blood®
Brief Report

PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Contribution of clonal hematopoiesis to adult-onset hemophagocytic lymphohistiocytosis

Peter G. Miller,[1,2] Adam S. Sperling,[1] Christopher J. Gibson,[1] Kaushik Viswanathan,[1] Cecilia Castellano,[1] Marie McConkey,[1] John Ceremsak,[3] Martin S. Taylor,[4] Sebastian Birndt,[5] Florian Perner,[1] Jon Arnason,[6] Mridul Agrawal,[1,2] Alison M. Schram,[7] Sarah Nikiforow,[1] German Pihan,[6] Robert P. Hasserjian,[4] Jon C. Aster,[8] Paul La Rosée,[9] Elizabeth A. Morgan,[8] Nancy Berliner,[2] and Benjamin L. Ebert[1,10]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, [2]Division of Hematology, Department of Medicine, Brigham and Women's Hospital, [3]Harvard-MIT Program in Health Sciences and Technology, and [4]Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA; [5]Klinik für Innere Medizin II, Universitätsklinikum Jena, Jena, Germany; [6]Department of Medical Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; [7]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY; [8]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [9]Klinik für Innere Medizin II, Schwarzwald-Baar-Klinikum, Villingen-Schwenningen, Germany; and [10]Howard Hughes Medical Institute, Dana-Farber Cancer Institute, Boston, MA

KEY POINTS

- **Clonal hematopoiesis is enriched in cases of adult-onset HLH.**
- ***Tet2* loss in hematopoietic cells amplifies the inflammatory response to TLR9 stimulation and phenotype in a mouse model of adult-onset HLH.**

Adult-onset hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening disease of immune hyperactivation. Unlike pediatric HLH, adult HLH is rarely driven by germline genetic variants. Although numerous precipitating etiologies have been identified, the reason that HLH occurs in only a subset of individuals and how other factors contribute to the disease remains unknown. We hypothesized that clonal hematopoiesis (CH), a state in which somatic mutations in blood cells cause an expanded population of mutant hematopoietic cells and drive an aberrant inflammatory state, could contribute to adult-onset HLH. In a highly annotated cohort of older adults with HLH we found that CH was more prevalent than in control cohorts. Using the adult-onset HLH mouse model in which repeated treatments of the TLR9 agonist, ODN1826, was delivered to the mouse, we observed that macrophages carrying mutations in *Tet2*, one of the most commonly mutated genes in CH, have an enhanced inflammatory response to TLR9 agonism. Finally, mice carrying *Tet2* mutations in the hematopoietic compartment (a common model for CH) displayed an exaggerated response to TLR9 agonism, including worse splenomegaly and anemia. Our data suggest that CH is more common in individuals with adult-onset HLH and can contribute to the pathophysiology of this disease. (*Blood*. 2020;136(26):3051-3055)

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening disease of aberrant immune activation.[1] In contrast to familial (or primary) HLH, which is associated with pathogenic germline variants, adult-onset HLH is rarely associated with germline variants and often occurs in the context of a precipitating etiology, such as malignancy, infection, or autoimmune disease, that leads to chronic immune stimulation with dysregulated regulatory feedback loops.[2,3] Because hematologic malignancies are strongly associated with HLH, we hypothesized that premalignant hematopoietic states would also predispose to HLH. Clonal hematopoiesis of indeterminate potential (CHIP) is an age-associated phenomenon in which individuals without a hematologic malignancy have a clonal population of blood cells bearing a leukemia-associated somatic mutation at a variant allele fraction (VAF) of at least 2%.[4] In addition to increasing the risk of developing a myeloid neoplasm, CHIP increases the risk of cardiovascular disease via aberrant inflammatory signaling in mutant immune cells.[5,6] We therefore examined whether clonal hematopoiesis is more common in patients with HLH than in those without and could contribute to inflammatory pathologies in experimental models of adult-onset HLH.

