Quality assurance of hematopoietic stem cells by macrophages determines stem cell clonality

Samuel J. Wattrus1,2, Mackenzie L. Smith1,2, Cecilia Pessoa Rodrigues1,2, Elliott J. Hagedorn1,2,†, Ji Wook Kim1,2, Bogdan Budnik3, Leonard I. Zon1,2,*

Tissue-specific stem cells persist for a lifetime and can differentiate to maintain homeostasis or transform to initiate cancer. Despite their importance, there are no described quality assurance mechanisms for newly formed stem cells. We observed intimate and specific interactions between macrophages and nascent blood stem cells in zebrafish embryos. Macrophage interactions frequently led to either removal of cytoplasmic material and stem cell division or complete engulfment and stem cell death. Stressed stem cells were marked by surface Calreticulin, which stimulated macrophage interactions. Using cellular barcoding, we found that Calreticulin knock-down or embryonic macrophage depletion reduced the number of stem cell clones that established adult hematopoiesis. Our work supports a model in which embryonic macrophages determine hematopoietic clonality by monitoring stem cell quality.

To better characterize macrophage-HSPC interactions, we tracked individual HSPCs in the CHT at 2 or 3 days postfertilization (dpf) and recorded macrophage interactions. We found that 70% of HSPCs experienced prolonged macrophage contact over a 3-hour imaging period (Fig. 1C). Within this time frame, 13% of these HSPCs were groomed and 13% were doomed. Some HSPCs were contacted by macrophages multiple times and underwent grooming or doomed after repeated interaction, suggesting that most HSPCs may eventually undergo grooming or dooming at some point during the 3 to 4 days that they occupy the CHT. Of HSPC divisions, 81% occurred within 30 min of grooming or prolonged contact (Fig. 1D). Using the Tg[EF1a:mAG-zGem(1/100)]2ruwa0440sh (Fucci) transgene (12) to label cells in S, G2, and M phases of the cell cycle, we found that ~65% of Fucci+ HSPCs contacted macrophages, compared with only 16% of Fucci− HSPCs (Fig. 1E). We next assessed the viability of HSPCs engulfed by macrophages. Staining for cell death with acridine orange or an annexin V–yellow fluorescent protein (YFP) construct (13) showed almost no apoptotic HSPCs in the CHT that were not already engulfed by macrophages (figs. S1, B and C). Only after full engulfment did HSPCs exhibit apoptosis (movie S3). Together, these data identify a set of macrophage-HSPC interactions that precede either HSPC division or death.

A subset of primitive macrophages regulates stem cell clone number

Because we saw proliferation after macrophage-HSPC interactions, we next sought to determine whether this might influence the number of stem cell clones that contribute to adult hematopoiesis. We used TWISTR (tissue editing with inducible stem cell tagging via recombinase) (14) to combine morpholino-mediated gene knock-down with Zebrabow HSC color labeling, Zebrabow-M;draculin:CreER<sup>2</sup> embryos enable unique lineage labeling of individual HSC clones at 24 hpf (Fig. 2A) (7, 15). To deplete embryonic macrophages, we injected the irf8 morpholino to block macrophage formation (16) or delivered clodronate liposomes to ablate macrophages at various time points: 28 hpf, before HSPC emergence in the VDA; 48 hpf, before HSPC lodgment in the CHT; 72 hpf, after HSPC lodgment in the CHT; 96 hpf, after HSPCs have doubled (4); or 120 hpf, as HSPCs start to colonize the marrow. Zebrabow analysis of adult marrow myelomonocytes revealed a consistent reduction in hematopoietic clonality compared with sibling controls when macrophages were depleted before 96 hpf (Fig. 2B). These results demonstrate that embryonic macrophages regulate HSC clone number
after VDA emergence and niche colonization until at least one round of amplification has completed.

