selectively expand HSCs from whole bone marrow cells (WBMCs) and whole  
33 spleen cells, while depleting mature hematopoietic cells including T cells. Depletion of allo-  
34 reactive T cells was demonstrated by transplantation of allogeneic cells, which confirmed that  
35 HSC-selective cultures prevented acute GvHD. Finally, we present evidence that these methods  
36 can be combined with antibody-mediated inhibition of graft rejection to achieve HSCT without  
37 genotoxic-conditioning.

## 38 39 METHODS: 40 Mice

1 All animal experiments were approved by the Administrative Panel on Laboratory Animal Care  
2 at Stanford University or performed in accordance with UK Home Office regulations. C57BL/6-  
3 CD45.2 mice (000664), C57BL/6-CD45.1 mice (PepboyJ; 002014), and Balb/c mice (000651)  
4 were purchased from the Jackson Laboratory or bred at the University of Oxford. C57BL/6-  
5 CD45.1/CD45.2 mice were bred from C57BL/6-CD45.1 and C57BL/6-CD45.2 at Stanford  
6 University. *Fancd2*<sup>-/-</sup> mice<sup>15</sup> were generously provided by Ken Weinberg and bred at Stanford  
7 University. All mice were 8-12 weeks at the experiment start point.

8

### 9 **Purified HSC cultures**

10 Immunophenotypic CD150<sup>+</sup>CD34<sup>-/lo</sup>c-Kit<sup>+</sup>Sca1<sup>+</sup>Lineage<sup>-</sup> HSCs were isolated from pelvic,  
11 femur, tibia, and vertebrae from C57BL/6-CD45.1 or C57BL/6-CD45.2 mice using an AriaII cell  
12 sorter (BD and cultured as described previously<sup>14</sup>. Cells were incubated at 5% CO<sub>2</sub> and indicated  
13 O<sub>2</sub> levels using a multigas incubator (ThermoFisher HERACell 150i or PHC MCO-170M-PE  
14 Incusafe), with complete media changes (performed in regular tissue culture hoods) every 2-3  
15 days after the initial 5 days. See Supplementary Information for further details.

16

### 17 **Unfractionated bone marrow and spleen cultures**

18 Unfractionated whole bone marrow or whole spleen were strained with a 100um filter and then  
19 plated on CellBIND (Corning) plates in HSC media (see composition above) and incubated at  
20 5% CO<sub>2</sub> and indicated O<sub>2</sub> levels, with complete media changes every 2-3 days.

21

### 22 **Cytometric analyses**

23 Flow cytometry and cytometry by time of flight (CyTOF) were performed at indicated  
24 timepoints. Cells were antibody stained (CD201-APC, cKit-BV421, Sca1-PE, Gr1-  
25 APC/eFluor780, Ter119-APC/eFluor780, CD4-APC/eFluor780, CD8-APC/eFluor780, CD45R-  
26 APC/eFluor780, CD127-APC/eFluor780) for 30 minutes at 4°C, washed and then analyzed using  
27 a LSRFortessa (BD) using PI as a live/dead cell stain. CyTOF was performed as detailed in the  
28 Supplementary Information.

29

### 30 **Transplantation assays**

31 For competitive transplantation assays, 5000 day-28 culture cells derived from C57BL/6-CD45.1  
32 mice were transplanted alongside 1x10<sup>6</sup> C57BL/6-CD45.1/CD45.2 competitor whole bone  
33 marrow cells (WBMCs) into 10Gy lethally irradiated C57BL/6-CD45.2 recipient mice.  
34 Secondary transplantation assays were performed as above but using 2x10<sup>6</sup> WBMCs isolated  
35 from primary recipients. For allogeneic transplantation assays into Balb/c mice, fresh or day-28  
36 cultured cells derived from WBMCs and/or whole spleen cells isolated from C57BL/6-CD45.1  
37 mice were transplanted into 7Gy irradiated Balb/c mice. Hematoxylin and eosin staining was  
38 performed on paraformaldehyde-fixed livers by the Stanford Animal Histology Service. For  
39 minor allele mismatch transplantation assays into *Fancd2*<sup>-/-</sup> mice, day-28 cultured cells derived  
40 from WBMCs or CD150<sup>+</sup>CD48<sup>+</sup>KSL HSCs, or fresh CD150<sup>+</sup>CD48<sup>+</sup>KSL HSCs isolated from

1 C57BL/6-CD45.1 mice were transplanted into *Fancd2*<sup>-/-</sup> mice. Where indicated, mice were pre-  
2 treated with 500ug of anti-CD4 (GK1.5; BioXCell) 7-days before transplantation.

#### 3 4 **RNA-seq analyses**

5 For bulk RNA-seq day-28 cultured cells were stained as indicated above and CD201<sup>+</sup>CD150<sup>+</sup>c-  
6 Kit<sup>+</sup>Scal<sup>+</sup>Lineage<sup>-</sup> and Kit<sup>+</sup>Scal<sup>-</sup>Lineage<sup>-</sup> cells FACS isolated and RNA extracted using a  
7 QIAGEN RNeasy micro kit. RNA-seq was performed by Novogene. Single cell (sc)RNA-seq  
8 was performed using 10X Genomics v3 reagents, as detailed in the Supplementary Information.

9  
10 A single cell RNA-seq website resource is available here: <http://128.232.227.172/Igarashi2022/>  
11 (username: BGLab; password: RHQoz89jc). Raw and processed data are available on GEO  
12 (GSE207743, GSE175400, GSE207740).

### 13 14 **RESULTS:**

#### 15 **Physiological O<sub>2</sub> concentrations reduce differentiation in long-term ex vivo HSC cultures**

16 The importance of O<sub>2</sub> concentration in cell culture conditions has come to the forefront in  
17 discussions around improving the physiological relevance and translational capacity of basic  
18 biological findings from ex vivo studies<sup>16-18</sup>. Recapitulating the pericellular oxygen partial  
19 pressure (pO<sub>2</sub>) found in specific tissue microenvironments has demonstrated significant value in  
20 producing data which is both more representative of in vivo physiology and more experimentally  
21 robust<sup>19</sup>. Direct measurement of pO<sub>2</sub> in the bone marrow of mice has identified a range of 9.9-32  
22 mm Hg O<sub>2</sub> (1.3-4.2%)<sup>20</sup>, of which LT-HSCs are not found in bone marrow niches with the  
23 deepest hypoxia, but rather reside in pO<sub>2</sub> of 18-19 mm Hg (2-3%)<sup>21</sup>. We hypothesized that  
24 calibration of O<sub>2</sub> concentration to levels more representative of the bone marrow might further  
25 improve our HSC cultures. In our previous studies, HSCs were grown in standard 20% O<sub>2</sub> tissue  
26 culture incubators, which yields a pericellular O<sub>2</sub> concentration of ~18.4%<sup>21-23</sup>. We compared  
27 these HSC culture conditions with cultures grown at 5% O<sub>2</sub>, and 1% O<sub>2</sub> (pericellular O<sub>2</sub>  
28 concentrations of ~3.5% and ~0.7%, respectively) (**Figure 1A**). Flow cytometry after 4-week  
29 culture identified striking differences in culture composition with significant increases in the  
30 frequency of phenotypic CD201<sup>+</sup>CD150<sup>+</sup>KSL populations (**Figure 1A-B**), which has recently  
31 been proposed as the ex vivo HSC fraction<sup>24-26</sup>. This high frequency of CD201<sup>+</sup>CD150<sup>+</sup>KSL  
32 cells was largely stable at 30-40% of live cells at low O<sub>2</sub> throughout the 4-week culture (**Figure**  
33 **1B**).