Methods

The study was approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board and was conducted in accordance with the Declaration of Helsinki. Genomic DNA was isolated from peripheral blood and bone marrow aspirate samples (DNeasy Kit; Qiagen, Hilden, Germany) of patients who met HLH-2004 diagnostic criteria treated at 5 clinical institutions between 2001 and 2018.[2] In the 2016 revised classification, analysis of histiocytoses and neoplasms of the macrophage-dendritic cell lineages, secondary HLH, or HLH of unknown origin were assigned to group H.[7] Hybrid capture of exons from 98 genes was performed on the genomic DNA samples with a custom SureSelect system (Agilent Technologies, Santa Clara,

Table 1. Characteristics of CH in adults with HLH

Characteristics	Total cohort	CHIP	No CHIP	*P* (CHIP vs no CHIP)
Patients without myeloid malignancy, n	80	21	59	
Median age (IQR)	52 (36.3-62.5)	58 (50.5-69.5)	48 (32-60)	.025*
Gender, n (%)				>.99
Male	43 (54)	11 (52)	32 (54)	
Female	37 (46)	10 (48)	27 (46)	
Prior cytotoxic therapy, n (%)				.77
Received	20 (25)	6 (29)	14 (24)	
Never received	60 (75)	15 (71)	45 (76)	
Specimen source, n (%)				.75
Bone marrow	64 (80)	16 (76)	48 (81)	
Peripheral blood	16 (20)	5 (24)	11 (19)	
Lymphoid malignancy, n (%)				.20
Present	35 (44)	12 (57)	23 (39)	
Absent	45 (56)	9 (43)	36 (61)	

Characteristics of patients in the cohort without myeloid malignancy, stratified by the presence or absence of CH. Cytotoxic therapy refers to the receipt of treatment directed at the malignancy associated with the HLH diagnosis.

*Fisher's exact test.

CA; supplemental Table 1, available on the *Blood* Web site) followed by library preparation and sequencing on the Illumina platform (San Diego, CA). Mutations were identified with Varscan 2.2.3 and annotated using ANNOVAR.[8] Mutations were scored based on the allele fraction, strand bias differential, local noise, mapping quality, and frequency in germline polymorphism databases. These variants were visually inspected in the Integrated Genome Viewer (Broad Institute, Cambridge, MA).

Bone marrow–derived macrophages were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with mouse macrophage colony-stimulating factor, as previously described and treated with phosphate-buffered saline vehicle or ODN1826 (Invivogen, San Diego, CA), and the conditioned medium was collected and analyzed by using a 44-plex mouse cytokine assay (MD44; Eve Technologies, Calgary, AB, Canada).[5] Bone marrow transplantation using the *Tet2*$^{fl/fl}$;*Vav-Cre* and *Tet2*$^{+/+}$;*Vav-Cre* mouse models, treatment of the recipients with ODN1826 and anti-IL10R antibody or control IgG, and analysis of the bone marrow were performed as previously described.[5,9,10]

Statistical comparisons were performed with the Holm-Sidak method for the cytokine analyses, and a 2-tailed Mann-Whitney *U* test or a 2-tailed Student *t* test for the mouse experiments. All analyses were performed with Prism (v8.4.3; GraphPad, San Diego, CA).

Results and discussion

We analyzed a cohort of 88 adult patients who met HLH-2004 diagnostic criteria, as described previously.[2] The average age of the cohort was 54 years (range, 18-81), and the majority were male (55%). A precipitating etiology was identified in most cases (77%), with some having multiple etiologies (16%), including malignancy (44% lymphoid, 9% myeloid, and 3% solid), infection (30%), and autoimmune disease (17%). Three patients had both a myeloid malignancy and lymphoid malignancy. Because CHIP is defined by the absence of any hematologic malignancy and some patients had lymphoid malignancies, we use the term clonal hematopoiesis (CH) in this report, with the same definition of somatic mutations and allele fraction, as in CHIP.[11] Although excluded from further CH analysis, myeloid neoplasms were highly enriched in the cohort, relative to the prevalence in the general population, occurring in 8 of the 88 patients with HLH, suggesting a potential relationship between somatic myeloid hematopoietic mutations and HLH.

