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1 # Heterogeneous Responses of Hematopoietic Stem Cells to Inflammatory
2 # Stimuli are Altered with Age
3

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15

16 **One sentence summary**
17 Murine hematopoietic stem cells display transcriptional heterogeneity that is quantitatively altered with
18 age and leads to the age-dependent myeloid bias evident after inflammatory challenge.

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19 **Summary**
20
21 Long-term hematopoietic stem cells (LT-HSCs) maintain hematopoietic output throughout an animal's
22 lifespan. With age, however, they produce a myeloid-biased output that may lead to poor immune
23 responses to infectious challenge and the development of myeloid leukemias. Here, we show that young
24 and aged LT-HSCs respond differently to inflammatory stress, such that aged LT-HSCs produce a cell-
25 intrinsic, myeloid-biased expression program. Using single-cell RNA-seq, we identify a myeloid-biased
26 subset within the LT-HSC population (mLT-HSCs) that is much more common amongst aged LT-HSCs
27 and is uniquely primed to respond to acute inflammatory challenge. We predict several transcription
28 factors to regulate differentially expressed genes between mLT-HSCs and other LT-HSC subsets. Among
29 these, we show that *Klf5*, *Ikzf1* and *Stat3* play important roles in age-related inflammatory myeloid bias.
30 These factors may regulate myeloid versus lymphoid balance with age, and can potentially mitigate the
31 long-term deleterious effects of inflammation that lead to hematopoietic pathologies.
32
33 **Keywords**
34 Hematopoietic stem cells, stem cell aging, inflammation, single-cell RNA-sequencing
35
36 **Highlights**
37 - LT-HSCs from young and aged mice have differential responses to acute inflammatory challenge.
38 - HSPCs directly sense inflammatory stimuli *in vitro* and have a robust transcriptional response.
39 - Aged LT-HSCs demonstrate a cell-intrinsic myeloid bias during inflammatory challenge.
40 - Single-cell RNA-seq unmasked the existence of two subsets within the LT-HSC population that
41 was apparent upon stimulation but not steady-state. One of the LT-HSC subsets is more prevalent
42 in young and the other in aged mice.
43 - *Klf5*, *Ikzf1* and *Stat3* regulate age- and inflammation-related LT-HSC myeloid-bias.

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44 **Introduction**
45
46 Long-term hematopoietic stem cells (LT-HSCs) must overcome the stresses of aging to maintain
47 appropriate immune cell output throughout a human's life (Akunuru and Geiger, 2016; Chen et al., 2016;
48 Denkinger et al., 2015; Dykstra et al., 2011; Geiger et al., 2013; Mehta et al., 2015; Morita et al., 2010;
49 Sawai et al., 2016). These stresses include replicative stress (Bernitz et al., 2016; Flach et al., 2014; Wang
50 et al., 2012), as well as acute and chronic infectious challenge (King and Goodell, 2011; Nagai et al.,
51 2006). Hematopoietic stem and progenitor cells (HSPCs) express innate immune receptors (King and
52 Goodell, 2011), such as toll-like receptors (TLRs), and respond to many inflammatory mediators,
53 including IFN-γ (Baldridge et al., 2010), M-CSF (Mossadegh-Keller et al., 2013), and the gram-negative
54 bacterial component lipopolysaccharide (LPS) (Nagai et al., 2006). In response to acute LPS exposure,
55 LT-HSCs increase proliferation, mobilize to the peripheral bloodstream (King and Goodell, 2011), and
56 initiate emergency myelopoiesis to increase the system's output of innate immune cells (Haas et al.,
57 2015). This increased output may be mediated by hematopoietic progenitors, such as multipotent
58 progenitors (MPPs) (Pietras et al., 2015; Young et al., 2016), in part due to direct secretion of cytokines
59 that drive myeloid differentiation (Zhao et al., 2014).
60
61 Physiologic aging in both humans and mice leads to permanent changes in LT-HSC function, such as
62 myeloid-biased hematopoietic output (Akunuru and Geiger, 2016). This is often accelerated by chronic
63 inflammation and, when dysregulated, can lead to replicative exhaustion and extramedullary
64 hematopoiesis (Esplin et al., 2011; Mehta et al., 2015). Several hypotheses have been proposed to explain
65 the age related changes in LT-HSC function (Kovtonyuk et al., 2016). First, cell-intrinsic changes within
66 each aged LT-HSC might make it inherently myeloid-biased (Grover et al., 2016). Second, the LT-HSC
67 population may be comprised of subsets of myeloid- and lymphoid-biased cells, the composition of which
68 changes with age such that myeloid-biased LT-HSCs are more prevalent within the aged LT-HSC
69 population (Dykstra et al., 2007; Yamamoto et al., 2013). The true nature of these age-related changes
70 may in fact be a combination of both of these hypotheses, such that with age there is a growing subset of
71 more intrinsically myeloid-biased LT-HSCs.
72
73 The transcriptional state of LT-HSCs in steady state and in response to inflammatory mediators may help
74 shed light on these questions, but is currently still poorly understood. A number of epigenomic and
75 transcriptomic changes have been observed during bulk and single-cell analysis of young and aged LT-
76 HSCs (Cabezas-Wallscheid et al., 2014; Grover et al., 2016; Kowalczyk et al., 2015; Mehta and
77 Baltimore, 2016; Sanjuan-Pla et al., 2013; Sun et al., 2014; Yu et al., 2016). However, it is unclear if and
78 how these changes lead to altered LT-HSC function, as seen with age-related myeloid bias (Dykstra et al.,
79 2011; Gekas and Graf, 2013). In particular, a recent study using single-cell RNA-seq (scRNA-seq)
80 (Kowalczyk et al., 2015) of steady-state, resting LT-HSCs has not identified a subpopulation structure;
81 however, an appreciation of the cell-intrinsic differences between young and aged LT-HSCs may become
82 apparent in the setting of acute stress, such as when analyzing their response to inflammatory challenge.
83 An understanding of how the response of LT-HSCs to inflammatory mediators changes with age may
84 therefore help elucidate the underlying mechanism of age-related myeloid bias. This may further provide
85 insight into age-related pathologies such as improper immune responses to vaccines or infectious
86 challenge, and the development of myeloid leukemia.
87

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88 In this work, we investigate the acute inflammatory response of mouse HSPCs *in vitro* and *in vivo*, and
89 how this response may be altered with age (**Figure 1A**). We demonstrate that major HSPC subtypes
90 respond transcriptionally to inflammatory stimuli and that this response is similar to the prototypical
91 response of mouse bone marrow derived dendritic cells (BMDCs). Using *in-vivo* experiments, we show
92 that the age-dependent myeloid bias after inflammatory challenge is intrinsic to LT-HSCs. Using single-
93 cell RNA-seq (scRNA-seq) we find that the LT-HSC compartment is comprised of at least two subsets
94 that become apparent only upon stimulation. One of these subsets has features consistent with myeloid-
95 bias, with distinct cell-intrinsic responses to inflammatory stimulation. The myeloid-biased subset
96 increases dramatically with age. We identify putative transcriptional regulators of these cell states, and
97 demonstrate the role of these regulators in age-related myeloid bias and differential responses to TLR
98 ligands.
99
100 **Results**
101
102 ***Differential response of young and aged mice to LPS in vivo***
103 To investigate the acute inflammatory response of hematopoietic progenitors from mice at different ages,
104 we challenged 8-12 week old ('young') and 20-24 month old ('aged') mice with a single intraperitoneal
105 injection of LPS (0.5μg/kg, **Figure 1B-E**). Young mice responded with a gradual increase in the
106 frequency of peripheral blood T cells over 72 hours, which was sustained 9 weeks after the LPS challenge
107 (**Figure 1B**). Conversely, aged mice had an over twofold lower baseline frequency of T cells in their
108 peripheral blood compared to young mice, and the frequency of these cells remained unchanged after the
109 LPS challenge (**Figure 1B**). Both young and aged mice showed a decrease in peripheral blood B cell
110 frequencies after LPS treatment. However, the acute response was particularly dramatic in aged mice,
111 which had a twofold loss in the frequency of B cells by 72 hours, and then recovered to levels comparable
112 to those seen in young mice by 6 weeks post-challenge (**Figure 1C**). In contrast to the milder increase in
113 the myeloid output of young mice, there was an over twofold increase in peripheral blood myeloid
114 frequencies in aged mice by 72 hours post-challenge (**Figure 1D,E**). This eventually normalized to
115 baseline frequencies by 9 weeks post-challenge. Aged mice therefore demonstrated a strong acute
116 increase in myeloid output in response to inflammatory challenge; this was not observed in young mice,
117 which conversely responded primarily with an increase in T cell output.
118
119 To evaluate the cumulative effect of acute inflammatory challenges on myeloid output, we performed an
120 LPS boost of all cohorts 2 months after the initial challenge. This resulted in a dramatic upregulation of
121 peripheral blood myeloid cells in aged mice lasting at least 12 days, whereas again, only a milder increase
122 in myeloid output was seen in young mice (**Figure S1A**). The spleens of stimulated aged mice revealed
123 increased myeloid cell frequencies and a dramatic loss of T cells compared to young mice (**Figure S1B**).
124 Finally, bone marrow of stimulated aged mice had a three-fold enrichment for LT-HSCs compared to
125 stimulated young mice, with a milder enrichment in short-term HSCs (ST-HSCs) and MPPs (**Figure**
126 **S1C**). This enrichment in LT-HSCs is higher than the two-fold enrichment seen between unstimulated
127 aged and young mice (Beerman et al., 2010; Mehta et al., 2015), suggesting that acute inflammatory
128 stimuli promote myeloid-biased output from HSPCs and that the differences in immune cell output might
129 originate from LT-HSCs.
130