34  
35 In terms of absolute cell numbers per well, 1% and 5% O<sub>2</sub> reduced the number of live cells by  
36 ~50% (**Figure 1C**), meaning that similar numbers of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells were generated  
37 in all cultures (**Figure 1D**). The increased frequency of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells was driven by  
38 a decrease in non-KSL cells at low O<sub>2</sub> (**Figure 1E**). Competitive transplantation assays  
39 confirmed the functional capacity of the cells cultured at all O<sub>2</sub> concentrations. Compared to  
40 20% O<sub>2</sub> cultured cells, 5% O<sub>2</sub> cultures displayed ~2-fold higher donor chimerism by the 16-week



1 endpoint (**Figure 1F, S1A**). By contrast, 1% O<sub>2</sub> cultured cells initially displayed similar  
2 chimerism to 5% O<sub>2</sub> cultures at 4-weeks, but dropped down to levels similar to 20% O<sub>2</sub> cultures  
3 by 16 weeks (**Figure 1F, S1A**). The cause of this lower engraftment rate at 1% O<sub>2</sub> is unclear.

4  
5 To characterise the molecular consequences of low O<sub>2</sub> culture conditions on HSC expansion, we  
6 performed RNA-sequencing (RNA-seq) analysis on CD201<sup>+</sup>CD150<sup>+</sup>KSL cells from 4-week  
7 cultures. The c-Kit<sup>+</sup>Sca1<sup>-</sup>Lineage<sup>-</sup> cells were also analyzed from the 20% O<sub>2</sub> cultures. Initial  
8 principal component analysis (PCA) separated CD201<sup>+</sup>CD150<sup>+</sup>KSL samples from c-Kit<sup>+</sup>Sca1<sup>-</sup>  
9 Lineage<sup>-</sup> samples via PC1 while 20% O<sub>2</sub> CD201<sup>+</sup>CD150<sup>+</sup>KSL samples separated from 5% and  
10 1% CD201<sup>+</sup>CD150<sup>+</sup>KSL samples via PC2 (**Figure S1B**). When compared against the c-  
11 Kit<sup>+</sup>Sca1<sup>-</sup>Lineage<sup>-</sup> samples, gene set enrichment analysis (GSEA) identified enrichment for LT-  
12 HSC gene sets in the CD201<sup>+</sup>CD150<sup>+</sup>KSL cell samples for all O<sub>2</sub> concentrations, while the c-  
13 Kit<sup>+</sup>Sca1<sup>-</sup>Lineage<sup>-</sup> samples were enriched for progenitor gene sets (**Figure S1C**). These results  
14 confirmed CD201<sup>+</sup>CD150<sup>+</sup>KSL cells as phenotypic (p)HSCs and c-Kit<sup>+</sup>Sca1<sup>-</sup>Lineage<sup>-</sup> cells as  
15 phenotypic hematopoietic progenitor cells (pHPCs).

16  
17 To investigate the transcriptional differences between these samples, we performed differential  
18 gene expression analysis. Between 5% and 1% O<sub>2</sub> pHSC samples, few differentially expressed  
19 genes were observed (**Figure 1G**). By contrast, large gene expression differences were observed  
20 between 20% and 5% O<sub>2</sub> pHSCs (**Figure 1G**). GO term analysis identified upregulation of  
21 transcriptional and endoplasmic reticulum stress pathways in 20% O<sub>2</sub> pHSCs (**Figure 1H**),  
22 which corresponded to the observed differential expression of the stress response factor *Atf5*  
23 (**Figure 1G**). Stress response pathway activity is known to induce HSC differentiation<sup>27,28</sup>, which  
24 may explain why 20% O<sub>2</sub> HSC cultures contained more differentiated cell types. Amino acid  
25 transporters were also enriched in 20% pHSCs (relative to 5% O<sub>2</sub> pHSCs), while sterol and  
26 cholesterol metabolism pathways were dominant among upregulated genes at 5% and 1% O<sub>2</sub>  
27 (relative to 20% O<sub>2</sub> pHSCs) (**Figure 1H**).

28  
29 To search for pathways that could be playing a role in the selectivity for CD201<sup>+</sup>CD150<sup>+</sup>KSL in  
30 the low O<sub>2</sub> cultures, we screened 116 small molecule inhibitors targeting various intracellular  
31 pathways (see **Table S1** for details) using 21-day expanded cells at 20% O<sub>2</sub>. One of the top hits  
32 was IACS-010759, an inhibitor of mitochondrial complex I, which plays an important role in  
33 aerobic respiration (**Figure 1I**). We next tested this compound on fresh HSCs. At 20% O<sub>2</sub>, we  
34 observed significant increases in CD201<sup>+</sup>CD150<sup>+</sup>KSL frequencies, and similar reductions in total  
35 cell numbers and non-KSL cells as our low O<sub>2</sub> cultures using 20 nM (**Figure 1J**). By contrast,  
36 little difference was seen when IACS-010759 was added to 5% O<sub>2</sub> or 1% O<sub>2</sub> HSC cultures.  
37 These results suggest that reduced mitochondrial respiratory chain activity may contribute to the  
38 inhibition of progenitor cells at low O<sub>2</sub>. However, further studies are warranted to assess the  
39 metabolic state and dependencies of HSCs and progenitor cells in these culture conditions.

40

## 1 **Low O<sub>2</sub> alters cellular heterogeneity within HSC cultures**

2 To further investigate the altered cellular heterogeneity within these HSC cultures, we performed  
3 single-cell (sc)RNA-seq on 4-week cultured 20% O<sub>2</sub>, 5% O<sub>2</sub>, and 1% O<sub>2</sub> cultures derived from  
4 LT-HSCs as well as a fresh c-Kit-enriched bone marrow sample (**Figure 2A**). Batch correction  
5 using Harmony was performed to integrate fresh and cultured scRNA-seq profiles, since  
6 matching populations between samples were highly divergent with clear changes in expression  
7 (**Figure S2A**). The combined data revealed matching lineage topology and differentiation stages  
8 between fresh and cultured cells (**Figure S2B**) and identified 21 cell clusters (**Figure 2B**). We  
9 performed manual annotation based on established lineage markers<sup>29</sup>, which highlighted  
10 differentiation trajectories towards monocytes, neutrophils, basophils/mast cells,  
11 megakaryocytes, erythroid and lymphoid/B cells (**Figure 2C, S3A**). To pinpoint the location of  
12 putative HSCs, we analyzed expression of known HSC markers (*Procr*, *Mecom*, *Mllt3*, *Ly6a*,  
13 *Hlf*) (**Figure S3A**) and HSC gene signatures (HSCscore<sup>30</sup> and RepopSig<sup>24</sup>) (**Figure S3B-C**),  
14 which showed the highest values within the cluster 1. Comparing cluster frequency for each  
15 sample, we observed striking differences in abundance between the high (20%) and low (5% and  
16 1%) O<sub>2</sub> cultures, whereas the 5% and 1% patterns were broadly similar (**Figure 2D, S3D**).  
17 Specifically, we identified increased abundance of low O<sub>2</sub> cultured cells in the HSC and  
18 intermediate progenitor clusters accompanied by relative depletion of the more differentiated  
19 clusters (**Figure 2E**). Cells with the highest RepopSig score were also much more abundant at  
20 low O<sub>2</sub> levels (**Figure S3E**).

21  
22 To probe the apparent block in differentiation caused by low O<sub>2</sub>, we used estimated pseudotime  
23 and the CellRank framework to infer cell fate probabilities for the main differentiation  
24 trajectories (**Figure 2F-G, S4A**). At low O<sub>2</sub> levels, cell density along pseudotime confirmed a  
25 sharp reduction in cell number at around the 0.02-0.021 pseudotime mark for both  
26 megakaryocyte and neutrophil trajectories (arrows in **Figures 2H** and **S4B**). At this stage, we  
27 observed almost no fate separation between the basophil, erythroid and megakaryocyte  
28 trajectories (**Figure S4C-D**), while the neutrophil trajectory had just separated from the other  
29 fates (**Figure S4E**). Thus, we asked which genes were dynamically expressed around the  
30 differentiation stage with the clear drop in cell numbers for the neutrophil and megakaryocyte  
31 trajectories.