CH with a VAF >0.02 was identified in 21 of the 80 patients (26%) without a known myeloid malignancy (Table 1; supplemental Tables 2 and 3). As expected, patients with CH were significantly older (median, 58 vs 48 years of age; $P = .025$). *DNMT3A* and *TET2* were the most commonly mutated genes (supplemental Figure 1A-C). The prevalence of CH was not significantly different between those who had or had not been previously treated with chemotherapy or radiation (30% vs 25%; $P = .77$). We compared cases with (n = 35) and without (n = 45) lymphoid malignancies. Patients with a lymphoid malignancy were older

Table 2. Frequency of CH in adults with HLH

Cohort	All (%)	<60 y (%)	>60 y (%)
Adult HLH	26	22	36
Acuna-Hidalgo et al[11]	2	1	5
Abelson et al[12]	11	7	14

Frequency of CH with a VAF >0.02 identified in the HLH cohort and healthy adults from published cohorts, determined by targeted sequencing of 71 genes[11] or 111 genes.[12]

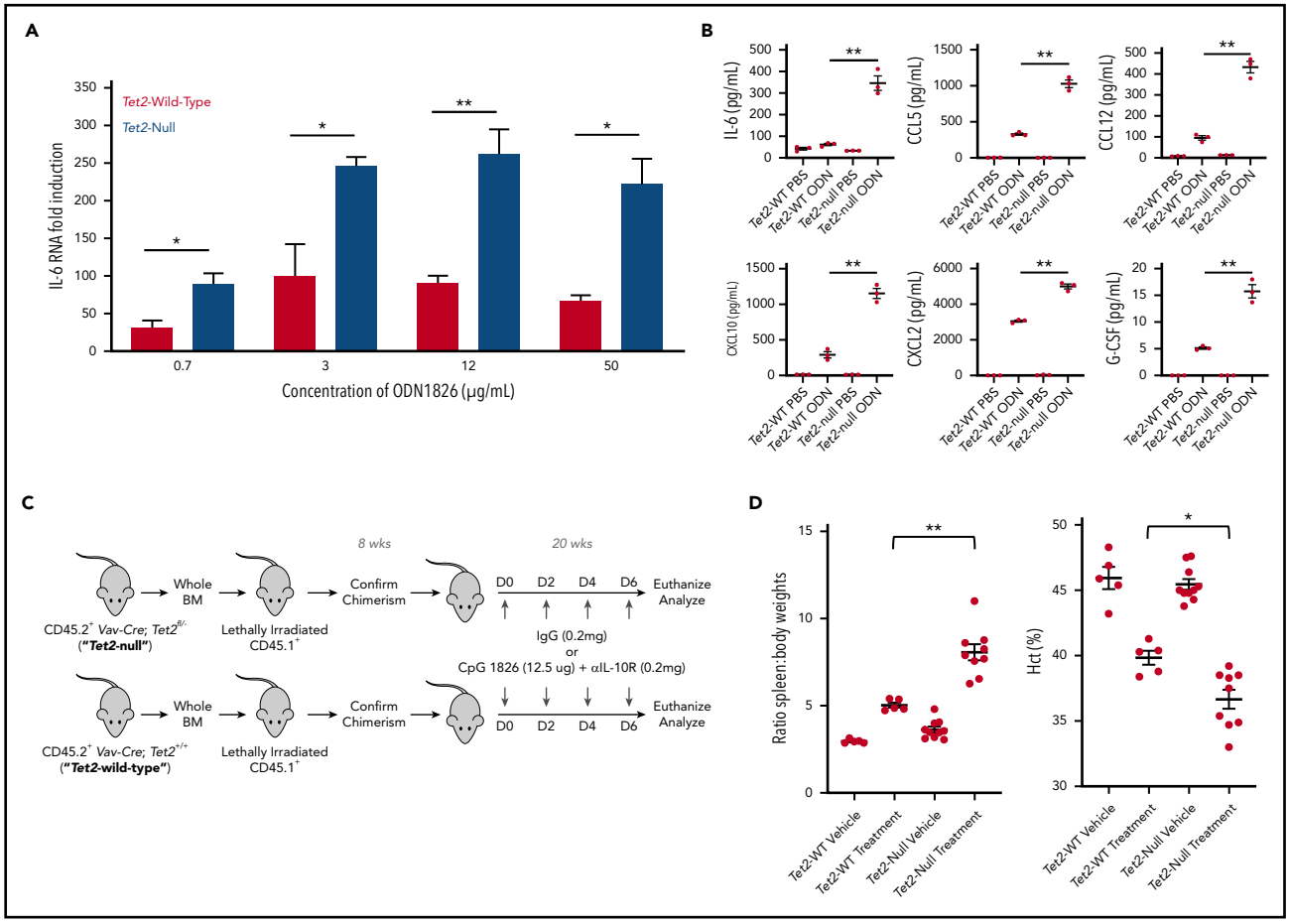

Figure 1. *Tet2* deletion in hematopoietic cells augments inflammatory phenotype in mouse models of adult HLH. (A) Il-6 transcript levels in *Tet2*-null (gray) or *Tet2*–wild-type (black) BMDMs 6 hours after stimulation with ODN1826 (expressed as standard error of the mean [SEM] of 3 replicates). Values were normalized to vehicle control. (B) Cytokine levels in supernatants of *Tet2*–wild-type (WT) or *Tet2*-null BMDMs 24 hours after stimulation with vehicle (phosphate-buffered saline) or ODN1826 (ODN), expressed as SEM of 3 replicates. (C) Schematic of the experimental setup. Spleen-to-body weight ratio (D) and hematocrit (Hct) (E) in each group. $*P < .05$; $**P < .01$.

(median, 60 vs 43 years of age; $P < .01$) but had similar prevalence of CH (34% vs 20%; $P = .20$).

To examine whether CH is more common in individuals with HLH than in healthy, age-matched controls, we compared our cohort to those in 3 published reports of CH, 2 of which used targeted panels in a large number of healthy controls and 1 that used whole-genome sequencing in the Trans-Omics for Precision Medicine cohort (TOPMed).