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131 ***Aged LT-HSCs stimulated with inflammatory signals yield a distinctive, long-term myeloid-biased***
132 ***output***
133 To examine whether the age-related myeloid bias in hematopoietic output after inflammatory challenge is
134 intrinsic to LT-HSCs, we tested the impact of stimulation on the ability of young and aged LT-HSCs to
135 reconstitute the immune system. Specifically, we first sorted LT-HSCs, ST-HSCs and MPPs from young
136 and aged CD45.2 C57BL/6 mice (**Figure S2**). The cells were then either maintained unstimulated or
137 stimulated with LPS and Pam3csk4 for 2 hours *in vitro*. They were subsequently transplanted together
138 with CD45.1 bone marrow helper cells into lethally irradiated young CD45.1 C57BL/6 mice. Peripheral
139 blood counts of CD45.2 expressing cells were monitored for several months (**Figure 1F-I** and **Figure**
140 **S3A,B**). Both unstimulated and stimulated, young and aged LT-HSCs demonstrated long-term
141 reconstitution of the immune system in these primary transplants. All transplanted ST-HSCs maintained
142 hematopoietic output for up to 3 months, whereas all MPPs failed long-term reconstitution (**Figure**
143 **S3A,B**), as expected. To validate which of these cells maintain long-term reconstitution potential, we
144 performed secondary transplants by taking bone marrow donor cells from primary transplant mice and
145 transplanting them to lethally irradiated young CD45.1 mice. Bone marrow from mice initially
146 transplanted with CD45.2 LT-HSCs successfully reconstituted the immune system with CD45.2 immune
147 cells while bone marrow from mice transplanted with CD45.2 ST-HSCs or MPPs failed to do so (**Figure**
148 **S3C,D**).
149
150 Notably, stimulated aged LT-HSCs led to a distinctive immune cell output in the reconstitution
151 experiments, with a particularly marked myeloid bias compared to all other conditions (**Figure 1F-I**). At
152 3 months post-reconstitution (*i.e.* 3 months after the *in vitro* LPS/Pam3csk4 challenge), no difference in
153 the frequencies of peripheral blood myeloid and lymphoid cells were seen between mice reconstituted
154 with either stimulated or unstimulated young LT-HSCs (**Figure 1F-I**). As previously reported (Beerman
155 et al., 2010; Pang et al., 2011), unstimulated aged LT-HSCs had higher peripheral blood myeloid output
156 and lower lymphoid output compared to unstimulated young LT-HSCs (**Figure 1F-I**). However,
157 stimulated aged LT-HSCs demonstrated a further decrease in the frequency of peripheral blood lymphoid
158 cells (**Figure 1F,G**) and a marked increase in the frequency of peripheral blood myeloid cells (**Figure**
159 **H,I**), even compared to the unstimulated aged LT-HSCs. Thus, aged LT-HSCs demonstrated myeloid-
160 biased 'memory' of the initial *in vitro* LPS/Pam3csk4 challenge that persisted for several months post-
161 reconstitution, a phenomenon not seen with stimulated young LT-HSCs. Interestingly, no significant
162 difference in LT-HSC frequency, including the previously identified myeloid-biased CD41+ LT-HSC
163 subpopulation (Gekas and Graf, 2013), was observed between cohorts (**Figure S3E-H**).
164
165 ***HSPCs demonstrate a canonical transcriptional response to TLR ligands***
166 We hypothesized that the differential effects of young and aged stimulated LT-HSCs may be due to a
167 variable immediate transcriptional response to inflammatory signals. To test this hypothesis, we measured
168 the transcriptional profiles of populations of HSPCs from young and aged mice during a 12-hour time-
169 course of LPS/Pam3csk4 stimulation *in vitro* (**Figure 2A**). LT-HSCs, ST-HSCs and MPPs from both
170 young and aged mice all demonstrated a robust and similar transcriptional response (**Figure 2B**), which
171 largely resembled that seen in mature cell types with different physiological functions, such as BMDCs
172 after LPS stimulation (**Figure 2B-D**) (Jovanovic et al., 2015). This includes the same temporal ordering
173 of induction in inflammatory gene clusters (**Figure 2C**) as in mature cell types (Amit et al., 2009;
174 Ramirez-Carrozzi et al., 2009), up-regulation of NF-κB-related genes (**Figure 2C**) (Bhatt et al., 2012;

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175 Hao and Baltimore, 2009), and induction of the expression of several effector cytokines (**Figure 2D**),
176 albeit at slightly lower levels (**Figure S4A-C**). Thus, the response of young and aged HSPCs to
177 inflammatory activation follows the canonical response of mature cells to similar stimulation, both in the
178 identity of the regulated genes and in the timescale of the response. This suggests that the differences in
179 the reconstitution outcome could not be resolved by the differences in the transcriptional response when
180 measured at the population level.
181
182 ***Single-cell RNA-seq reveals two subsets of LT-HSCs with distinct responses to stimulation and***
183 ***compositional changes with age***
184 Next, we considered the possibility that there are different subsets of LT-HSCs either in steady state or
185 post-stimulation ("cell intrinsic changes"), and that their relative proportions may change with age
186 ("compositional changes"). Early hematopoietic progenitors are comprised of heterogeneous functional
187 subpopulations (Benz et al., 2012; Gekas and Graf, 2013; Morita et al., 2010; Sanjuan-Pla et al., 2013),
188 which often reveal themselves in response to inflammatory stimuli (Haas et al., 2015; Zhao et al., 2014).
189 While a previous scRNA-seq study of LT-HSCs has mostly revealed age-related differences in the cell
190 cycle (Kowalczyk et al., 2015), we hypothesized that stimulation could unveil additional cell intrinsic
191 distinctions that cannot be observed in resting cells.
192
193 To determine the composition of HSPCs in each age group and condition, we performed full-length
194 scRNA-seq (Picelli et al., 2013) of young and aged LT-HSCs, ST-HSCs and MPPs, with and without 2
195 hours of *in vitro* LPS/Pam3csk4 stimulation. As we aimed to distinguish cell intrinsic, possibly subtle,
196 stimulus-specific states within a very well-defined cell population, we opted for the deeper-coverage full
197 length scRNA-Seq approach over massively parallel approaches (Tanay and Regev, 2017; Wagner et al.,
198 2016). We profiled 2046 individual cells from nine mice (5 young, 4 aged), with 124-186 cells for each
199 given cell type and condition. In order to eliminate sources of variability resulting from known
200 confounding factors, we removed 611 cells as low-quality and 58 as possible contaminants (**STAR-**
201 **Methods**). In addition, 578 of the cells were actively cycling (**STAR-Methods**). Overall, we retained 949
202 cells for subsequent analysis, comprised of 187 MPPs, 404 ST-HSCs, and 358 LT-HSCs.
203
204 We identified three major groups of cells using unsupervised clustering (see **STAR-Methods**) (**Figure**
205 **3A**; denoted clusters 1, 2 and 3). Cluster 1 (311 cells) contained most (302 of 354) of the unstimulated
206 HSPCs of all types, forming a continuum from MPPs to LT-HSCs (**Figure 3B**), with aged and young LT-
207 HSCs clustering together (**Figure 3D**), and hardly any stimulated cells. Clusters 2 (103 cells) and 3 (421
208 cells) almost exclusively contained stimulated HSPCs (**Figure 3C**), and had opposing patterns with
209 respect to aged and young LT-HSCs (**Figure 3E**): cluster 3 contained 77% of the aged stimulated LT-
210 HSCs and only 13% of the young stimulated LT-HSCs (**Figure 3E**), whereas cluster 2 had 72% of the
211 young stimulated LT-HSCs, and only 10% of the aged stimulated LT-HSCs (**Figure 3E**). This suggests
212 that there are distinct subsets of LT-HSCs in the bone marrow that can be discerned by their different cell
213 intrinsic responses to stimulation, and that the relative frequencies of these subsets appear to change with
214 age. Of note, the distinction between these LT-HSC subsets could only be discerned with stimulation.
215 Given these findings, we focused further analysis on LT-HSCs.
216