32  
33 For the megakaryocyte trajectory, we identified 75 differentially expressed genes, which showed  
34 stereotypic pattern and order of gene expression changes (**Figure 2I**). Many of these were known  
35 hematopoietic regulators<sup>31</sup>. For instance, several HSC-associated genes (e.g., *Mecom*, *Hlf*, *Ly6a*)  
36 dropped off early on (by pseudotime value 0.015). On the other hand, a different set of genes  
37 including several known markers of differentiation (e.g., *Gata1*, *Gata2*, *Ms4a2*, *Ms4a3*) were  
38 beginning to be upregulated around the putative differentiation block stage.

39

1 The dynamic genes in the neutrophil trajectory showed a limited overlap with the megakaryocyte  
2 trajectory, although we observed a similar decrease in *Mecom* expression in both (**Figure S4F**).  
3 Instead, the list of dynamic genes featured genes associated with very early stage of myeloid  
4 differentiation, including *Mpo*, *Ctsg* and chemokine *Ccl9* appearing just before the region of  
5 interest and more typical neutrophil markers including *Elane*, *Cebpe* appearing later (**Figure**  
6 **S4F**). Together, these results pinpoint critical stages in the transition from the HSC-like state to  
7 more differentiated progenitors that are suppressed in low O<sub>2</sub> cultures, and suggest specific  
8 molecular events associated with this transition.

9  
10 Finally, we performed differential gene expression analysis to interrogate O<sub>2</sub> concentration  
11 specific-transcriptomes specifically within our cluster 1 HSCs. We identified upregulation of the  
12 interferon response specifically at 5% O<sub>2</sub> (**Figure 2J, S4G**). This higher inflammatory signaling  
13 could relate to the recently described link between inflammatory signaling and ex vivo HSC self-  
14 renewal<sup>32</sup>. To allow for the community to interrogate these datasets, we have generated a website  
15 portal for interrogating gene expression within these clusters  
16 (<http://128.232.227.172/Igarashi2022/>).

### 17 18 **Low O<sub>2</sub> affords HSC expansion from unfractionated hematopoietic cell populations**

19 The selectivity of these low O<sub>2</sub> cultures prompted us to consider whether culture-based  
20 enrichment of HSCs could be achieved from WBMCs (**Figure 3A**). While we have previously  
21 succeeded in expanding LT-HSCs from c-Kit<sup>+</sup> hematopoietic stem and progenitor cells (HSPCs),  
22 LT-HSC selection from WBMCs was not possible at 20% O<sub>2</sub><sup>33</sup>. Compared to 20% O<sub>2</sub> WBMC  
23 cultures, similar number of live cells were generated after four weeks at 5% O<sub>2</sub>, but lower  
24 numbers were generated at 1% O<sub>2</sub> (**Figure 3B**). Both 5% O<sub>2</sub> and 1% O<sub>2</sub> cultures contained ~3-  
25 fold more CD201<sup>+</sup>CD150<sup>+</sup>KSL cells than the 20% O<sub>2</sub> cultures (**Figure 3C, S5A**). In primary  
26 competitive transplantation assays, 5% O<sub>2</sub> and 1% O<sub>2</sub> cultured cells performed better than 20%  
27 O<sub>2</sub> cultures (**Figure 3D, S5B**). However, only 5% O<sub>2</sub> cultures achieved robust multilineage PB  
28 chimerism in secondary recipients (**Figure 3E, S5C**).

29  
30 To track the cellular heterogeneity within these WBMC cultures over time, we performed  
31 cytometry by time of flight (CyTOF) analysis using a hematopoiesis antibody panel (**Figure 3F**;  
32 **Table S2**). Given the superior functional results at 5% O<sub>2</sub>, we focused our analysis on this  
33 culture condition. We used unsupervised clustering to resolve cell populations within the  
34 cultures, and manually annotated these immunophenotypic clusters (**Figure S5D**). Clusters at  
35 less than 1% of the cell cultures were grouped as “Other”. Over the first week, the CD201<sup>+</sup>KSL  
36 fraction was initially very rare (0.5%), but increased after day 7 and reached over 50% by day 28  
37 (**Figure 1F**). The inverse was seen for granulocytes/monocytes and erythroid cells, which were  
38 initially high and dropped down between day 7 and 14.

39

1 To assess a second hematopoietic cell population, we also evaluated whole spleen cells in 5% O<sub>2</sub>  
2 culture using this CyTOF time course assay (**Figure 3G, S5A**). Similar to WBMC cultures,  
3 CD201<sup>+</sup>KSL frequencies were initially very low and rose between day 7 and day 14, while  
4 erythroid cells displayed the inverse. In fresh spleen, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were initially  
5 present at a frequency of 27% and were gradually depleted over time in culture. By day 28, T  
6 cells were just 2% of the culture. Compared with the WBMC cultures, lower CD201<sup>+</sup>KSL  
7 frequencies were observed in spleen cultures at day 28 (23% vs 50%; **Figure 3F-G**).

8  
9 Within both the WBMC and whole spleen cell cultures, we could identify two KSL  
10 compartments, one marked by CD201 (CD201<sup>+</sup>KSL) and a second marked by CD41  
11 (CD41<sup>+</sup>KSL) (**Figure 3F-G, S5D**). Interestingly, while the CD201<sup>+</sup>KSL expanded progressively  
12 over 28 days, the CD41<sup>+</sup>KSL was most abundant at day 14 and 21 before dropping down at day  
13 28. Further interrogation of the Kit<sup>+</sup>Scal<sup>+</sup>Lineage<sup>-</sup> population specifically on day 28 suggested  
14 the major population (expressing CD201<sup>+</sup>), also co-expressed CD31 (Pecam) and low levels of  
15 CD11b (Mac1) (**Figure S5E**). This analysis suggests additional markers that may help to resolve  
16 the HSC compartment *ex vivo*.

17  
18 Given the selectivity of these culture conditions, we wondered whether we could use this system  
19 to assay for HSC expansion capacity. We therefore turned to embryonic development, to assay  
20 when expandable potential could be first observed. As definitive HSCs are reported to be first  
21 seen from embryonic day (E)10.5<sup>4</sup>, we evaluated expansion potential from various embryonic  
22 organs at E11.5 to E16.5 (**Figures 3H**). Our results largely mirrored the reported localization of  
23 HSCs in the developing embryo, with pHSCs expanding from the fetal liver from E13.5 onwards  
24 but were not observed from the yolk sac<sup>34</sup> (**Figure 3I**). Consistent with the reported transient  
25 HSC activity in the placenta<sup>35,36</sup>, pHSCs expanded from E12.5 and E13.5 placenta, but not at  
26 later timepoints. Total live cell numbers (**Figure S5F**) also correlated with pHSCs presence in  
27 these cultures (**Figure 3I**), demonstrating culture selectivity. However, we did not detect  
28 expansion potential from the embryo at E11.5-12.5, suggesting our assay is selective for a subset  
29 of mature HSCs. These results suggest that this culture system may provide a useful assay for  
30 HSC expansion potential, although further work is needed to functionally validate these findings.

### 31 **Ex vivo selective culture depletes GvHD-causing T cells**

32 In allogeneic HSCT, allo-reactive T cells are a major cause of GvHD. Based on the robust  
33 concomitant depletion of T cells and expansion of HSCs in our whole spleen cell cultures, we  
34 hypothesized that our low O<sub>2</sub> *ex vivo* HSC expansion culture conditions would allow for  
35 engraftment post-allogeneic transplantation while avoiding GvHD. We therefore tested this in an  
36 acute GvHD model, where C57BL/6 donor cells were transplanted into irradiated allogeneic  
37 Balb/c recipients (**Figure 4A**). To induce acute GvHD in this model, a mixture of WBMCs and  
38 whole spleen cells were co-transplanted into irradiated recipients. Consistent with other reports<sup>37</sup>,  
39 transplantation of fresh spleen/WBMC led to acute GvHD in 2 weeks (**Figure 4B, S6A**). By  
40

1 contrast, recipients of cultured spleen/WBMCs, or even expanded spleen only survived long-  
2 term (**Figure 4B-C**) and displayed multilineage donor peripheral blood chimerism (**Figure S6B**).  
3 We further confirmed that the loss of T cells was responsible for the survival of these recipients  
4 by transplantation of fresh T cells with cultured spleen/WBMCs. Addition of fresh T cells led to  
5 rapid GvHD (**Figure S6C**). Together, these results confirm that low O<sub>2</sub> HSC-selective media  
6 conditions lead to depletion of mature immune cells and enrichment of transplantable HSPCs.