[12-14] In the targeted sequencing cohorts, the prevalence of CH with a VAF >0.02 was ~2- to 15-fold higher in the HLH cohort (Table 2). Similarly, using a VAF >0.1 cutoff in TOPMed (required, given the lower sequencing depth) and VAF >0.1 cutoff in our cohort, CH was approximately threefold more prevalent in the patients with HLH (supplemental Figure 1D). Taken together, these data indicate that the prevalence of CH in patients with HLH is higher than in the general population and is not driven merely by the age of the cohort.

The high prevalence of CH in HLH could be causally related, with somatically mutated myeloid cells contributing directly to the dysregulated inflammatory response in HLH. Alternatively, or in addition, the immunologic state of HLH could contribute to expansion of mutant hematopoietic cells. To address this question, we examined whether macrophages bearing *Tet2* mutations have a hyperinflammatory response to Toll-like receptor (TLR) signaling. We focused on TLR9, the receptor for the DNA-encoded Epstein-Barr virus, which has been implicated in adult-onset HLH.[1,15] We generated bone marrow–derived macrophages (BMDMs) from mice with or without hematopoietic deletion of *Tet2*, by using *Vav1-Cre* recombinase (*Tet2*-null and *Tet2*–wild-type, respectively). After treatment with the mouse TLR9 agonist ODN1826, we observed a dose-dependent increase in interleukin-6 (*Il6*), a previously established mediator of the aberrant immune response in CH, by quantitative polymerase chain reaction.[16,17] Expression of the *Il6* gene was significantly higher at every ODN1826 dose in the *Tet2*-null BMDMs (Figure 1A). After 24 hours of ODN1826 stimulation, 6 inflammatory cytokines had significantly higher expression in conditioned cell culture supernatant of *Tet2*-null BMDMs (Il6, Ccl5, Ccl12, Cxcl10, Cxcl2, and G-CSF), whereas no cytokines had significantly higher levels in the *Tet2*–wild-type BMDMs (Figure 1B; supplemental Figure 2). These findings suggest that loss of *Tet2* in macrophages causes an exaggerated inflammatory response to TLR9 stimulation.

