thrombosis with its use.[6] More data on patients on anticoagulation are needed.

The negative impact of AUB on the health and QoL of individuals with inherited bleeding disorders, notably von Willebrand disease and disorders of platelet function, is well documented.[2] AUB is also reported in women and girls with hemophilia A or B and rare factor deficiencies,[3] consistent with an impact similar to what would be seen in those prescribed anticoagulation. HMB impacts physical health by producing iron deficiency, anemia, and the need for treatments that may have side effects. In individuals with bleeding disorders, HMB is documented to have a significant impact on QoL with more days lost from school or work, increased rates of depression, and worse scores using validated measures of QoL compared with individuals without HMB.[2] While useful in identifying individuals with underlying bleeding disorders,[7] in the study by de Jong and colleagues, the International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool did not correlate with AUB. However, it may still be useful in screening for underlying bleeding disorders in women and girls with bleeding symptoms in addition to HMB.

Menstrual bleeding has been recommended as a vital sign given its impact on the health and well-being of individuals who menstruate.[8] We must be better at designing studies of anticoagulants that collect prospectively defined data on menstrual blood loss, as well as other reproductive tract bleeding such as hemorrhagic ovarian cysts and secondary postpartum hemorrhage. Recommended definitions of relevant clinical bleeding for use in studies have focused on new bleeding[9] and do not capture well worsening of normal bleeding, such as with regular menstruation.

Further research is needed to define optimal anticoagulant choice and management for individuals who menstruate. For now, when initiating anticoagulation in our clinical practices, we must educate our patients about the risk of heavier menstrual blood loss, ask them about their menses before and after initiating anticoagulation, initiate treatment for HMB as needed, and monitor them for iron deficiency and other associated sequelae.

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HEMATOPOIESIS AND STEM CELLS

Comment on Shin et al, page 1774

Clonal hematopoiesis transcending species barriers

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In this issue of *Blood*, Shin et al expand our understanding of clonal hematopoiesis (CH) by showing its natural emergence in aged non-human primates (rhesus macaques), and by demonstrating robust expansion of *TET2*-mutated clones causing hyperinflammation in a CRISPR-Cas9–based autologous transplantation model in macaques.[1]

CH describes the overrepresentation of the progeny of a single hematopoietic stem cell (HSC), or clone, in the peripheral blood cell pool. A wealth of data published in recent years has established CH as a universal phenomenon associated with human aging.[2-4] Studies have further demonstrated that larger clones bearing leukemia-associated mutations are associated with an array of adverse outcomes in humans, including development of hematologic malignancies and cardiovascular disease[2,5]; this condition has been termed clonal hematopoiesis of indeterminate potential (CHIP) when the variant allele fraction (VAF) exceeds 2%.[6] Murine models have proven to be important tools in understanding underlying mechanisms, indicating that the association of CHIP with inflammatory diseases is causal and a product of increased inflammation in terminally differentiated myeloid cells.[5,7] Embarking on the study presented here, the authors hypothesized that rhesus macaques might present a faithful model organism for CH because they closely resemble humans in many central attributes of hematopoiesis, while still allowing for experimental engineering of CH that would not be ethical in humans.