7  
8 Finally, we evaluated whether we could combine this GvHD-free HSC expansion method with  
9 antibody-based inhibition of immune rejection in a relevant allogeneic HSCT mouse model  
10 (**Figure 4D**). Given the toxicities of bone marrow conditioning regimens and GvHD in Fanconi  
11 Anemia patients receiving allogeneic HSC transplantation, we selected a minor allele mismatch  
12 *Fancd2*<sup>-/-</sup> HSCT mouse model for this proof-of-concept study. Minor mismatches between  
13 wildtype C57BL/6 and *Fancd2*<sup>-/-</sup> mice lead to rejection of C57BL/6 donor cells when  
14 transplanted without conditioning<sup>38</sup>. However, the immune rejection can be inhibited with anti-  
15 CD4 antibody-based conditioning. We initially confirmed that anti-CD4 antibody treatment was  
16 necessary for engraftment of C57BL/6 cells (expanded from 5x10<sup>6</sup> WBMCs) in *Fancd2*<sup>-/-</sup> mice  
17 (**Figure S6D**). This experiment confirmed donor chimerism was dependent on anti-CD4-  
18 conditioning, but only achieved ~50% donor chimerism.

19  
20 We repeated the transplantation experiments using cells expanded from 20x10<sup>6</sup> WBMCs in anti-  
21 CD4-conditioned *Fancd2*<sup>-/-</sup> mice. We achieved robust levels (>80%) of myeloid donor chimerism  
22 even within four weeks, which remained stably high over 20 weeks (**Figure 4E-F**). Notably, we  
23 observed lower levels of donor chimerism in the bone marrow HSC compartment than  
24 downstream progenitor and peripheral blood compartments. These results suggest a selective  
25 advantage in the ability of wild-type HSCs to support hematopoiesis, as compared to *Fancd2*<sup>-/-</sup>  
26 HSCs, and correspond with the reported reduced fitness of *Fancd2*<sup>-/-</sup> HSCs<sup>38</sup>. By contrast, 5000  
27 freshly purified HSCs failed to engraft in *Fancd2*<sup>-/-</sup> mice while 4-week expanded cultures derived  
28 from 500 HSCs also displayed robust donor chimerism (**Figure 4E-F**). It is currently unclear  
29 why freshly-purified HSCs failed to engraft, but it is likely due to the low efficiency of  
30 engraftment in the non-conditioned setting and the need for supraphysiological HSC numbers for  
31 engraftment without bone marrow conditioning<sup>39</sup>. Together, these results highlight the potential  
32 translational implications for selective HSC expansion cultures.

### 33 34 **DISCUSSION:**

35 In this report, we demonstrate that we can improve the selectivity of PVA-based mouse HSC  
36 expansion cultures by optimizing O<sub>2</sub> concentrations. Furthermore, we have discovered that HSCs  
37 expand at the expense of GvHD-causing T cells under optimized low O<sub>2</sub> conditions.  
38 Additionally, we show proof-of-concept data for the combined use of expanded HSCs and  
39 antibody-mediated inhibition of immunologic rejection for allogeneic HSCT. However, further  
40 work will be needed to determine the translational potential of this work. For example, we will

1 need to investigate whether antibody conditioning for HLA-mismatched allo-HSCT could be  
2 combined with these approaches<sup>40</sup>. Additionally, our acute GvHD assays used T-cell-containing  
3 splenocytes, while peripheral blood products are used in the clinical setting.

4  
5 The risks of GvHD that are associated with allo-HSCT have led to a major research effort in  
6 autologous HSCT gene therapies, where a patient's HSCs are collected, gene corrected, and then  
7 returned to reconstitute a healthy hematopoietic system<sup>41,42</sup>. However, this therapeutic strategy is  
8 still challenging, expensive, and patient access is usually limited to a few specialized centers.  
9 Our results suggest that if HSC selective expansion methods can be translated to humans and the  
10 risk of GvHD becomes avoidable, GvHD-free allogeneic HSCT could provide a competitive  
11 approach to autologous HSCT gene therapies. These results also highlight the potential caveats  
12 for allogeneic HSCT. In hematological malignancies, allo-T cell-mediated graft-vs-tumor effects  
13 are essential for long-term remission following HSCT<sup>43</sup>. However, expanded HSC products  
14 could be combined with T cell add-back strategies and there may be advantages of being able to  
15 separate the dose of HSPCs from the dose of T cells.

16  
17 Besides the translational implications of this work, we also hope that our findings will support  
18 further investigations into HSC biology and hematopoiesis. The long-term ex vivo HSC culture  
19 system provides a tractable ex vivo model system to study HSC self-renewal and lineage  
20 commitment. In summary, we have identified physioxia as optimal for PVA-based HSC  
21 expansion cultures and demonstrated that these methods are highly selective for HSCs over more  
22 mature hematopoietic cell types, with implications for both basic and translational stem cell  
23 biology.

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#### 37 **AUTHORSHIP CONTRIBUTION:**

38  
39

1 KJI, IK, SC, DK, TKT, JB, IH, PYH, HMK, JWH, and KN designed experiments, performed  
2 experiments, analyzed the data, and reviewed and edited the manuscript. GPN, KB, AC, BG, and  
3 HN designed experiments, analyzed the data and reviewed and edited the manuscript. ACW  
4 designed experiments, performed experiments, analyzed the data, and wrote the manuscript.  
5

#### 6 **CONFLICT OF INTEREST DISCLOSURES:**

7 HN is a co-founder and shareholder in Megakaryon, and Century Therapeutics. ACW is a  
8 consultant for Graphite Bio and ImmuneBridge. AC discloses financial interests in the following  
9 entities working in the rare genetic disease space: Beam Therapeutics, Decibel Therapeutics,  
10 Editas Medicines, Global Blood Therapeutics, GV, Lyrik Therapeutics, Magenta Therapeutics,  
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12 execution, interpretation, or publication of the work in this manuscript.  
13

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- 20

1 **FIGURE LEGENDS:**

2 **Figure 1: Improved stability of PVA-based HSC cultures in low O<sub>2</sub>**

- 3 A. Schematic of low O<sub>2</sub> HSC cultures (left). Mouse CD150<sup>+</sup>CD34<sup>-</sup>KSL HSCs were sorted into  
4 PVA-based media in 96-well plates (50 cells per well) in 200ul of media and cultured for four  
5 weeks at 20%, 5%, or 1% O<sub>2</sub>. Representative flow cytometry plots for 4-week HSC-derived  
6 cultures (right). Upper panels display c-Kit and Sca1 expression within the Lineage<sup>-</sup> cell fraction  
7 while lower panels display CD201 and CD150 expression within the KSL cell fraction.  
8 B. Mean frequency of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells within the HSC-derived cultures described in  
9 A. n=4.  
10 C. Mean number of live cells per well within the HSC-derived cultures described in A. n=4.  
11 D. Mean number of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells per well within the HSC-derived cultures  
12 described in A. n=4.  
13 E. Mean number of non-KSL cells per well within the HSC-derived cultures described in A. n=4.  
14 F. 16-week donor peripheral blood chimerism from 4-week-old HSC-derived cultures incubated  
15 at 20%, 5%, or 1% O<sub>2</sub>. 5000 cells from each culture were transplanted alongside 1x10<sup>6</sup> WBMCs  
16 into lethally irradiated recipients. Mean ± standard deviation (SD); n=8-9.  
17 G. Differential gene expression analysis between 5% O<sub>2</sub> pHSC and 1% O<sub>2</sub> pHSC samples (left),  
18 or 5% O<sub>2</sub> pHSC and 20% O<sub>2</sub> pHSC samples (right). Results displayed as Log<sub>2</sub>(fold-change) vs -  
19 Log(adjusted P value).  
20 H. GO term enrichment analysis for underexpressed and overexpressed genes between 5% O<sub>2</sub>  
21 pHSC and 20 % O<sub>2</sub> pHSC samples.  
22 I. Fold change in CD201<sup>+</sup>CD150<sup>+</sup>KSL cells relative to control wells following a 7-day culture  
23 with the indicated compounds at 20% O<sub>2</sub>. Of 116 compounds tested, only the 74 that supported  
24 cell survival/growth are displayed (see **Supplementary Table 1** for a full list). Cell cultures  
25 were initiated with 50 CD201<sup>+</sup>CD150<sup>+</sup>KSL cells resorted from 3-week HSC cultures. Mean of 4  
26 wells (from 2 biological replicates) displayed.  
27 J. Mean frequency of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells (left) and mean number of live cells per well  
28 (left), within 7-day HSC-derived cultures at 20% O<sub>2</sub>, 5% O<sub>2</sub>, or 1% O<sub>2</sub>, cultured with either  
29 DMSO or the mitochondrial complex I inhibitor IACS-01-759 (20 nM). n=6.  
30 Statistical analysis was performed by ANOVA. n.s. denotes not significant; \* denotes p<0.05;  
31 \*\*\*\* denotes p<0.0001.