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217 ***Stimulated aged LT-HSCs express a myeloid-biased gene expression signature***
218 To identify the differences between the stimulated LT-HSCs in cluster 3 and cluster 2, we examined
219 genes that were differentially expressed between the two clusters. Cluster 3-specific genes were enriched
220 for genes related to myeloid function and inhibiting lymphoid differentiation, including pathways related
221 to NF-κB localization, negative regulation of lymphocyte development, macrophage proliferation, cell
222 migration and localization, and platelet derived growth factor signaling (**Figure S4D**). Cluster 2-specific
223 genes were enriched for genes involved in lymphocyte development, cell proliferation, and the acute
224 inflammatory response (**Figure S4E**). Of note, cells in any given cluster, regardless of age, were similar
225 to each other and distinctly different from cells in the other cluster. Interestingly, we observed some
226 differences in the level of expression of inflammatory genes such as IL6 and TNFα in stimulated LT-
227 HSCs in both clusters (**Figure S4F,G**). This data suggests that aged and young LT-HSCs have different
228 proportions of cells that display unique lineage-biased pathway preferences in response to inflammatory
229 signals.
230
231 **Myeloid-biased LT-HSCs can be identified by a distinct signature in unstimulated LT-HSCs and**
232 **their proportion increases with age**
233 To test whether the LT-HSC subsets exist also in unstimulated cells in steady-state conditions, we
234 identified the 47 genes that were differentially expressed both when comparing cluster 3 *vs.* cluster 2
235 (SCDE FDR < 0.01) and when comparing unstimulated aged *vs.* young LT-HSCs within cluster 1 (**Figure**
236 **S5A; STAR-Methods**, SCDE FDR < 0.1). We then tested whether these 47 genes coherently co-vary
237 across the 149 unstimulated LT-HSCs, and thus might reflect a variable cell state within these cells.
238 Indeed, we identified three distinct co-varying gene clusters (**FIgure 3F**), two of which contained genes
239 involved in myeloid and platelet differentiation, including *Selp*, *Vwf*, *Gpr64*, *Plscr2*, and *Wdfy1*. Notably,
240 recent studies have reported myeloid-biased CD41, Vwf or CD150-high expressing LT-HSC
241 subpopulations (Dykstra et al., 2011; Gekas and Graf, 2013; Sanjuan-Pla et al., 2013); in our analysis,
242 aged LT-HSCs have increased yet variable expression of CD150 and Vwf, and to a lesser extent
243 expression of CD41 (**Figure 3H and Figure S4H-J**). A recent report has also suggested Hoxb5 can be
244 used as a marker for truly long-term reconstituting subsets of LT-HSCs (Chen et al., 2016); our results
245 show no significant difference in Hoxb5 expression among the various subsets (**Figure 3H**).
246
247 Next, we generated a refined gene signature by first scoring unstimulated LT-HSCs with the initial set of
248 47 genes (**Figure S5A**), identifying two putative cell subsets (**STAR Methods**). We then used these
249 subsets to initialize *k*-means clustering (k=2) within the unstimulated LT-HSCs. We used the identities of
250 the cells based on this clustering to designate them as myeloid-biased LT-HSCs, or “mLT-HSCs”, and
251 non-myeloid biased LT-HSCs, or “nmLT-HSCs”. We next tested these two final clusters for differentially
252 expressed genes, finding 365 upregulated genes and 34 downregulated genes in the mLT-HSC cluster,
253 which we use to define our “mLT-HSC signature” (SCDE FDR < 0.1 and the same direction of change as
254 for stimulated mLT-HSCs) (**Table S1**).
255
256 In the final *k*-means clusters, 92% of cells in the myeloid biased cluster were aged cells and only 8% were
257 young cells (**Figure 3G**, to the right of the dashed line), while only 20% of cells in the non-myeloid
258 biased cluster were aged cells and 80% were young cells (**Figure 3G**, to the left of the dashed line). This
259 is consistent with our findings that the frequency of stimulated mLT-HSCs increases with age (**Figure**
260 **3E**). Applying the same signature to our stimulated LT-HSCs or to an independent dataset of

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261 unstimulated aged and young LT-HSCs from two mouse strains (Kowalczyk et al., 2015) showed
262 consistent results: while the LT-HSC population is inherently heterogenous, more aged LT-HSCs score
263 highly for the mLT-HSC signature (**Figure S5B,C**). Thus, the mLT-HSC signature allowed us to identify
264 the subtle portion of myeloid-biased like cells among the young unstimulated LT-HSCs, and to show that
265 the proportion of high-scoring mLT-HSC cells rises with age.
266
267 ***Identification of transcription factors regulating LT-HSC subpopulations***
268 To identify transcription factors (TFs) that may regulate differentially expressed genes between the mLT-
269 HSC and nmLT-HSC subsets, we looked for enriched TF motifs in the enhancer sequences associated
270 with these genes (Lara-Astiaso et al., 2014) (**Figure 4A,B**). In particular, we focused on TFs which
271 themselves were differentially expressed between mLT-HSCs and nmLT-HSCs (**Figure 4A,B**), since
272 altered transcriptional regulation of a TF is likely to affect its target genes.
273
274 Among the 10 significant TFs (**Figure 4A,B**; blue dots) were *HoxA9*, *Klf4*, *Klf5*, *Ikzf1*, and *Stat3* (**Figure**
275 **4C-E and Figure S5D-F**). *HoxA9* is known to potentiate LT-HSC function (Lebert-Ghali et al., 2016)
276 and lead to LT-HSC proliferation (Lebert-Ghali et al., 2016; Smith et al., 2011); interestingly, it was
277 upregulated in the aged stimulated mLT-HSCs (comparing aged LT-HSCs between cluster 3 and 2;
278 **Figure 4B**). The role of *Klf4*, *Klf5* and *Ikzf1* in LT-HSC function is less well established, although it has
279 been suggested that *Klf5* may play a role in LT-HSC homing to the bone marrow niche (Taniguchi
280 Ishikawa et al., 2013). All four TFs had their motifs enriched in the enhancers of genes upregulated in
281 mLT-HSCs (**Figures 4A-D**). Among these, *Klf4* and *Klf5* were transcriptionally upregulated in mLT-
282 HSCs, whereas *Ikzf1* was down-regulated in mLT-HSCs, and had motif instances enriched in genes
283 upregulated in these cells (**Figure 4A,E**), consistent with its known role as a repressor (Koipally et al.,
284 1999). *Stat3* is a known regulator of HSC self-renewal, especially under stress conditions, (Chung et al.,
285 2006) and loss of *Stat3* is associated with a LT-HSC aging phenotype (Mantel et al., 2012). *Stat3* binding
286 sites were enriched at enhancers of genes upregulated in mLT-HSCs (**Figures 4A,E**).
287
288 ***Klf5, Ikzf1 and Stat3 play a role in age-related inflammatory myeloid bias of LT-HSCs***
289 We tested the predicted role of these TFs in the age-related myeloid bias of LT-HSCs by shRNA
290 knockdowns of each of *HoxA9*, *Klf4*, *Klf5*, *Ikzf1* or *Stat3* in young and aged HSPCs, leading to a 50-75%
291 reduction in mRNA expression of each gene (**Figure S6A**). We compared these knockdowns to a control
292 empty vector (MG) and knockdown of *Zbtb4*, a gene that is expressed in LT-HSCs but not differentially
293 expressed between mLT-HSCs and nmLT-HSCs. HSPCs were first isolated from young and aged mice,
294 and subsequently transduced with a retroviral vector expressing an shRNA construct for a particular TF.
295 These cells were then used to reconstitute lethally irradiated young C57BL/6 recipient mice. As expected,
296 at 3-months post reconstitution, when all the cells produced are progeny of transplanted LT-HSCs, we
297 found that mice reconstituted with control (MG vector) young LT-HSCs had higher lymphoid output
298 (**Figure 5A**, compare black bars to dark red bars), and lower myeloid (CD11b+) and granulocyte (Gr-1+)
299 output (**Figure 5B,C**, compare black bars to dark red bars) than mice reconstituted with control aged LT-
300 HSCs.
301
302 Of the tested TFs, *Klf5 and Ikzf1* had significant, age-dependent effects. Consistent with the upregulated
303 expression of *Klf5* in mLT-HSCs, we found that knockdown of *Klf5* in aged LT-HSCs resulted in
304 increased lymphoid output (**Figure 5A**) and decreased myeloid output to levels seen with control young
305 LT-HSCs (**Figure 5B,C**), whereas its knockdown in young LT-HSCs had no discernable effect compared