Out of 60 aged macaques analyzed with a median age of 25 years, 12 were found

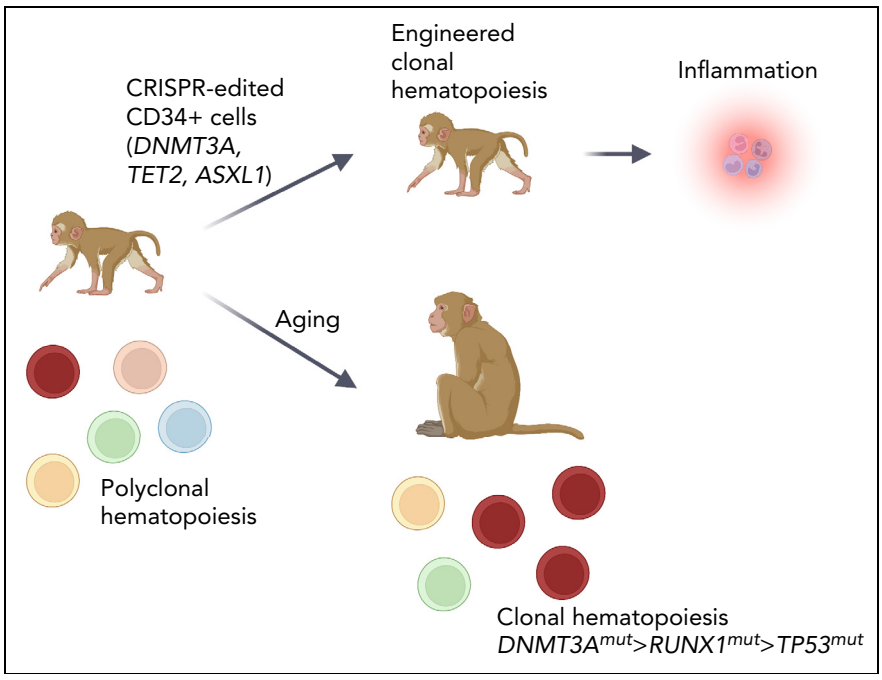
to carry naturally occurring somatic mutations that fulfilled the criteria for CH with a known driver mutation present at a VAF > 1% (see figure). This contrasts to aged mice (24 months old) whereby naturally occurring mutations could only be detected in 2% of animals, and at a lower VAF threshold.[8] As in humans, the most commonly mutated gene was *DNMT3A*, accounting for 4 cases. The next most frequent alterations were mutations in *RUNX1*, *TP53*, *NOTCH1*, *CREBBP*, and *TET2*, a list similar, yet not identical, to the findings in human cohorts. The limited number of cases precludes definitive conclusions about the biological significance of the differences.

Using CRISPR-Cas9–based editing of mobilized peripheral blood $CD34^+$ cells, the authors went on to develop an autologous transplantation model of CH in macaques. The authors transplanted autologous $CD34^+$ cells engineered to carry loss-of-function mutations in *DNMT3A*, *TET2*, and *ASXL1* into macaques after myeloablative irradiation (see figure). This model demonstrated robust expansion of *TET2*-mutated and, to a lesser degree, *DNMT3A*-mutated clones, consistent with murine models and findings in humans indicating that clone growth rates vary by mutation.[3,4] Moreover, this model produced convincing evidence that *TET2*-mutated CH in macaques causes interleukin-1β (IL-1β)– and IL-6–driven inflammation. This was shown with several orthogonal assays, including elegant gene expression analysis in genotyped bone-marrow derived colony forming units, functional studies in isolated macrophages, and in the serum. These findings were concordant with prior findings in mice.[5,7]

The authors then harnessed the strengths of this model to conduct a treatment study with the Food and Drug Administration–approved IL-6 receptor antibody tocilizumab, testing the hypothesis that this treatment might impact clone outgrowth. In 1 out of the 3 treated macaques, treatment resulted in a decrease in clone size as assessed by mutant allele fraction. Although the biological interpretation of this part of the study is limited by the small sample size, it does demonstrate that preclinical testing of pharmacologic interventions in this model is feasible.

In sum, this fascinating study advances the field in several ways. It demonstrates the emergence of natural CH in non-human primates, supporting a model in which somatic mutations in specific epigenetic regulators cause CH across species barriers, at least within primates. The experiments add to several recent observations in mice and humans highlighting inflammation as a consequence of CHIP.[5,7] A particularly notable finding in humans was obtained from a subgroup analysis of the CANTOS trial. CANTOS showed that treatment with the IL-1β-targeting antibody canakinumab reduced recurrent cardiovascular events in high-risk patients, albeit at the cost of increasing rates of severe infection.[9] Subgroup analysis by CHIP status demonstrated that a large part of the reduction in event rates achieved by treatment was attributable to the subgroup of patients with *TET2*-mutated CHIP.[10] Also, the present study reports the first engineered model of CH in primates that is poised to validate interventions targeting CHIP in the future.