33 **Figure 2: Single-cell transcriptomics identifies molecular consequences of low O<sub>2</sub> on HSC**  
34 **cultures**

- 35 A. Schematic of the scRNA-seq experiment analyzing HSPCs cultured at different O<sub>2</sub>  
36 concentrations.  
37 B. UMAP projections of all samples with color-coded cluster membership.  
38 C. Manual annotation of clusters in B based on marker gene expression. Bas, basophil; Ery,  
39 erythroid; ILC, innate lymphoid cell; Ly/DC, lymphoid/dendritic cell; Meg, megakaryocyte; MC,  
40 mast cell; Mono typ, typical monocyte; Neu, neutrophil; prog, progenitor.

- 1 D. UMAP projections of all cells (grey) and cells from indicated conditions (blue). In each case,  
2 an equal cell number was randomly selected for each sample.
- 3 E. Bar plot indicating relative cell abundance in areas of the landscape for each sample. Areas  
4 were chosen as follows: HSC - cluster 1, Intermediate prog - clusters 0 and 2-4,  
5 Ery/Bas/MC/Meg prog - clusters 6, 7 and 17, Neu/Mono/DC - clusters 5, 11 and 14, Other -  
6 remaining clusters. \* a indicates statistically significant change in cell abundance (FDR <0.05)  
7 compared to the 20% O<sub>2</sub> condition.
- 8 F. UMAP projection color-coded with diffusion pseudotime values, overlaid with arrows  
9 indicating putative paths of differentiation using random walks estimation with CellRank  
10 Pseudotime Kernel.
- 11 G. UMAP projection color-coded by cell fate probability of cells differentiating into the tip of  
12 cluster 17 (Megakaryocytes).
- 13 H. Cell density along the trajectory shown in G for each sample, only pseudotime values  
14 between 0 and 0.05 are shown. Vertical lines indicate the regions of interest, where cells at 5%  
15 and 1% O<sub>2</sub> disappear.
- 16 I. Heatmap of genes differentially expressed between the beginning and end of the region of  
17 interest shown in H.
- 18 J. Enrichr gene enrichment analysis of upregulated genes within cluster 1 at 5% O<sub>2</sub>, compared to  
19 20% (left), and 1% O<sub>2</sub> (right).

20

21 **Figure 3: Low O<sub>2</sub> cultures expand HSCs from unfractionated WBMCs and embryonic**  
22 **tissues**

- 23 A. Schematic of low O<sub>2</sub> WBMC cultures. Unfractionated mouse WBMCs were seeded into  
24 PVA-based media in 24-well plate wells (5x10<sup>6</sup> cells per well) in 1ml of media and cultured for  
25 four weeks at 20%, 5%, or 1% O<sub>2</sub>.
- 26 B. Mean number of live cells per well within the WBMC-derived cultures described in A at day-  
27 28. n=6.
- 28 C. Mean number of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells per well within the WBMC-derived cultures  
29 described in A at day-28. n=6.
- 30 D. 16-week donor peripheral blood chimerism from 4-week-old WBMC-derived cultures  
31 incubated at 20%, 5%, or 1% O<sub>2</sub>. 5000 cells from each culture were transplanted alongside 1x10<sup>6</sup>  
32 WBMCs into lethally irradiated recipients. Mean ± SD; n=3-5.
- 33 E. 12-week donor peripheral blood chimerism following secondary transplantation of WBMCs  
34 from primary recipient mice described in D. Mean ± SD; n=3-4.
- 35 F. Schematic of CyTOF time course during 5% O<sub>2</sub> WBMC cultures (left) and frequency of  
36 immunophenotypic cell populations at indicated timepoints (right). See **Figure S5B** for cell  
37 immunophenotypes.
- 38 G. Schematic of CyTOF time course during 5% O<sub>2</sub> whole spleen cell cultures (left) and  
39 frequency of immunophenotypic cell populations over 28-day cultures (right). See **Figure S5B**  
40 for cell immunophenotypes.

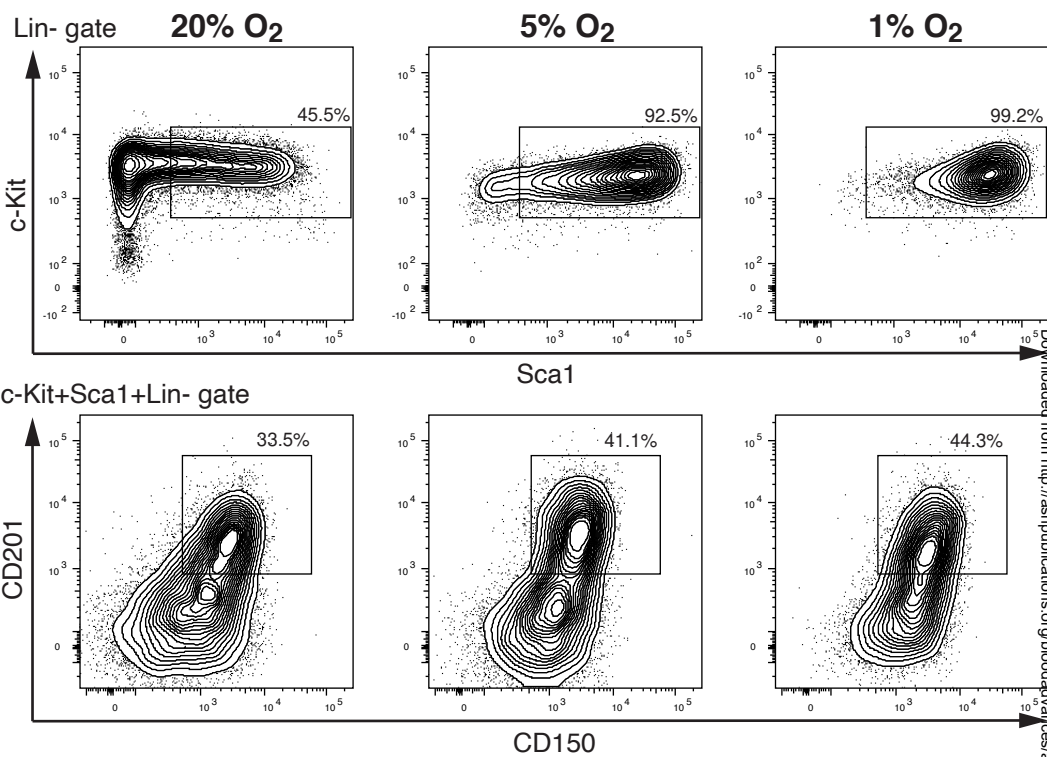
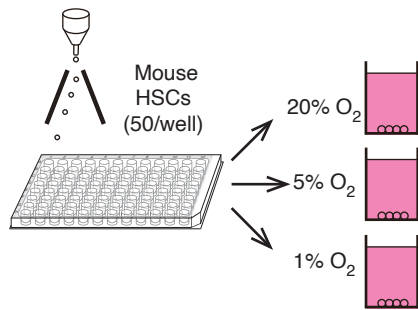
1 H. Schematic of selective HSC expansion assay from mouse embryonic tissues.  
2 I. Mean number of CD201<sup>+</sup>CD150<sup>+</sup>KSL cells generated from E11.5-12.5 embryonic tissue  
3 (excluding extraembryonic tissues and fetal liver), E11.5-16.5 heart, yolk sac, placenta, and fetal  
4 liver-derived cultures. Starting cell number indicated in brackets seeded in 1ml of media.  
5 Statistical analysis was performed by ANOVA. n.s. denotes not significant; \* denotes p<0.05;  
6 \*\*denotes p<0.01; \*\*\*\* denotes p<0.0001.