To better understand the mechanism and cellular consequences of macrophage-HSPC interactions, we pursued transcriptomic analysis of niche macrophages. Because macrophages can take up fluorescent material from HSPCs, we reasoned that it would be possible to identify interacting macrophages by their fluorescence profile. Indeed, flow cytometry of dissociated mpeg1:EGFP;runx1+23:mCherry embryos revealed a rare population of EGFP+mCherry+ cells over several hours in the CHT that was morphologically consistent with macrophages containing HSPC fragments (Fig. 2C). We dissected embryonic zebrafish tails and purified interacting macrophages (EGFP+mCherry+) and noninteracting macrophages (EGFP−mCherry−) for single-cell RNA sequencing (scRNA-seq). We identified a single population of macrophages that segregated by both gene expression and mCherry fluorescence (Fig. 2D). These cells were enriched for genes associated with engulfment, lysosomal degradation, and cholesterol transport and were marked by genes including hmox1a, ctsl1, slc40a1, lbp1ab, and clqa (Fig. 2D and fig. S2A). We validated these data with a fluorescent cholesterol mimic, LysoTracker dye, and in situ hybridization (fig. S2, B to D). Together, these data show that a transcriptionally distinct and relatively homogeneous subset of macrophages engage HSPCs in the CHT.

Surface Calreticulin drives macrophage-HSPC interactions

To gain insight into the proteinaceous material taken up by macrophages, we pursued a modified form of single-cell proteomics called few-cell proteomics (17) to compare interacting macrophages with noninteracting macrophages.
We identified 203 peptides enriched in interacting macrophages, potentially representing a repertoire of proteins either involved in the process of macrophage-HSPC interaction or taken directly from HSPCs. To identify molecular patterns recognized on HSPCs, we excluded peptides with enriched transcripts in interacting macrophages and compared the remaining peptides with the Cell Surface Protein Atlas (18). Notably, surface peptides enriched in interacting macrophages included three Calreticulin paralogs: calr, calr3a, and calr3b (Fig. 3A). Though Calreticulin is widely expressed and typically functions as a chaperone protein in the endoplasmic reticulum (ER),...
it can also sometimes be displayed on the cell surface as an “eat-me” signal (9, 10, 19). Based on our proteomic results, we hypothesized that HSPCs could display surface Calreticulin, stimulating macrophage interactions. We found that 30% of HSPCs at 72 hpf exhibited classic punctate surface Calreticulin staining (20) (Fig. 3B), similar to the percentage of HSPCs interacting with macrophages in vivo (Fig. 1B). Additionally, the canonical surface Calreticulin binding partners, lrp1ab and c1qa, were transcriptionally enriched in interacting macrophages (Fig. 2D). Together, Lrp1ab and C1qa contact Calreticulin and form a bridging complex to initiate phagocytic activity (10, 20, 21).

**Fig. 3. Calreticulin drives HSPC-macrophage interactions to regulate clonality.**

(A) Analysis of differentially enriched potential surface proteins from interacting macrophages identifies three paralogs of Calreticulin. (B) Flow cytometry shows that ~30% of runx1+23:mCherry+ HSPCs stain for surface Calreticulin. (C) Morpholino knock-down of calr3a or calr3b significantly reduces the fraction of HSPCs interacting with macrophages at any one time. Data are means ± SD. Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test; ns is not significant, *P < 0.05, and ***P < 0.001. MO, morpholino. (D) Calreticulin paralogs without the ER-retention KDEL sequence were fused to EGFP, driven by the HSPC-specific runx1+23 enhancer, and injected into stable runx1+23:mCherry;mpeg1:BFP embryos. Mosaic animals overexpress Calreticulin in a random subset of HSPCs. The arrow indicates an HSPC overexpressing calr3a engaged by a macrophage. (E) HSPCs overexpressing calr, calr3a, or calr3b are more frequently engaged by macrophages compared with non-overexpressing HSPCs in the same embryos. Overexpressing egfp alone has no effect. Data are means ± SD. Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test; ns is not significant, *P < 0.05, **P < 0.01, and ****P < 0.0001. (F) Knock-down of calr3a or calr3b reduces the number of HSC clones that contribute to adult hematopoiesis. Data are means ± SD. Data were analyzed by unpaired Student’s t test; *P < 0.05 and ****P < 0.0001.
These results show that Calreticulin decorates the surface of HSPCs and may promote macrophage interaction.

To study the role of Calreticulin in macrophage-HSPC interactions, we used morpholinos to knock down Calreticulin gene expression. Knockdown of calr3a or calr3b significantly reduced the percentage of HSPCs engaged by macrophages (Fig. 3C). This effect was reversed in genetic rescue experiments (Fig. S3, A and B). We then generated parabiotic fusions of embryos with or without Calreticulin knock-down and found that knock-down HSPCs had reduced interactions with control macrophages.