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306 to controls. Knockdown of *Ikzf1* in young LT-HSCs resulted in decreased lymphoid output (**Figure 5A**).
307 Interestingly, loss of *Ikzf1* in aged LT-HSCs had no significant effect on lymphoid output, but like
308 knockdown of *Klf5*, it resulted in decreased myeloid output to levels seen with control young LT-HSCs
309 (**Figure 5B,C**).
310
311 We next tested whether these TFs regulate myeloid output of LT-HSCs under conditions of inflammatory
312 stress. To do this, we challenged with LPS the aforementioned mice, each expressing an shRNA of a
313 different TF in the bone marrow compartment (as in **Figure 1B-E**). The frequency of peripheral blood
314 cells was tracked for 3 weeks after the LPS challenge (**Figure 5D-I and Figure S6B,C**). As expected,
315 mice transplanted with aged LT-HSCs expressing the control vector showed a sustained upregulation of
316 myeloid output over the 3 weeks (**Figure 5D-I**, solid red lines); whereas mice transplanted with young
317 LT-HSCs expressing the control vector showed only a transient increase in myeloid output 72 hours after
318 the initial challenge, followed by rapid recovery to baseline peripheral blood myeloid frequencies (**Figure**
319 **5D-I**, solid black lines).
320
321 In mice transplanted with aged LT-HSCs expressing the *Klf5* shRNA, the response to LPS phenocopied
322 that of mice transplanted with young control LT-HSCs (**Figure 5G**). These mice responded with a
323 transient increase in myeloid output at 72 hours and rapid recovery to baseline myeloid output, which was
324 lower than that seen with mice transplanted with aged control LT-HSCs (**Figure 5G**). Similarly, mice
325 transplanted with aged LT-HSCs expressing the *Ikzf1* shRNA had a muted response compared to mice
326 transplanted with aged control LT-HSCs (**Figure 5E**). These mice sustained myeloid output for 1 week
327 after the initial challenge before returning to their baseline myeloid output, which was equivalent to that
328 seen in mice transplanted with young control LT-HSCs (**Figure 5E**). Thus, both *Klf5* and *Ikzf1* may play
329 a critical role in regulating inflammatory myelopoiesis in aged LT-HSCs. Mice transplanted with aged
330 LT-HSCs expressing the *Stat3* shRNA also had lower baseline myeloid output than mice transplanted
331 with aged control LT-HSCs, and showed minimal response to LPS (**Figure 5I**), suggesting *Stat3* may also
332 play an important role in the overall inflammatory response of LT-HSCs.
333
334 To identify the cell types responsible for the changes observed after *Ikzf1*, *Stat3* or *Klf5* knockdown in
335 aged HSPCs, we analyzed the bone marrow compartment of all mice 3 months post transplantation. Mice
336 transplanted with aged control cells had higher LT-HSC and lower common lymphoid progenitor (CLP)
337 frequencies compared to mice reconstituted with young control cells (**Figure 5J-L**, compare black bars to
338 dark red bars). Interestingly, knockdown of either *Klf5*, *Ikzf1* or *Stat3* in transplanted aged LT-HSCs
339 resulted in a decreased frequency of LT-HSCs compared to control transplanted aged LT-HSCs, although
340 the effect of *Klf5* knockdown was only marginally statistically significant (**Figure 5J**). These
341 knockdowns resulted in LT-HSC frequencies and numbers that were comparable to mice transplanted
342 with young control LT-HSCs (**Figure 5J**). No significant effect on LT-HSC bone marrow frequency was
343 seen in mice transplanted with young LT-HSCs expressing any of these knockdown constructs (**Figure**
344 **5J,** grey bars).
345
346 Some decrease in bone marrow CMP frequency and an increase in CLP frequency was seen in mice
347 transplanted with aged LT-HSCs expressing the *Ikzf1* shRNA when compared with control aged LT-
348 HSCs, however these differences were not statistically significant (**Figure 5K,L**). This trend may suggest
349 that *Ikzf1* knockdown in aged LT-HSCs phenocopies control young LT-HSCs. No statistically significant
350 changes were observed in the frequencies of other progenitors, including ST-HSCs, MPPs and MEPs

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351 when comparing mice transplanted with aged LT-HSCs expressing one of the knockdown constructs
352 compared to aged control LT-HSCs (**Figure S6D-F**). These data therefore suggest that *Klf5*, *Ikzf1* and
353 *Stat3* regulate inflammatory myeloid bias in aged LT-HSCs and may do so by altering the function and
354 frequency of LT-HSCs in the bone marrow compartment.
355
356 **Discussion**
357
358 In this work, we demonstrate that LT-HSCs have a heterogeneous response to inflammatory stimuli that
359 is altered with age. We show that even the most multipotent of HSPCs directly respond *in vitro* to TLR
360 ligands with a potent transcriptional response. Using scRNA-seq, we demonstrate that both the young and
361 aged LT-HSC compartments are comprised of at least two distinct subsets of cells with a defined
362 molecular signature, and with age, the LT-HSC population is enriched for myeloid-biased-like LT-HSCs
363 (mLT-HSCs). Of note, it cannot be verified if the mLT-HSCs identified in this work represent irreversible
364 state or plastic/reversible state within this subpopulation. We posit that an increased proportion of mLT-
365 HSCs in the bone marrow is a key driver of emergency myelopoiesis and further identify several
366 transcription factors that regulate steady state and inflammatory myeloid bias in aged LT-HSCs.
367 Together, our data suggests a revised model (**Figure 6**) of age-related inflammatory myelopoiesis that
368 highlights important contributions from LT-HSCs, the earliest hematopoietic progenitor.
369
370 Whereas we found an approximately 30% increase in myeloid cell output in young mice after LPS
371 challenge, this bias is dramatically higher in aged mice, which have a two-fold increase in myeloid output
372 (**Figure 6**). Interestingly, we found that after inflammatory challenge, aged mice also have a relative
373 increase in LT-HSC frequency in the bone marrow. These effects are likely due to both cell-intrinsic
374 changes in LT-HSCs and changes in the environmental signals present in the bone marrow of aged mice
375 after LPS challenge. We demonstrate a cell-intrinsic component to this differential response between
376 young and aged mice by stimulating LT-HSCs with TLR ligands *in vitro* and transplanting them to
377 lethally irradiated recipients. While it has been demonstrated that TLR stimulation of LT-HSCs induces
378 their proliferation (Zhao et al., 2013), our results suggest that it does not alter their long-term
379 reconstitution potential. Importantly, aged LT-HSCs maintained a memory of the *in vitro* inflammatory
380 challenge and had increased myeloid output 3 months after transplant compared to unstimulated aged LT-
381 HSCs. These data therefore confirms that LT-HSCs directly sense TLR ligands (Nagai et al., 2006), and
382 in response to this, the aged LT-HSC population has an amplified cell-intrinsic myeloid bias. Given that
383 the LT-HSC population is heterogeneous, it is yet to be determined whether inflammation plays a role in
384 either enriching for cells in the mLT-HSC state or in changing the state of nmLT-HSCs to an mLT-HSC-
385 like state.
386
387 We show that the various types of HPSCs respond transcriptionally to TLR ligands *in vitro* in a similar
388 way to that seen in BMDCs after LPS stimulation (Ramirez-Carrozzi et al., 2009). Given the different
389 functional roles of HSPCs and mature immune cells, this similarity in transcriptional response is
390 particularly surprising. It has been suggested that, as in mature cell types, inducing the expression of NF-
391 κB with LPS/Pam3csk4 in HSPCs may affect cytokine secretion and proliferation (Zhao et al., 2011,
392 2014). We observed that the dynamics of expression of NF-κB-driven genes was largely similar between
393 HSPCs and DCs. Thus, the majority of NF-κB-responsive genes appear to be regulated similarly in both
394 HSPCs and mature cells.
395