#### 8 **Figure 4: Low O<sub>2</sub> selective HSC cultures avoid GvHD**

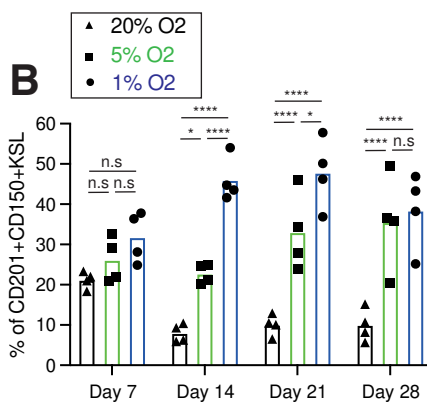
9 A. Schematic of allogeneic transplantation assay. Unfractionated WBMCs and/or spleen cells  
10 from C57BL/6 mice were transplanted into irradiated Balb/c mice before or after 4-week PVA-  
11 based culture.  
12 B. Survival of Balb/c recipients in the assay described in B, following transplantation of 5x10<sup>6</sup>  
13 WBMCs and 5x10<sup>6</sup> whole spleen cells (fresh or cultured) from C57BL/6 mice. n=7-8. Statistical  
14 analysis was performed by Mantel-Cox test. \*\*\*\* denotes p<0.0001.  
15 C. Survival of Balb/c recipients in the assay described in B, following transplantation of 5x10<sup>6</sup>  
16 whole spleen cells (fresh or cultured) from C57BL/6 mice. n=7-8. Statistical analysis was  
17 performed by Mantel-Cox test. \*\*\*\* denotes p<0.0001.  
18 D. Schematic of antibody conditioning transplantation assay. 4-week cultured WBMCs (derived  
19 from 20x10<sup>6</sup> WBMCs), 4-week cultured HSCs (derived from 500 HSCs), or 5000 fresh HSCs  
20 from C57BL/6-CD45.1 mice were transplanted into Fancc2<sup>-/-</sup>-CD45.2 mice 7 days after  
21 treatment with anti-CD4 (GK1.5) antibody.  
22 E. Donor chimerism in Fancc2<sup>-/-</sup> recipient mice at 4-20 weeks with peripheral blood Mac1<sup>+</sup>Gr1<sup>+</sup>  
23 myeloid cells (top left panel), B220<sup>+</sup> B cells (top right panel), CD4<sup>+</sup>CD3<sup>+</sup> T cells (bottom left  
24 panel), and CD8<sup>+</sup>CD3<sup>+</sup> T cells (bottom right panel). n=4.  
25 F. Donor chimerism in Fancc2<sup>-/-</sup> bone marrow and peripheral blood compartments for recipients  
26 described in F. n=4.

# Figure 1

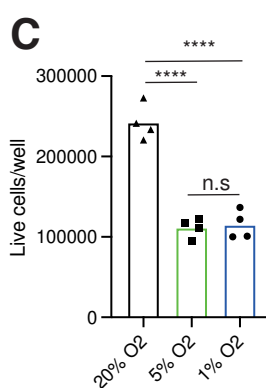
## A



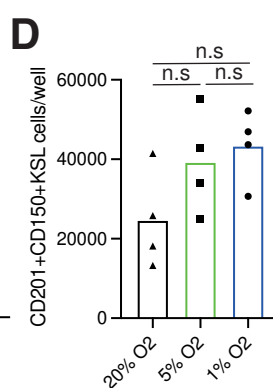
## B



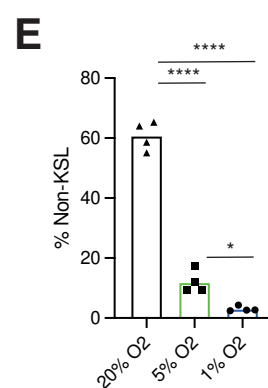
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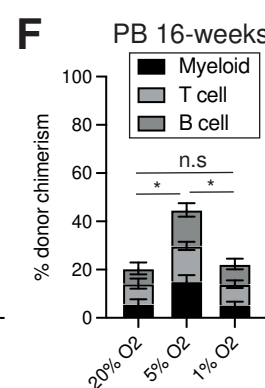
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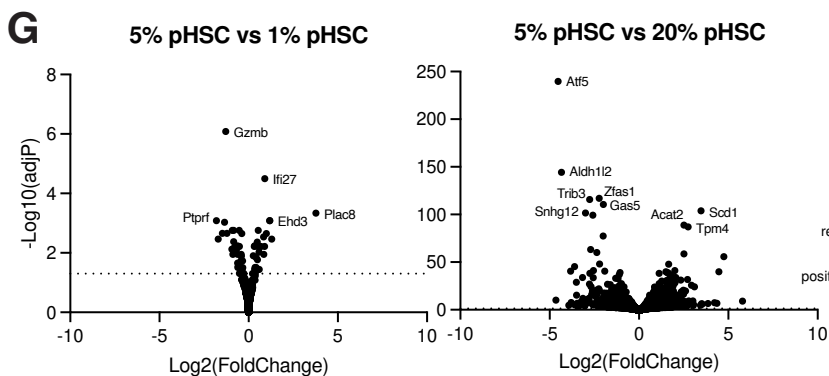
## E



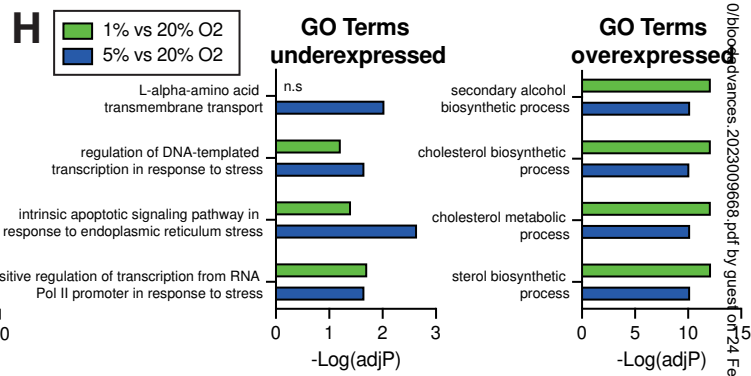
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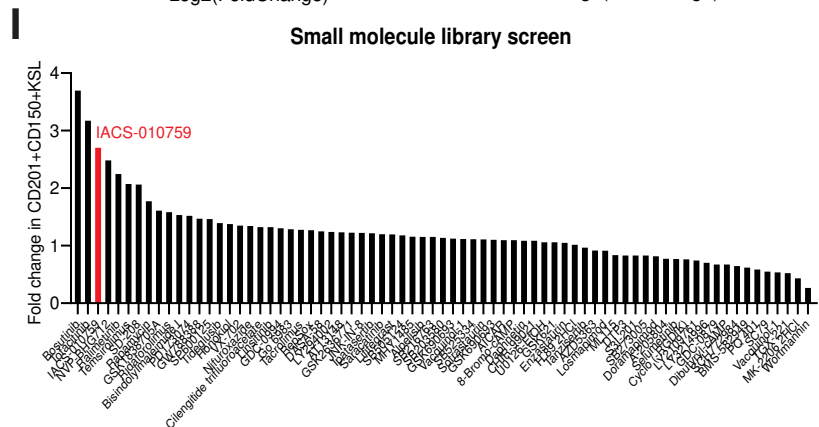
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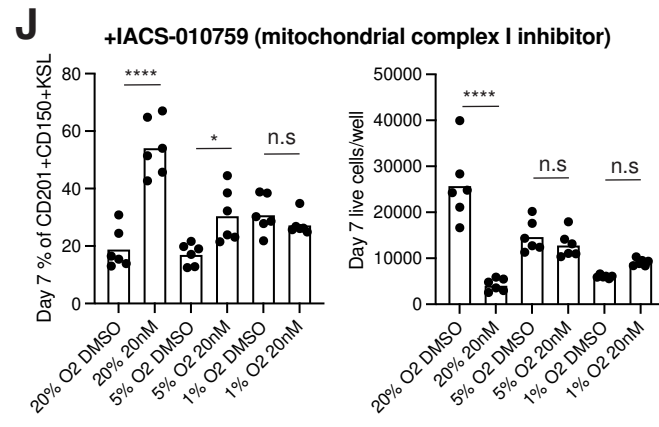
## H