By contrast, control HSPCs had normal levels of interaction with Calreticulin knock-down macrophages, indicating that Calreticulin presentation is HSPC autonomous (fig. S3, C and D). Next, we tested the effect of constitutively surface-translocated Calreticulin expressed under the HSPC-specific runx1+23 enhancer.
(4) (Fig. 3D). Injecting this construct into early embryos generated mosaic animals, which permitted direct comparison of HSPCs with or without Calreticulin overexpression. HSPCs overexpressing calr3a or calr3b were 3- to 5-fold more likely to interact with macrophages compared with non-overexpressing HSPCs in the same embryo (Fig. 3E). When calr3a or calr3b were knocked down, prolonged contact, grooming, and dooming interactions all decreased, with a more severe decrease in dooming (Fig. S3E). By contrast, nearly all cells overexpressing Calreticulin were doomed (Fig. S3F). Taken together, these data show that surface Calreticulin promotes macrophage-HSPC interactions and suggest that differing levels of Calreticulin determine whether an HSPC experiences prolonged contact, is groomed, or is doomed.

To determine whether Calreticulin-dependent interactions during development were responsible for regulating HSC clonality into adulthood, we knocked down calr3a or calr3b and color labeled HSCs at 24 hpf. Adult Zebrafish analysis of morphants showed a significant reduction in the number of HSC clones compared with sibling controls (Fig. 3F). These data show that Calreticulin-dependent interactions in development support a greater number of long-lived HSC clones.

**Macrophages buffer HSPC stress and promote divisions**

We next assessed Calreticulin function in HSPC development. To analyze changes to HSPC emergence, we injected the irf8, calr3a, or calr3b morpholino into ed41:mCherry; kdrl:mCherry embryos to visualize the endothelial-to-hematopoietic transition (22). Quantification of EGFP+ mCherry+ cells in the VDA revealed no significant difference in HSPC budding (Fig. S4A). Serial imaging of ed41:mCherry+ cells over early development revealed that knockdown of calr3a or calr3b did not affect HSPC numbers through 60 hpf but later reduced HSPCs at 72 and 84 hpf (Fig. S4, B and C). This was due to apoptosis or altered trafficking to the kidney marrow (Fig. S4, D and E). Rather, depletion of macrophages or knockdown of calr3a or calr3b significantly reduced the fraction of proliferative HSPCs in the CHT at 72 hpf, as measured by 5-ethyl-2'-deoxyuridine (EdU) incorporation (Fig. 4A). This corroborates the association of macrophage-HSPC interactions with HSPC division that was identified by live imaging (Fig. 1, D and E).

These data show that Calreticulin-dependent macrophage-HSPC interactions serve to expand and maintain HSPCs during early development by promoting proliferation in the CHT.

To molecularly evaluate the effect of macrophage interactions on HSPCs and the qualities that lead to surface Calreticulin, we injected runx1+23:mCherry embryos with the irf8 or control morpholino and performed scRNA-seq on HSPCs at 72 hpf. This analysis identified a population of HSPCs enriched in irf8 morphants marked by genes associated with FoxO activity and cellular senescence (Fig. 4, B and C). FoxO activity initiates in response to increased reactive oxygen species (ROS) and mediates detoxification of ROS and repair of ROS-induced damage (23, 24). In murine HSCs, FoxO deletion and ROS accumulation results in dysregulation of apoptosis, cell cycling, and colony formation (25). The enrichment of HSPCs with FoxO activity in irf8 morphants suggested potential ROS accumulation, which is ordinarily resolved by macrophages. In agreement with this, flow cytometric analysis showed higher ROS levels in HSPCs marked by surface Calreticulin, with significant correlation between ROS levels and surface Calreticulin intensity (Spearman’s correlation; **R > 0.2 x 10^(-6)**) (Fig. 4D). Inhibiting ROS with diphenylene iodonium reduced macrophage-HSPC interactions (Fig. 4E), whereas increasing ROS with hydrogen peroxide or D-glucose (26) increased macrophage-HSPC interactions (Fig. S5A). Consistent with prior work linking ROS, ER stress, and surface Calreticulin (27), ER stress inhibition by perk knock-down also decreased macrophage-HSPC interactions (Fig. S5B). These data show that without macrophages, a population of HSPCs with increased ROS accumulates and that higher ROS levels correlate with surface Calreticulin.