To investigate whether CH can contribute to HLH phenotypes in vivo, we adopted an established model of adult-HLH that uses repeated injections of the mouse TLR9 agonist, ODN1826, in combination with an interleukin-10 receptor (IL-10R) antibody.[8,18] Within 6 to 10 days of this treatment, mice developed an HLH-like phenotype including cytopenias, splenomegaly, weight loss, and hemophagocytosis. To examine the impact of *Tet2* loss on this

phenotype, we transplanted bone marrow from CD45.2$^+$ *Tet2*-null or *Tet2*–wild-type donor mice into lethally irradiated CD45.1$^+$–wild-type recipients (Figure 1C). Greater than 90% chimerism was achieved at 8 weeks. Starting at 20 weeks after transplantation, ODN1826 (12.5 μg) and IL-10R antibody (0.2 mg) or vehicle and IgG control antibody (0.2 mg) were administered by intraperitoneal injection every 2 days, for a total of 4 doses.

All treated animals had weight loss, splenomegaly, and anemia, but mouse recipients of *Tet2*-null cells had significantly worse splenomegaly, as measured by total spleen weight, spleen-to-body weight ratios, and more severe anemia (Figure 1D-E). Peripheral blood counts and cellular composition of blood were not significantly different between the groups (supplemental Figure 3A). Treatment of both genotypes caused similar changes in the bone marrow hematopoietic stem cell (HSC) and progenitor compartments, including expansion of Lin^{lo}Sca1$^+$c-Kit$^+$ cells and an increase in the multipotent population 3 (MPP3) at the cost of short-term HSCs, long-term HSCs, and MPP2 and MPP4 cells. (supplemental Figure 3B-C).

The pathophysiology of adult-onset HLH remains obscure, with increasing evidence suggesting that the disease may be influenced by multiple inputs with complicated interplay between myeloid and lymphoid cells.[3,19] The rarity of HLH suggests that neither myeloid malignancies nor CH alone causes HLH, but instead that both may increase hyperinflammatory effects of other sources of immune activation. In our cohort of 88, myeloid malignancies were present in ~10% of cases, and CH was present in an additional 26%.

Functionally, CH has been associated with increased expression of inflammatory cytokines.[5,14,20,21] We found that *Tet2*-mutant macrophages have an exaggerated inflammatory response to TLR9 stimulation in vitro and that *Tet2* loss exacerbates the HLH phenotype in a TLR9-mediated mouse model in vivo. Taken together, our human genetic and experimental results support a multiple-hit model in which CH can enhance the hyperimmune activation seen in adult-onset HLH.

Acknowledgments

The authors thank Aaron Thorner and Anwesha Nag (Dana-Farber Center for Cancer Genome Discovery) for help in sequencing the samples.

This work was supported by National Institutes of Health (NIH), National Heart, Lung, and Blood Institute (NHLBI) fellowship training grant 4T32HL116324-04, an American Society of Hematology Research Training Award for Fellows, and an Edward P. Evans Young Investigator Award (P.G.M); Dana Farber/Harvard Cancer Center (DF/HCC), National Cancer Institute (NCI) Career Development award K12CA087723 (A.S.S.); Deutsche Forschungsgemeinschaft (DFG) grant AG252/1-1 (M.A.); NIH/NHLBI grant R01HL082945 and NIH/NCI grant P01CA108631, the Howard Hughes Medical Institute, the Edward P. Evans Foundation, and the Leukemia and Lymphoma Society (B.L.E.).

Authorship

Contribution: P.G.M., N.B., and B.L.E. initiated the project, designed the research, and wrote the paper with input from other authors; P.G.M., A.S.S., C.J.G., J.C., M.A., M.M., K.V., and C.C., performed the research; P.G.M, C.J.G., and A.S.S. analyzed the data; and S.B., F.P., J.A., R.P.H., A.M.S., M.S.T., G.P., J.C.A., E.A.M., P.L.R., and S.N. contributed vital new reagents or analytical tools.