## I

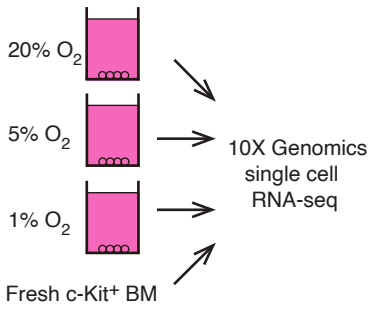


## J

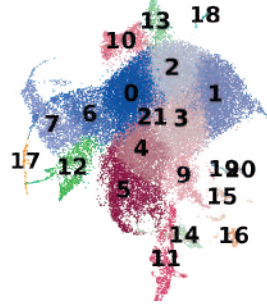


# Figure 2

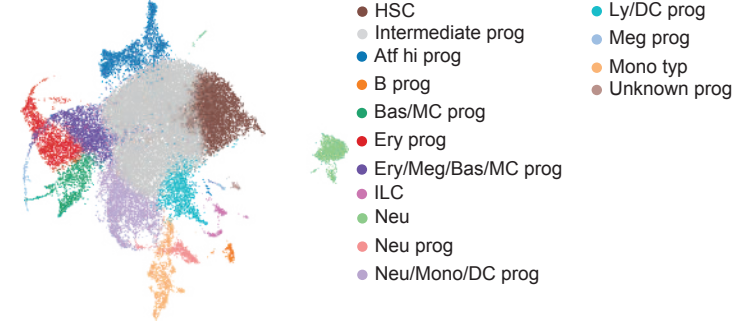
**A**



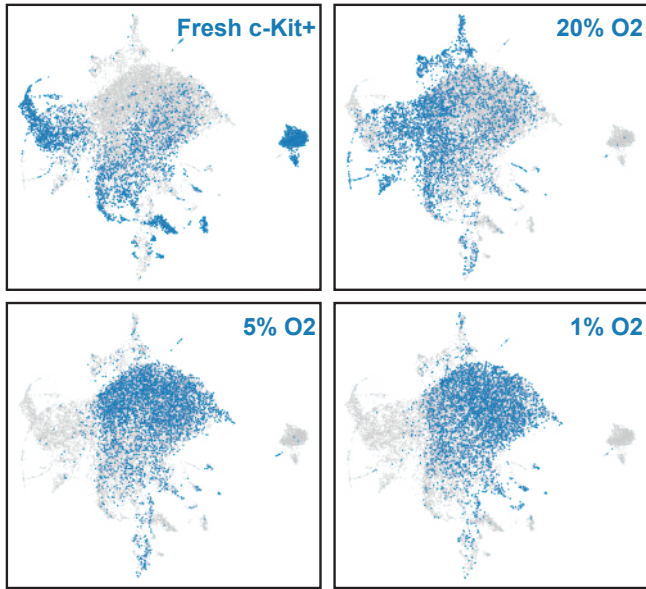
**B Clusters**



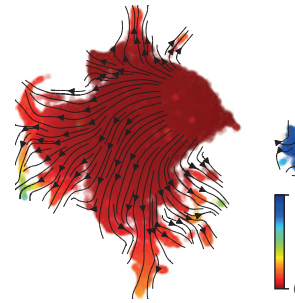
**C Annotation**



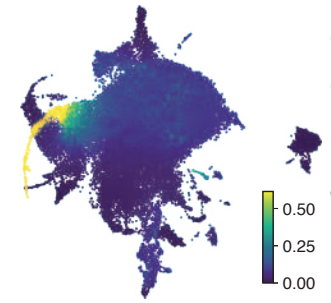
**D**



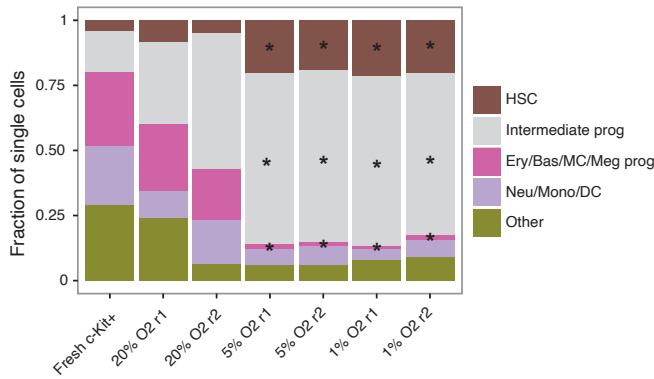
**F Pseudotime progression**



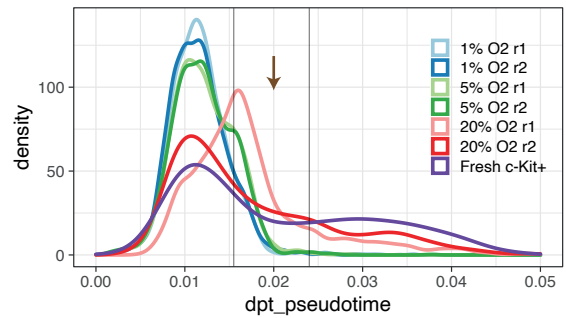
**G Meg-fate probability**



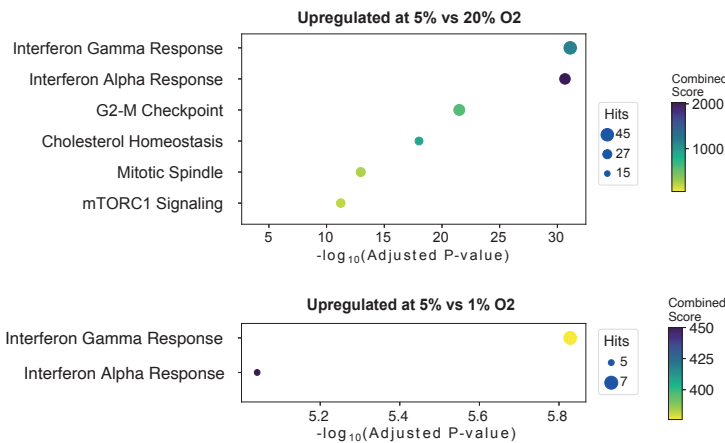