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396 Using scRNA-seq, we identified subsets of LT-HSCs with distinct transcriptional responses to
397 inflammatory signals. Previous efforts have successfully identified phenotypic markers for
398 megakaryocyte biased LT-HSC subpopulations (Dykstra et al., 2011; Gekas and Graf, 2013). The gene
399 signature in this study provides functional insight into the basis of myeloid bias in the context of aging
400 and inflammation. Using this myeloid gene signature from stimulated LT-HSCs, we uncovered a subset
401 of mLT-HSC-like cells enriched in the unstimulated aged LT-HSC compartment that have the potential to
402 respond uniquely to acute inflammatory signals. This is consistent with recent results suggesting that
403 there is an epigenetically primed subset of LT-HSCs that is uniquely poised to respond to LPS (Yu et al.,
404 2016). We show herein that such a subset may also be identified using transcriptomic data.
405
406 While mLT-HSCs express a gene-signature reflective of myeloid bias, it remains unclear that they
407 preferentially produce myeloid cells in transplant experiments. Since these cells are defined by their
408 transcriptional patterns, and not phenotypic markers, we are unable to perform single-cell transplant
409 experiments to validate their preferential myeloid output with currently available techniques. We do
410 know, however, that the relative distribution of mLT-HSCs as defined by our myeloid-biased gene
411 signature qualitatively reflects the age-related changes in myeloid output we expect to see in LT-HSCs.
412
413 Our data supports the model that the increased proportion of these mLT-HSCs with age correlates with an
414 increase in baseline myeloid output in aged mice, which is in turn exacerbated during inflammatory
415 challenge. Accordingly, in the setting of *in vitro* TLR stimulation prior to transplantation (**Figure 1F-I**),
416 we hypothesize that stimulation of aged LT-HSCs preferentially expands mLT-HSCs or selects cells in an
417 mLT-HSC-like state. This mLT-HSC enrichment then results in a sustained increase in myeloid output
418 for several months post-reconstitution (**Figure 1H-I**). In the context of physiologic aging, it might be that
419 the accumulation of inflammatory challenges over the lifetime of an animal results in selection and
420 expansion of mLT-HSCs, partially due to direct sensing of these inflammatory signals by these cells. This
421 hypothesis is supported by the fact that chronic inflammatory stimulation of young mice, either by
422 repeated LPS challenge or increased activation of NF-κB, leads to a myeloid-biased output (Esplin et al.,
423 2011; Zhao et al., 2013). However, a direct test of this notion is difficult because even germ-free animals
424 will experience inflammatory events throughout their lifetime. It is therefore possible that there is an
425 intrinsic, inflammation-independent process that drives LT-HSCs towards a myeloid bias over the
426 lifetime of an animal.
427
428 Using the inferred myeloid-biased gene signature in our study, we were also able to identify several
429 transcriptional regulators of inflammatory myelopoiesis in aged stimulated mLT-HSCs. Members of the
430 Kruppel-like factor (Klf) family of TFs were among those that were predicted to regulate genes in the
431 myeloid-biased signature and were themselves differentially regulated in mLT-HSCs versus nmLT-HSCs.
432 Among these were *Klf4* and *Klf5*, both of which are required for embryonic stem cell self-renewal (Jiang
433 et al., 2008). *Klf4*, in particular, was one of the key reprogramming factors first used to dedifferentiate
434 somatic cells to induced pluripotent stem cells (Takahashi and Yamanaka, 2016). The enrichment of both
435 of these TFs in mLT-HSCs may therefore play a role in the increased symmetric self-renewal divisions
436 seen in aged LT-HSCs (Geiger et al., 2013; Sudo et al., 2000). Indeed, knockdown of *Klf5* or *Ikzf1* in
437 aged LT-HSCs, but not in young LT-HSCs, results in decreased myeloid output and decreased LT-HSC
438 bone marrow frequency. This suggests that both of these factors may play important roles in regulating
439 LT-HSC myeloid versus lymphoid balance with age. Consistent with these results, it has recently been

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440 shown that deficiency of *Klf5* in LT-HSCs leads to decreased bone marrow homing of these cells in
441 transplant experiments and reduced output of myeloid cells, especially neutrophils (Shahrin et al., 2016;
442 Taniguchi Ishikawa et al., 2013). Our results suggest that, in addition to aging, the physiological role of
443 *Klf5* in regulating myeloid output becomes particularly relevant during inflammatory challenge.
444
445 The role of *Ikzf1* in LT-HSC function is less well understood. *Ikzf1* is known to regulate early lymphoid
446 differentiation at the level of lymphoid-primed MPPs (LMPPs) and early B and T cell progenitors (Ng et
447 al., 2009; Yoshida et al., 2006). Previous studies have suggested that *Ikzf1* does not play a role in myeloid
448 versus lymphoid lineage commitment of young LT-HSCs (Ng et al., 2009), though some evidence exists
449 that its expression is upregulated in young lymphoid-primed LT-HSCs (Challen et al., 2010). Our results,
450 however, suggest that with age and in the context of inflammation, *Ikzf1* may indeed have a positive role
451 in myeloid fate decisions. Consistent with this, *Ikzf1* has been shown to bind enhancer elements of both
452 myeloid and lymphoid genes in human HSPCs (Novershtern et al., 2011).
453
454 Knockdown of *Stat3* in aged LT-HSCs also severely hampered myeloid output after inflammatory
455 challenge. This is consistent with the role of *Stat3* as a major inflammatory TF. In particular, some studies
456 suggest that *Stat3* is induced in response to TLR4 signaling in certain cell types (Kortylewski et al.,
457 2009). Interestingly, complete knockout of *Stat3* in LT-HSCs has been shown to result in a premature
458 aging phenotype (Mantel et al., 2012); our results suggest that partial loss of *Stat3* is not enough to
459 recapitulate this phenotype.
460
461 Since decreasing the expression of these TFs can alter the balance of myeloid and lymphoid cells during
462 emergency myelopoiesis, manipulating them or other aspects of the unstimulated or stimulated mLT-HSC
463 programs may provide new therapeutic avenues for re-establishing appropriate lymphoid versus myeloid
464 balance to improve immune function and prevent myeloid leukemias with age.

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626 **Author contributions**

627 M.M., A.M., C.D., M.S.K., A.R. and D.B. designed the experiments. M.M., A.M., M.S.K. and K.L.
628 performed the experiments. C.D. analyzed the sequencing data. A.M., M.M., and D.B. wrote the
629 manuscript, with all authors contributing to writing and providing feedback.

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637 **Figure legends**
638
639 **Figure 1. Aged hematopoietic stem cells exposed to inflammatory signals demonstrate increased**
640 **myeloid output in a cell-intrinsic manner.** (A) Schematic overview of the approach. (B)-(C) Young (8-
641 12 weeks) and aged (20-24 months) mice were exposed to single sub-lethal dose of LPS and peripheral
642 blood (B) T cell, (C) B cell, (D) myeloid cell and (E) granulocyte frequencies were measured by flow
643 cytometry at the indicated time points after LPS exposure (n = 4-14 per group). (F)-(I) LT-HSCs (Lin-
644 cKit+Sca1+CD150+CD48-) sorted from young and aged CD45.2 mice were stimulated with LPS and
645 Pam3csk4 for 2 hours prior to competitive transplant into CD45.1 recipients. Peripheral blood (F) CD3+,
646 (G) CD19+, (H) CD11b+ and (I) Gr-1+ frequencies were measured by flow cytometry at 3 months post-
647 reconstitution (n = 11-12 per group). Data represent at least two independent experiments and are
648 presented as mean ± SEM. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$. P-values
649 were corrected for multiple hypothesis testing by Bonferroni's multiple comparison test.
650
651 **Figure 2. Early hematopoietic progenitors demonstrate a rapid transcriptional response to**
652 **inflammatory signals.** (A) Schematic of LPS and Pam3CSK4 time-course experiment. LT-HSCs, ST-
653 HSCs and MPPs from young (8-12 weeks) and aged (20-24 months) mice were exposed to LPS and
654 Pam3CSK4 *in vitro* for the indicated time after which RNA was harvested for bulk RNA-sequencing. (B)
655 Heatmap of differentially expressed genes in young and aged hematopoietic progenitors alongside an
656 expression map of mature bone-marrow derived dendritic cells (DCs) challenged with LPS for
657 comparison. (C) Heatmap of NF-kB regulated inflammatory genes clustered by temporal expression
658 patterns described previously (Bhatt et al., 2012). (D) Heatmap of cytokines expressed in early
659 progenitors.
660
661 **Figure 3. Single-cell RNA-sequencing reveals heterogeneity in the hematopoietic stem cell**
662 **compartment that is altered with age.** (A)-(E) LT-HSCs, ST-HSCs and MPPs from young and aged
663 mice were stimulated with LPS and Pam3CSK4 and sorted for single-cell RNA-seq. Single cell RNA-seq
664 data for all cells, projected on two t-SNE axes. (A) Density-based clusters. (B)-(E) Single-cell t-SNE plots
665 (as before) indicating all cell types among (B) unstimulated and (C) stimulated cells, and mouse ages
666 among (D) unstimulated and (E) stimulated cells. (F) Correlation across cells between DE genes common
667 to both simulated cluster 3 vs 2 and unstimulated aged vs young LT-HSCs. Three clusters of correlated
668 genes are identified. (G) Heat-map represents the expression values of genes in the unstimulated myeloid-
669 biased gene signature for each single unstimulated LT-HSC. The panels below show the myeloid
670 signature score for each cell and is the basis for the ordering of the x axis. The color coded bar at the
671 bottom shows the age of the animals from which the cells were derived. (H) Violin plots of all LT-HSCs
672 showing the mRNA expression of *Hoxb5*, *CD41*, *CD150* and *Vwf*.
673
674 **Figure 4. Several transcriptional factors may be involved in the underlying myeloid-bias of aged**
675 **LT-HSCs.** (A) Enrichment of transcription factor motifs in enhancers of cluster 3-vs-2-specific genes (x-
676 axis) and differential expression of the TF genes themselves in the same comparison (y-axis). Significant
677 genes (FDR<0.1) are indicated. An enrichment score > 0.5 indicates that the TF motif is enriched among
678 genes that are expressed more highly in cluster 3, while a score below 0.5 indicates that the TF is
679 expressed more highly in cluster 2. (B) As in (A), but considering only aged LT-HSCs in cluster 3 and
680 cluster 2. (C,D) Observed/expected enrichment of enhancers associated with DE genes (indicated by