Embryos injected with the irf8 morpholino also had fewer HSPCs marked by genes associated with cell cycling and ERK/MAPK signaling (Fig. 4, B and C). In accordance with this, depleting macrophages or inhibiting ERK/MAPK without reducing macrophage interactions decreased HSPC proliferation. ERK/MAPK inhibition in the context of macrophage depletion did not further reduce proliferation, indicating that macrophages likely stimulate division through this pathway (Fig. S5C). Because inflammation is a critical developmental determinant of HSPC proliferation (28, 29), we reasoned that cytokines expressed by the macrophages, such as il1b, could be responsible for HSPC divisions (Fig. S5D) (29, 30). To investigate this possibility, we generated parabiotic fusions of control-injected embryos to il1b morpholino-injected embryos (31) and evaluated interactions between HSPCs and macrophages from both parabionts (Fig. S5E). Interactions with control macrophages led to significantly more HSPC divisions than interactions with il1b knock-down macrophages, showing that macrophage-produced Il1b promotes HSPC division (Fig. S5F). Additionally, heat-shock overexpression of il1b rescued HSPC proliferation after macrophage depletion (29) (Fig. 4F). ERK/MAPK inhibition abolished the effect of il1b (Fig. S5G), indicating that Il1b-mediated HSPC divisions act through ERK/MAPK, matching the proliferation signature identified by scRNA-seq (Fig. 4, B and C). These results show that HSPC cycling in the CHT is mediated through ERK/MAPK activity induced by macrophage-derived Il1b.

**Discussion**

Our data support a model in which macrophages of the embryonic niche vet the quality of newly formed HSPCs through prolonged physical contact, leading to either expansion or engulfment. This process is mediated by the display of surface Calreticulin, which is associated with increased ROS. It has previously been reported that metabolic shifts during HSPC generation in the VDA increase ROS to mediate HIF1α stabilization (26). Cells with high ROS are also at increased risk for DNA damage and dysfunction (23, 24). Our work suggests that although ROS promotes stem cell emergence in the VDA, titration of ROS is ultimately required for normal hematopoiesis. Although we see no evidence for veam1 expression in embryonic macrophages, previous studies have indicated a role for macrophages in HSPC homing (6). Macrophages are involved in HSPC mobilization in the VDA (32), and murine macrophage subpopulations facilitate HSPC engraftment (33). By contrast, our study finds that macrophages in the CHT remove clones with high surface Calreticulin that have not down-regulated ROS. Healthy HSPCs with low-to-moderate ROS and Calreticulin experience prolonged macrophage contact and grooming, avoid complete engulfment, and respond to pro-proliferative Il1b enabling competition for marrow colonization.