Conflict-of-interest disclosure: B.L.E. has received research funding from Celgene and Deerfield Management and consulting fees from GRAIL and is on the scientific advisory boards of Exo Therapeutics and Skyhawk Therapeutics. P.G.M. has received consulting fees from Foundation Medicine Inc. P.L.R. is on the advisory boards of SOBI and Novartis. The remaining authors declare no competing financial interests.

ORCID profiles: P.G.M., 0000-0002-6797-9335; P.L.R., 0000-0002-6758-7809; E.A.M., 0000-0001-5880-9337; B.L.E., 0000-0003-0197-5451.

Correspondence: Benjamin L. Ebert, Dana-Farber Cancer Institute, 450 Brookline Ave, D1610A, Boston, MA 02215; e-mail: benjamin_ebert@dfci.harvard.edu.

Footnotes

Submitted 14 July 2020; accepted 31 August 2020; prepublished online on *Blood* First Edition 22 September 2020. DOI 10.1182/blood.2020008206.

The original sequencing data are available upon request.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

REFERENCES

1. Schram AM, Berliner N. How I treat hemophagocytic lymphohistiocytosis in the adult patient. *Blood*. 2015;125(19):2908-2914.
2. Miller PG, Niroula A, Ceremsak JJ, et al. Identification of germline variants in adults with hemophagocytic lymphohistiocytosis. *Blood Adv*. 2020;4(5):925-929.
3. Carvelli J, Piperoglou C, Farnarier C, et al. Functional and genetic testing in adults with HLH do not reveal a cytotoxicity defect but rather an inflammatory profile. *Blood*. 2020; 136(5):542-552.
4. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. *Science*. 2019; 366(6465):eaan4673.
5. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. *N Engl J Med*. 2017; 377(2):111-121.
6. Fuster JJ, MacLauchlan S, Zuriaga MA, et al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science*. 2017;355(6327): 842-847.
7. Emile JF, Abla O, Fraitag S, et al; Histiocyte Society. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672-2681.
8. Wang K, Mingyao L, Hakonarson H. ANNOVAR. Functional annotation of genetic variants from next-generation sequencing data. *Nucleic Acids Res*. 2010;38:e164.
9. Behrens EM, Canna SW, Slade K, et al. Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice. *J Clin Invest*. 2011;121(6):2264-2277.
10. Pronk CJ, Rossi DJ, Månsson R, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell*. 2007;1(4):428-442.
11. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126(1):9-16.
12. Acuna-Hidalgo R, Sengul H, Steehouwer M, et al. Ultra-sensitive Sequencing Identifies High Prevalence of Clonal Hematopoiesis-Associated Mutations throughout Adult Life. *Am J Hum Genet*. 2017;101(1):50-64.
13. Abelson S, Collord G, Ng SWK, et al. Prediction of acute myeloid leukaemia risk in

healthy individuals. *Nature*. 2018;559(7714):400-404.

14. Bick AG, Weinstock JS, Nandaiumar SK, et al. Inherited causes of clonal hematopoiesis of indeterminate potential in TOPMed whole genomes. *BioRxiv*. 2019;782748.

15. Guggemoos S, Hangel D, Hamm S, Heit A, Bauer S, Adler H. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J Immunol*. 2008;180(1):438-443.

16. Bick AG, Pirruccello JP, Griffin GK, et al. Genetic Interleukin 6 Signaling Deficiency Attenuates Cardiovascular Risk in Clonal Hematopoiesis. *Circulation*. 2020;141(2):124-131.

17. Zhang Q, Zhao K, Shen Q, et al. Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. *Nature*. 2015;525(7569):389-393.

18. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995;374(6522):546-549.

19. Liu P, Pan X, Chen C, et al. Nivolumab treatment of relapsed/refractory Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in adults. *Blood*. 2020;135(11):826-833.

20. Busque L, Sun M, Buscarlet M, et al. High-sensitivity C-reactive protein is associated with clonal hematopoiesis of indeterminate potential. *Blood Adv*. 2020;4(11):2430-2438.

21. Cook EK, Izukawa T, Young S, et al. Comorbid and inflammatory characteristics of genetic subtypes of clonal hematopoiesis. *Blood Adv*. 2019;3(16):2482-2486.