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681 heatmap), when sorted by decreasing motif strength for (C) Ikzf1 and (D) Klf5. (E) Violin plots of all LT-
682 HSCs showing the mRNA expression of *Ikzf1*, *Klf5* and *Stat3*.
683
684 **Figure 5**. **Klf5, Ikzf1, and Stat3 regulate steady-state and inflammatory age-related myeloid bias.**
685 (A)-(L) Bone marrow cells from young (8-12 weeks) and aged (20-24 months) C57BL/6 mice were
686 transduced with constructs to knock-down the indicated transcription factors. These cells were
687 subsequently reconstituted into lethally irradiated young C57BL/6 recipient mice. Shown are the
688 frequencies of peripheral blood (A) CD3e+, (B) CD11b+ and (C) Gr-1+ cells at 2-months post-
689 reconstitution. (D)-(I) These mice were subsequently challenged with a single sub-lethal dose of LPS and
690 peripheral blood immune cells were tracked over time by flow cytometry. Shown are peripheral blood
691 myeloid cells for mice with knockdown of (D) Hoxa9, (E) Ikzf1, (F) Klf4, (G) Klf5, (H) Zbtb4 and (I)
692 Stat3. (J)-(L) These mice were subsequently harvested and the bone marrow compartment was analyzed
693 for the frequency of (J) LT-HSCs, (K) CMPs and (L) CLPs. Data represent at least two independent
694 experiments and are presented as mean ± SEM. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes p
695 < 0.001. P-values was corrected for multiple hypothesis testing by Bonferroni's method.
696
697 **Figure 6. Model of LT-HSC aging and inflammatory myeloid-bias.** Shifts in clonal heterogeneity
698 during LT-HSC aging affects the inflammatory response of LT-HSCs. The LT-HSC compartment is
699 comprised of unbiased and myeloid-biased LT-HSCs. With age, the clonal distribution of LT-HSCs shifts
700 towards myeloid-biased variants. During acute inflammatory challenges, myeloid-biased LT-HSCs
701 preferentially expand, leading to increased myeloid output. Several cell-intrinsic factors, including the
702 transcriptional regulators Klf5, Ikzf1 and Stat3 may play a role in establishing a myeloid-biased
703 differentiation program during aging and inflammation. Extrinsic factors, including inflammatory
704 cytokines and growth factors secreted from other cell types may also play a role.

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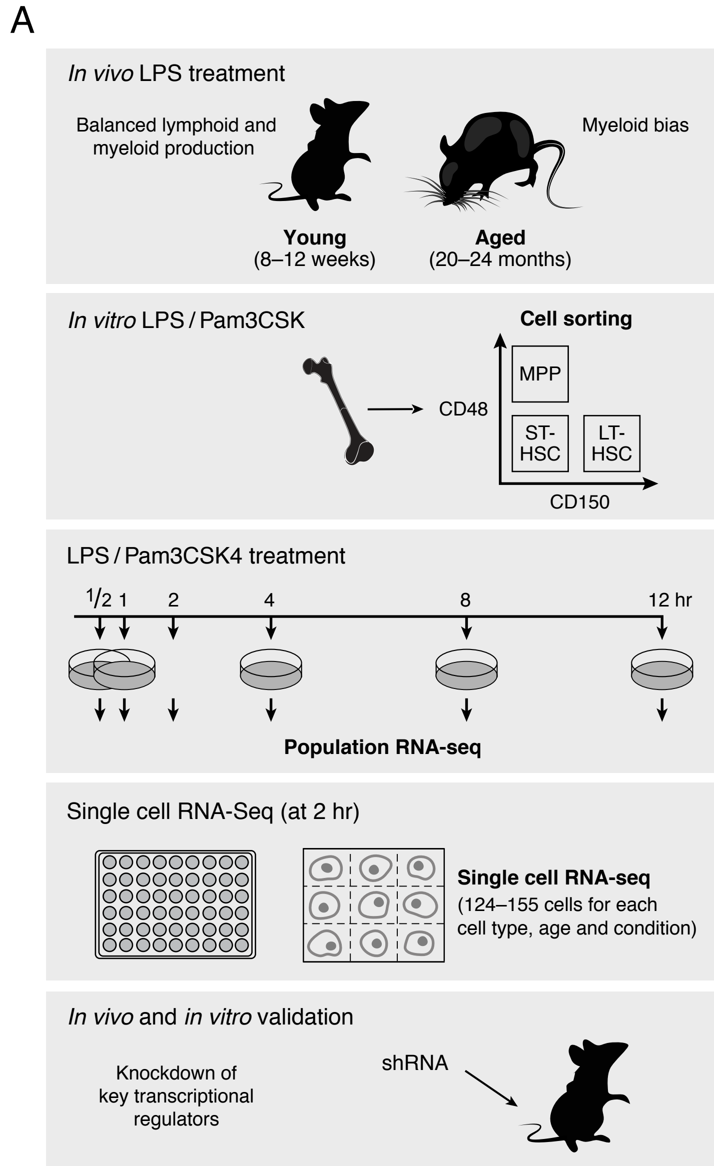