The study also shines renewed light on some of the major unanswered questions in the field. What is special about the recurrent mutations in CH, most prominently those in epigenetic modifiers, that make them so much more common than other mutations? What are the factors, both cell-intrinsic and extrinsic, that determine clonal outgrowth? What are the cellular and molecular underpinnings of hyperinflammation in CHIP? Combining longitudinal studies in humans with perturbation in innovative model systems will be required to fully answer these questions and realize the vast potential of targeting CHIP with therapeutic intention.



CRISPR-Cas9 engineered and naturally occurring CH position rhesus macaques as a faithful primate model of CH. Figure was created with BioRender.com.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Zhao et al, page 1790

Optimized CD19/CD22/CD3 antibody

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In this issue of *Blood*, Zhao et al[1] presented an optimized CD19/CD22/CD3 trispecific antibody and demonstrated its antitumor potential in immune escape and patient derived xenograft (PDX) mouse models of B-cell malignancy.

Bispecific antibodies retargeting T cells to tumor cells have become a recognized tool for cancer therapy, especially for hematological malignancies. Blinatumomab (CD19/CD3) is a prominent example.[2] This development has spurred the engineering of bispecific antibodies with diverse formats.[3] However, tumor heterogeneity and antigen loss, as part of the tumor escape and resistance mechanism, remain major challenges.[4,5] The generation of trispecific antibodies, targeting two different tumor-associated antigens, is an extension of the concept, expected to overcome these problems by improving tumor selectivity and specificity.[6] However, the design of trispecific antibodies forming immunological synapse is challenging, due to the individual differences in target size and epitope position. In consequence, there is no "one best format," and individual solutions are required.

Zhao et al reported here the generation of a novel (CD19/CD22/CD3) trispecific antibody with optimized configuration by a site-specific fusion approach. Furthermore, the therapeutic advantage of the trispecific antibody over corresponding bispecific antibodies was demonstrated in xenograft tumor mouse models with varied CD19/CD22 expression profiles. In the first part of the study, Zhao et al generated bi- and trifunctional antibodies composed of a CD3-directed Fab fragment, a CD19-directed single-chain Fv (scFv), and a CD22-directed nanobody. The scFv and the nanobody were fused to the heavy and light chain of the Fab fragment, respectively. Fusion either to the N-terminus or C-terminus of the Fab chains led to bi- and trispecific antibody variants differing in the relative position and proximity of the binding units. In vitro, all variants with one exception performed as intended, mediating targeting dependent cytotoxicity of activated T cells. Fusion of the CD22- and CD19-binding units to the C-terminus of the Fab fragment (ie, opposing the CD3-binding site) appeared most favorable. Quantitative analysis of immunological synapses and cytokine release (interleukin 2 [IL-2]/interferon γ [IFN-γ]/tumor necrosis factor α [TNF-α]) from retargeted T cells supported this finding. Interestingly, the authors opted for a design without Fc region, combining diverse small antibody formats, where linker design becomes a crucial factor. Linker length and configuration can be expected to influence the distance and orientation of the binding units and impact the stability, expression, and bioactivity of the molecule.[7] For N-terminal fusion a rigid peptide linker from pyruvate dehydrogenase was used, and for C-terminal fusion a flexible $(G_4S)_3$ linker was used. The rationale of different linker usage was not stated. Comparative analysis of the optimized trispecific antibody with the corresponding bifunctional antibodies showed similar binding and cytotoxic activity on single-target expressing cell lines and superior retargeting and stimulatory activity on dual target expressing cell lines. Thus, the trispecific antibody not only showed the capacity to replace the bispecific antibodies, but also added value by enhancing the retargeting potency on tumor cells expressing both antigens. Importantly, the in vitro studies were conducted with a panel of tumor cell lines and primary B-cell acute lymphoblastic leukemia (B-ALL) tumor cells with different target expression levels. Thus, the retargeting-activity of the antibodies was shown in a broad range of target densities.

Next, the antitumor activity of the optimized trispecific antibody was assessed in humanized tumor mouse models. Immunodeficient NCG mice were injected with