Our work establishes that stem cells are quality assured for stress levels during development, and this affects the clones that contribute to blood formation in adulthood. Calreticulin functions as an “eat-me” molecule that initiates macrophage-HSPC interaction and leads to programmed cell clearance or stem cell expansion. Orthologs of CD47 and SIRPa, “don’t-eat-me” signals, have not been identified in zebrafish, but other primitive signals could influence macrophage behaviors. This quality assurance mechanism may also operate in adulthood in response to environmental stress, such as during marrow transplantation or in clonal stem cell disorders, including myelodysplasia and leukemia. Macrophages may selectively expand or remove clones of tissue-specific stem cells in other systems similar to our findings. Other tissue stem cells rely on macrophages to assure adequate tissue regeneration (34), which could occur through selective proliferation of certain clones. Manipulating this quality assurance mechanism may have important therapeutic implications for stem cell disorders and tissue regeneration.
REFERENCES AND NOTES
1. K. Kissa, P. Herbomel, Nature 454, 112–115 (2010).
2. J. Y. Bertrand et al., Nature 454, 106–111 (2010).
3. E. Murayama et al., Immunity 25, 963–975 (2006).
4. O. J. Tamplin et al., Cell 160, 241–252 (2015).
5. E. J. Hagedorn et al., bioRxiv 2021.11.03.467105 [Preprint] (2021); https://doi.org/10.1101/2021.11.03.467105.
6. D. Li et al., Nature 564, 119–124 (2018).
7. J. Henninger et al., Nat. Cell Biol. 19, 17–27 (2017).
8. P. Herbomel, B. Thisse, C. Thisse, Development 126, 3735–3745 (1999).
9. M. Feng et al., Proc. Natl. Acad. Sci. U.S.A. 112, 2145–2150 (2015).
10. M. Feng et al., Nat. Commun. 9, 3194 (2018).
11. F. Ellett, L. Pase, J. W. Hayman, A. Andrianopoulos, G. J. Lieschke, Blood 117, e49–e56 (2011).
12. M. Sugiyama et al., Proc. Natl. Acad. Sci. U.S.A. 106, 20812–20817 (2009).
13. T. J. van Ham, J. Mapes, D. Kokel, R. T. Peterson, Proc. Natl. Acad. Sci. U.S.A. 112, 11471–11476 (2015).
14. S. Avagyan et al., Science 374, 768–772 (2021).
15. Y. A. Pan et al., Development 140, 2835–2846 (2013).
16. L. Li, H. Jin, J. Xu, Y. Shi, Z. Wen, Blood 117, 1359–1369 (2011).
17. B. Budnik, E. Levy, G. Harmange, N. Slavov, Genome Biol. 19, 161 (2018).
18. D. Bausch-Fluck et al., PLOS ONE 10, e0121314 (2015).
19. M. P. Chao et al., Sci. Transl. Med. 2, 63ra64 (2010).
20. S. J. Gardai et al., Cell 123, 321–334 (2005).
21. C. A. Ogden et al., J. Exp. Med. 194, 781–796 (2001).
22. K. Kissa et al., Blood 111, 1147–1156 (2008).
23. G. J. Kops et al., Nature 413, 316–321 (2002).
24. H. Tran et al., Science 296, 530–534 (2002).
25. Z. Tothova et al., Cell 128, 325–339 (2007).
26. J. M. Harris et al., Blood 121, 2483–2493 (2013).
27. T. Panaretakis et al., EMBO J. 28, 578–590 (2009).
28. R. Espin-Palazon et al., Cell 159, 1070–1085 (2014).
29. J. M. Frame et al., Dev. Cell 55, 133–149.e6 (2020).
30. E. M. Pietras et al., Nat. Cell Biol. 18, 607–616 (2016).
31. A. Lopez-Munoz et al., Mol. Immunol. 48, 1073–1083 (2011).
32. J. Travnickova et al., Nat. Commun. 6, 6227 (2015).
33. A. Chow et al., J. Exp. Med. 208, 261–271 (2011).
34. D. Ratnayake et al., Nature 591, 281–287 (2021).

ACKNOWLEDGMENTS
We thank the Boston Children’s Hospital (BCH) veterinary staff, the BCH flow cytometry core, Single Cell Discoveries, and the Harvard Center for Mass Spectrometry. We also thank A. Han for her assistance with fetal mouse dissection, B. Miller for her help preparing cryosections, and colleagues for critical reading of the manuscript. S.J.W. thanks P. Chen for her continued support in preparing this manuscript. S.J.W. thanks P. Chen for her continued support in all things. Funding: This work was supported by National Institutes of Health grants 1F31HL149154-01 (S.J.W.); 5T32HL007574-40 (C.P.R.); R01HL144780-01 (L.I.Z.). This work was also supported by Stand Fund (L.I.Z.). L.I.Z. is a Howard Hughes Medical Institute investigator. Author contributions: Conceptualization: S.J.W., L.I.Z.; Methodology: S.J.W., M.L.S., B.B.; Investigation: S.J.W., M.L.S., C.P.R.; Writing – original draft: S.J.W.; Writing – review and editing: S.J.W., M.L.S., C.P.R., J.K., E.J.H., B.B., L.I.Z.; Competing interests: L.I.Z. is a founder and stockholder of Fate Therapeutics, CAMP4 Therapeutics, Amagena Therapeutics, and Scholar Rock and is a consultant for Celularity. B.B. is on the scientific advisory board of Preverna, Inc. All other authors declare that they have no competing interests. Data and materials availability: All data are available in the main text or the supplementary materials. The scRNA-seq data are available in the NCBI Gene Expression Omnibus (GSE196553). Proteomic data are available in the MassIVE database (MSV000088780) and PRIDE repository (PXD034434). License information: Copyright © 2022 the authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original US government works. https://www.science.org/about/science-licenses-journal-article-reuse

SUPPLEMENTARY MATERIALS
science.org/doi/10.1126/science.abo4837
Materials and Methods
Figs. S1 to S5
Tables S1 and S2
References (25–56)
MADR Reproducibility Checklist
Movies S1 to S3
View/request a protocol for this paper from Bio-protocol.

Submitted 6 February 2022; resubmitted 11 July 2022
Accepted 19 August 2022
10.1126/science.abo4837