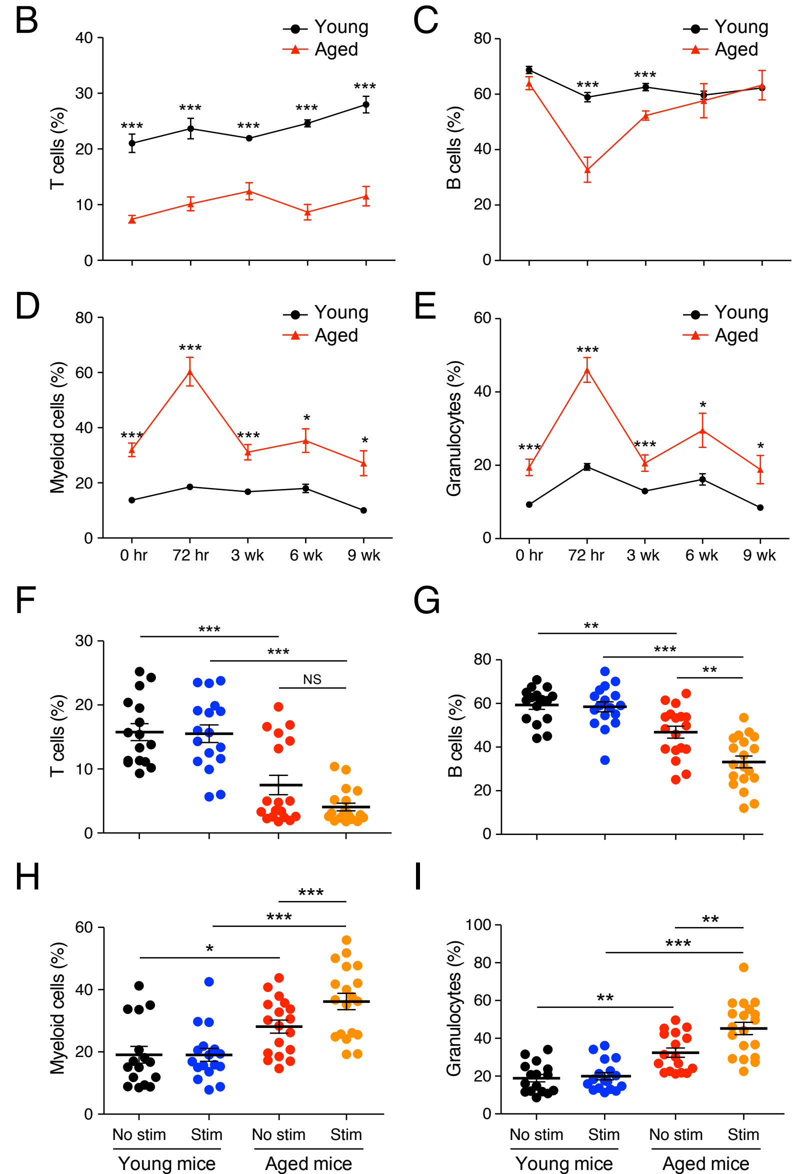

Figure 1

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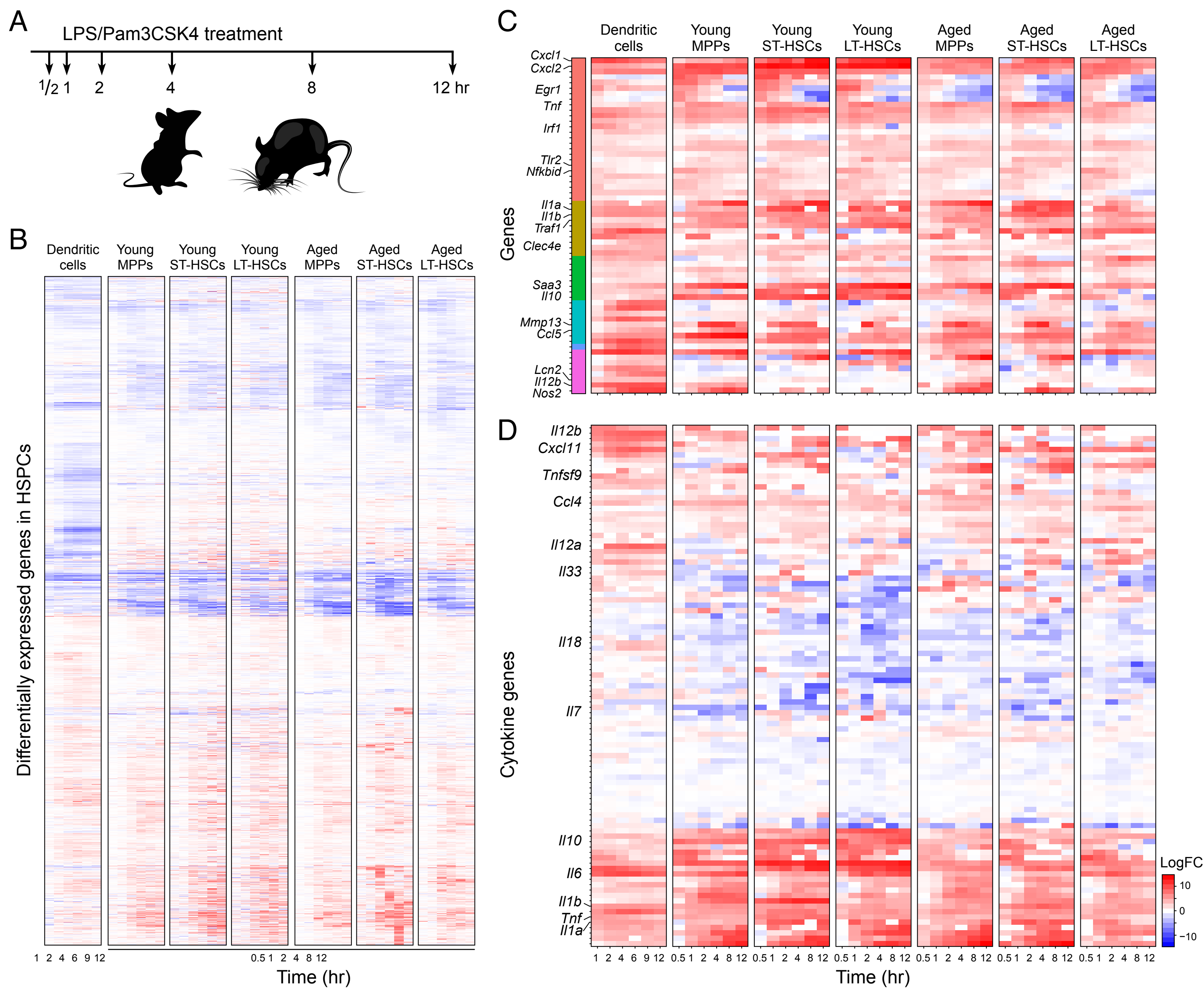

Figure 2

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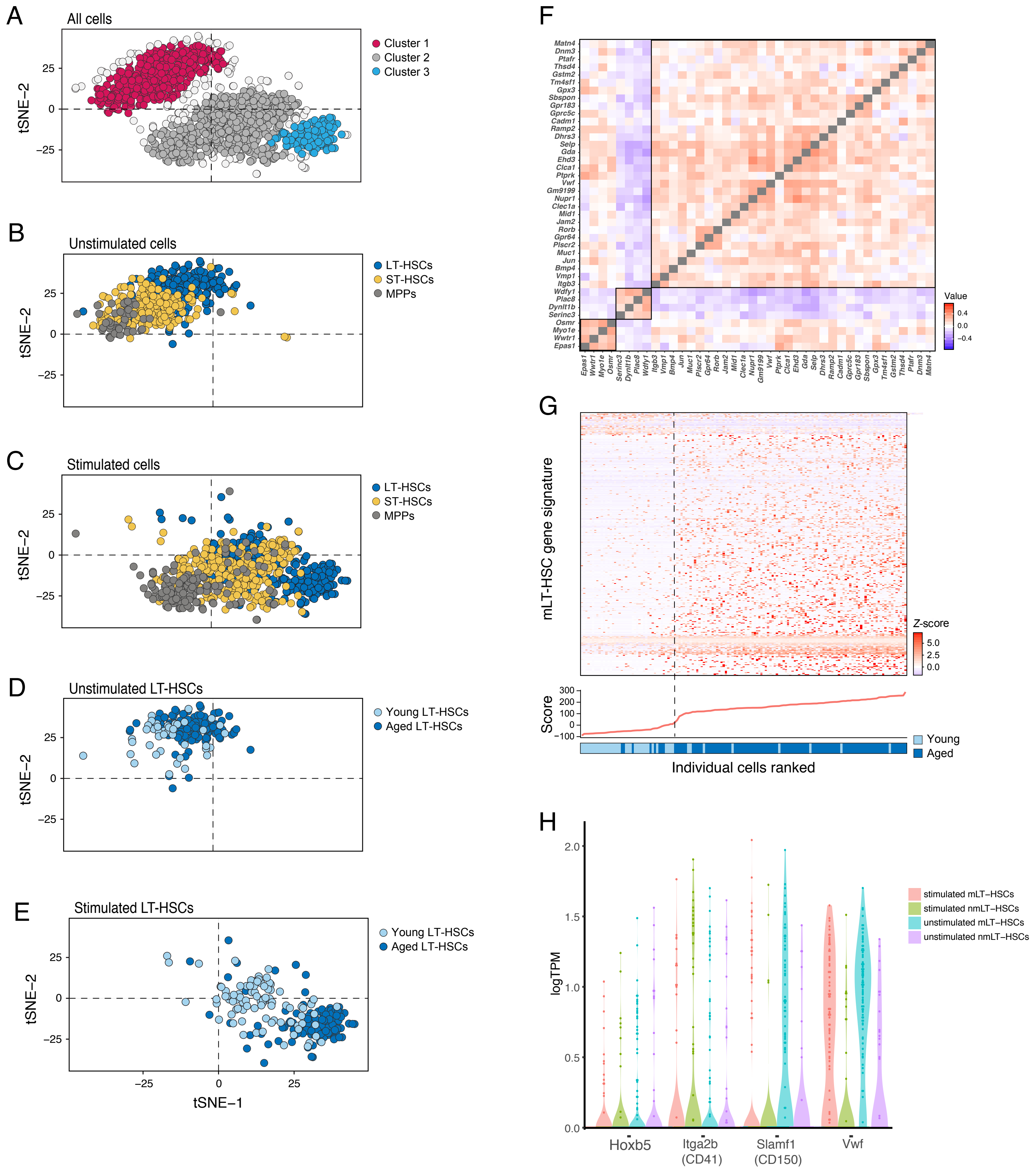