**E**



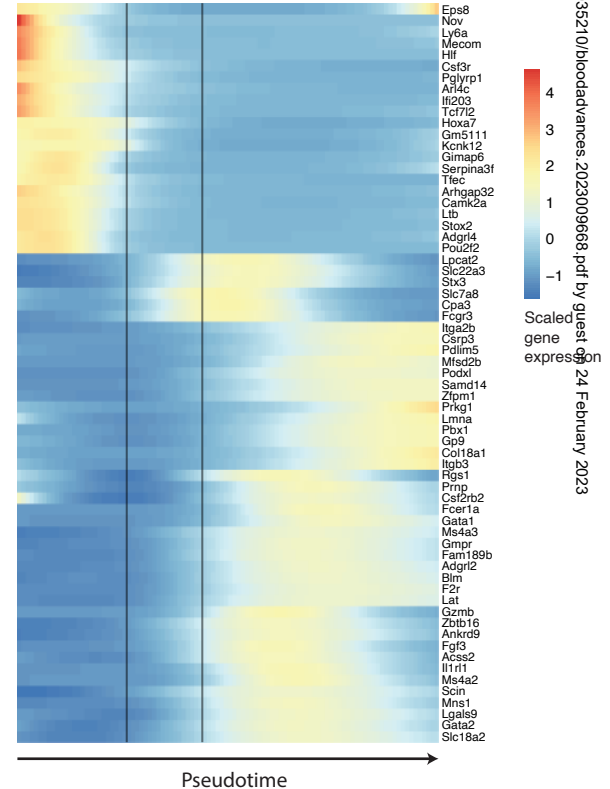
**H**



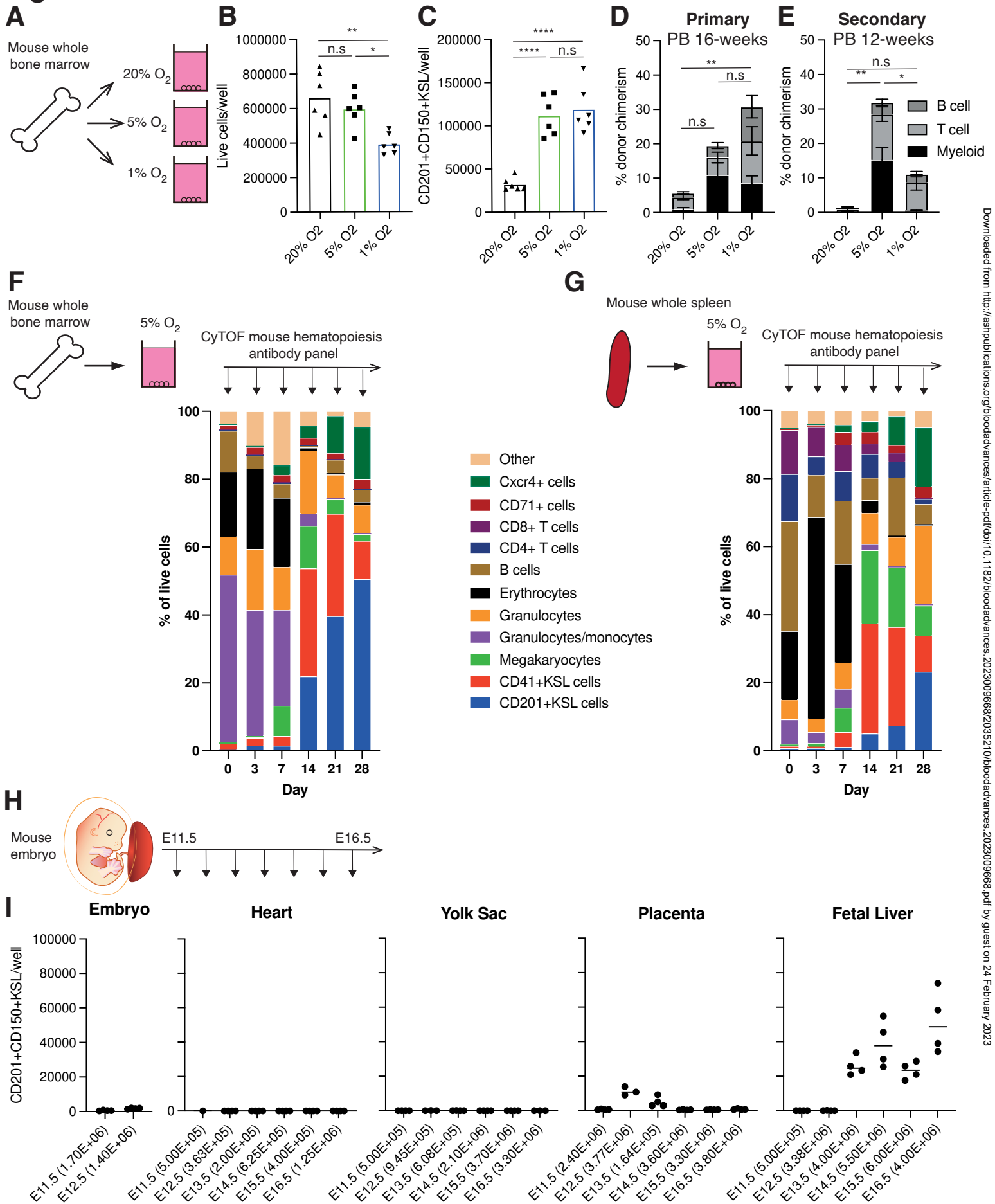
**J Cluster 1 differential gene expression (MSigDB Hallmark genesets)**



**I**

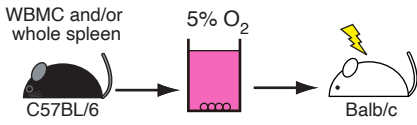


**Figure 3**

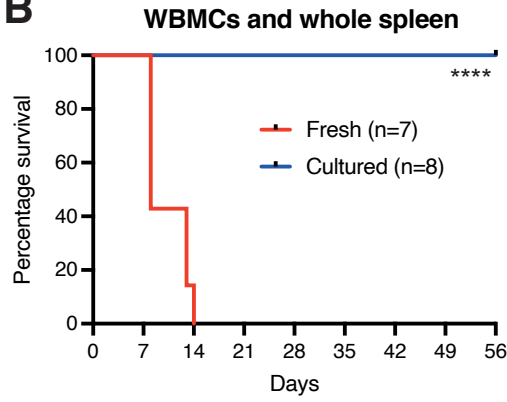


**Figure 4**

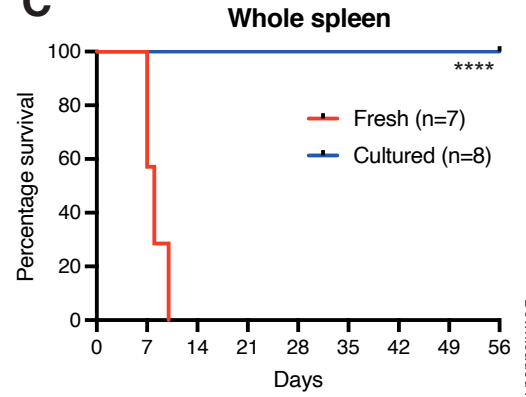
**A**



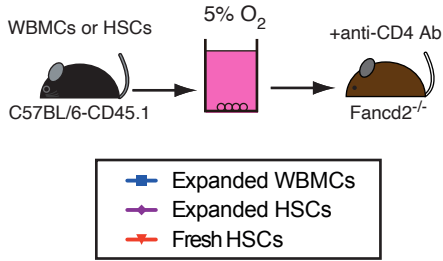
**B**



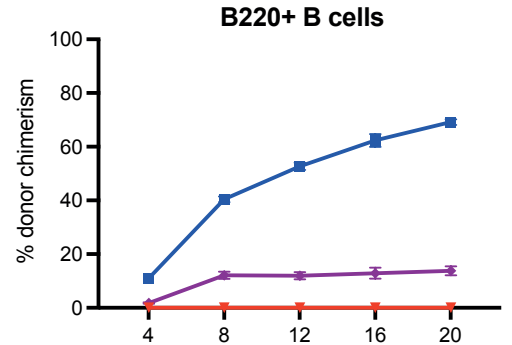
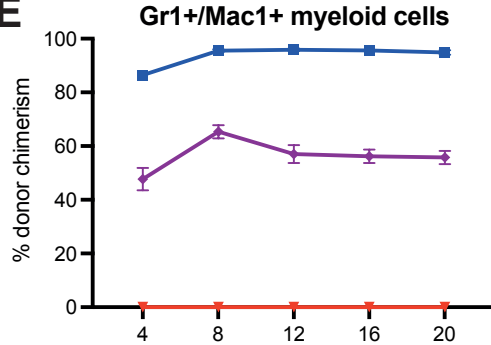
**C**



**D**



**E**



**F**