Figure 3

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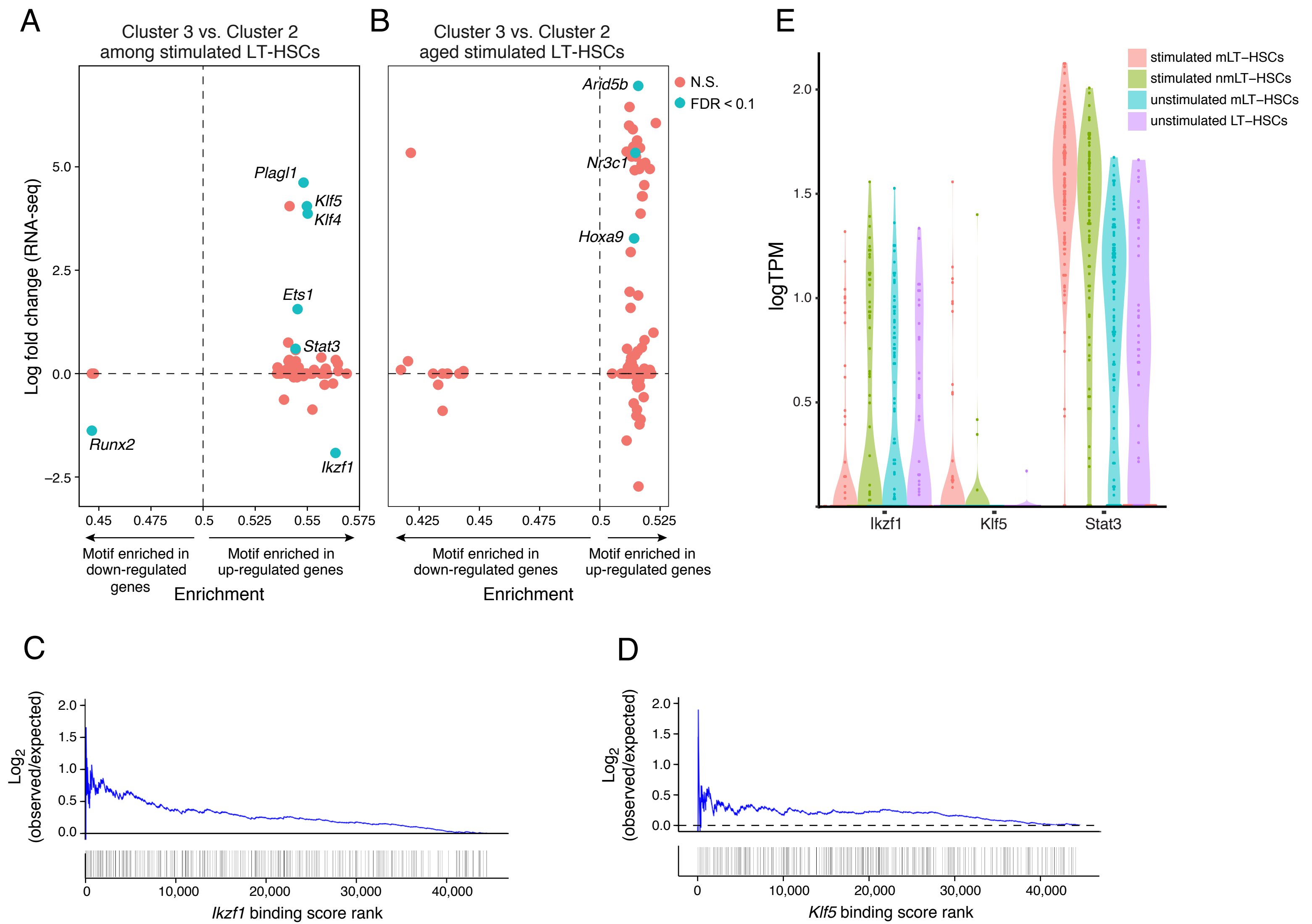

Figure 4

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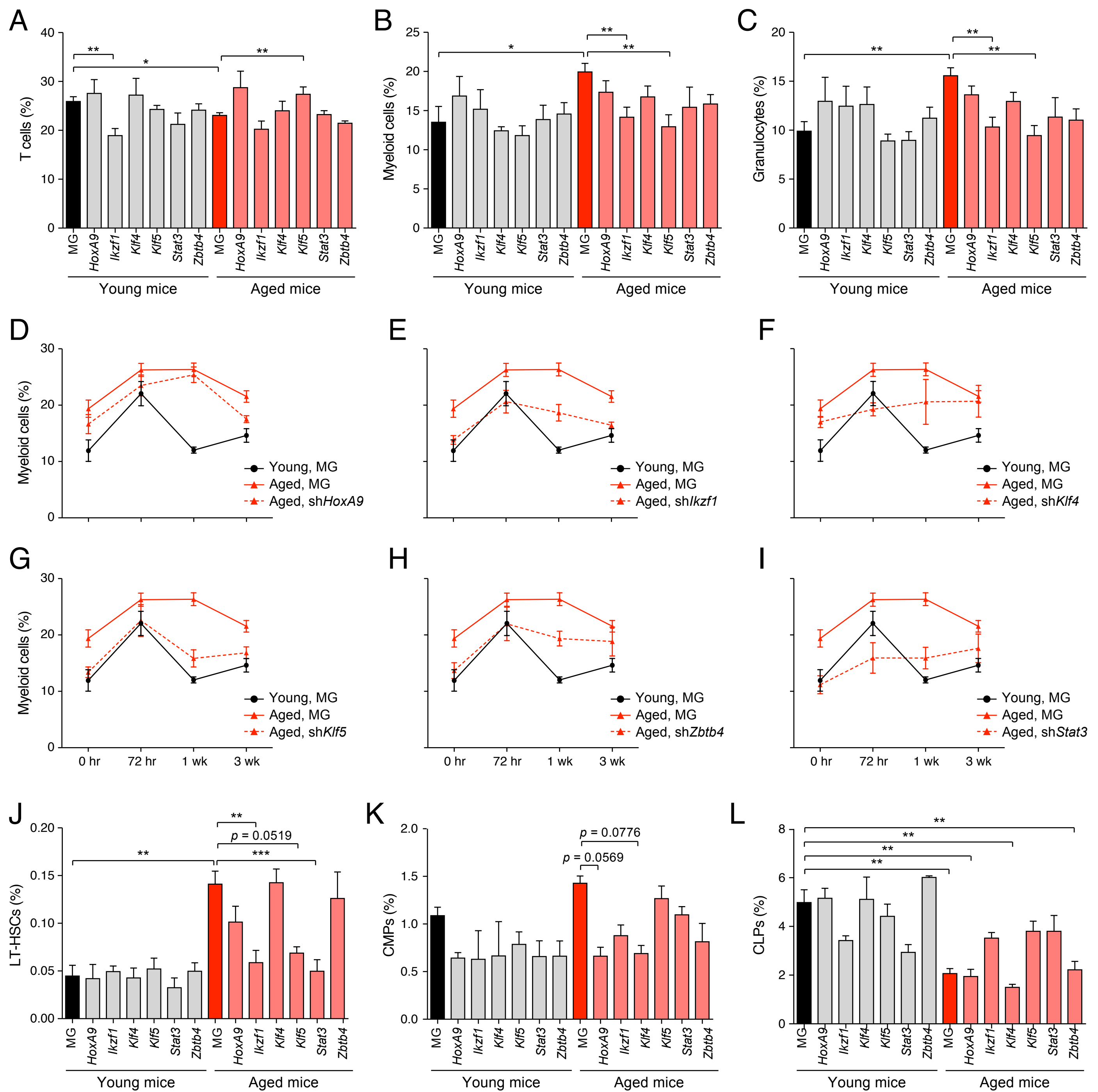

Figure 5

YOUNG

AGED

non-myeloid biased LT-HSCs

replicative stress
chronic inflammation

myeloid biased LT-HSCs

ACUTE INFLAMMATORY CHALLENGE

selective expansion of myeloid biased LT-HSCs

intrinsic factors *extrinsic factors*