those patients in the high-risk group who received ID-MTX had a lower risk of CNS relapse (P = .03), but there was no difference in the low-risk group. These findings were not replicated in the validation cohort; however, the overall CNS risk was lower. Thus, power was limited to detect a small difference. Furthermore, although the optimal prophylaxis dose is unknown, it is widely accepted that a minimum dose of 3 g/m^2 is needed to achieve adequate CSF levels and is considered "high dose." There is very limited evidence that this strategy is protective in other aggressive lymphomas, including DLBCL, and a larger study specifically in high-risk patients is awaited. Complicating the analysis is the inclusion of other cytotoxic agents that can also penetrate the CNS, such as ifosfamide and etoposide, making it difficult to determine the independent effect of ID-MTX. Taken together, the current study suggests that there may be a protective effect, but larger confirmatory studies are needed. As gemcitabine-based combinations are also used but lack CNS penetrant agents, there may be an opportunity to compare the risk of CNS relapse in larger cohorts treated with these regimens.

In summary, the CNS-PINK is the first step forward to a CNS risk model in ENKTL. Further validation studies and investigation of the protective effect of MTX and other CNS penetrant agents are eagerly awaited.

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PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Comment on Dabek et al, page 2574

Wnt to the rescue! A new role in granulopoiesis

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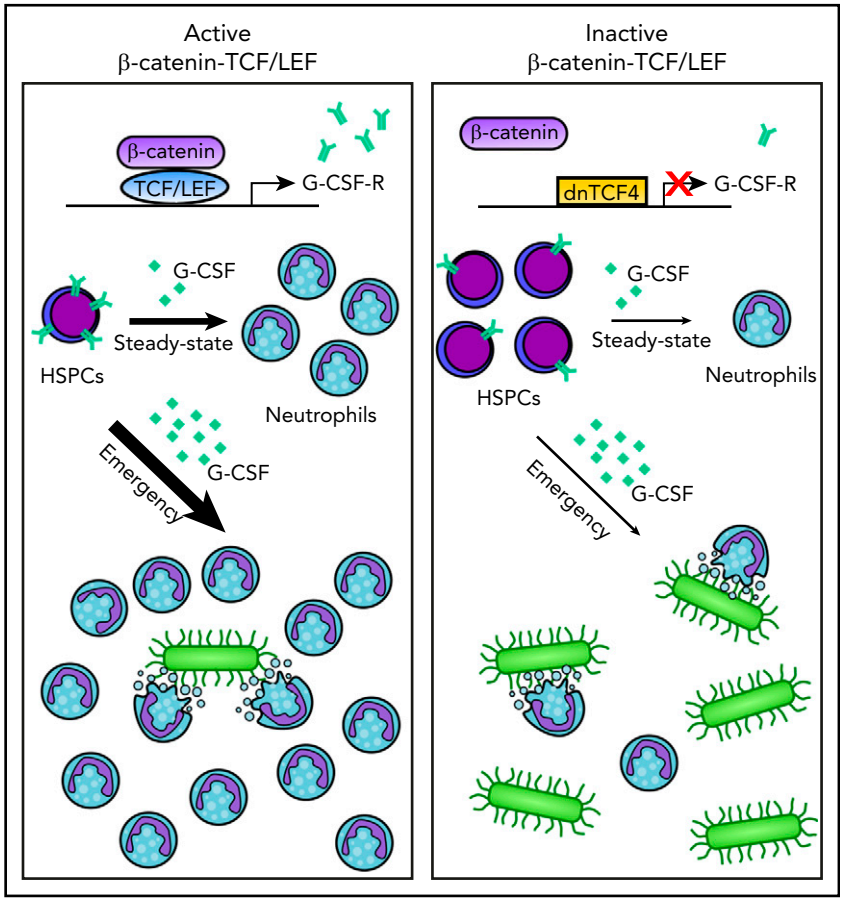
In this issue of *Blood*, Danek et al reveal a previously unknown role for the canonical Wnt signaling pathway in the regulation of granulocyte production in both steady-state and during emergency granulopoiesis.[1]

Neutrophilic granulocytes serve as a first line of defense against pathogens. Adequate numbers are critical for survival, as demonstrated by the severe and often fatal infection in patients with congenital or iatrogenic neutropenia.[2] Given their short half-life, neutrophils need to be constantly replenished to maintain steady-state neutrophil counts. Moreover, upon severe systemic infection, when neutrophils are being consumed, granulopoietic output is massively enhanced to meet the high demand. Consequently, an intricate and redundant regulatory network consisting of cytokines/growth factors, their cognate receptors, and downstream transcriptional programs has evolved to sustain appropriate, demand-adapted granulocyte numbers.[3]

Danek et al now identify the canonical Wnt signaling pathway as yet another important regulator of granulopoiesis. The canonical Wnt pathway signals via various secreted Wnt ligands that bind to the Frizzled receptors, leading to stabilization and accumulation of β-catenin and its translocation to the nucleus, where it ultimately acts as transcriptional coactivator by interacting with members of the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors such as TCF4.[4] The role of the canonical Wnt signaling pathway in hematopoietic stem and progenitor cell (HSPC) biology has been extensively studied, yielding conflicting results.[5] Stimulatory, inhibitory or even neutral effects have been described, and these discrepancies are likely due to use of different experimental model systems that interfere with canonical Wnt signaling at various levels of the pathway and to varying degrees.[6]

In an attempt to overcome these likely limitations, Danek et al use a novel mouse model that employs a dominant-negative form of TCF4 (dnTCF4) whereby transcriptional activity of β-catenin-TCF4 in HSPCs is impaired, whereas the non-transcriptional activities of β-catenin as well as noncanonical Wnt signaling are left intact. The authors show that mice expressing dnTCF4 have reduced peripheral blood and bone marrow (BM) neutrophils and increased immunophenotypically defined HSPC subsets with the exception of long-term hematopoietic stem cells HSCs (LT-HSCs). This accumulation of HSPCs is due to impaired



Active β-catenin-TCF/LEF signaling drives expression of the G-CSF receptor on HSPCs which, in turn, renders HSPCs more responsive to G-CSF thereby promoting steady-state and emergency granulopoiesis (left panel). By contrast, disruption of the β-catenin-TCF/LEF signaling results in impaired steady-state and emergency granulopoiesis (right panel). See graphical abstract in the article by Danek et al that begins on page 2574.

granulocytic differentiation. To elucidate the mechanistic basis for the observed differentiation defect of dnTCF4-expressing HSPCs, gene expression analyses were performed and compared with WT HSPCs, thereby identifying a number of genes known to be important in regulating granulopoiesis. Among these downregulated genes in dnTCF4-expressing HSPCs is *Csf3r*, encoding the receptor for granulocyte colony-stimulating factor (G-CSF), a master regulator of steady-state and emergency granulopoiesis. Accordingly, cell surface G-CSF receptor (G-CSF-R) expression is reduced on murine HSPCs expressing dnTCF4. These findings suggest that the lack of β-catenin-TCF4–mediated transcription of *Csf3r* and the resulting reduced expression of the G-CSF-R is responsible for the observed defect in granulocytic differentiation (see figure). Indeed, Danek et al provide evidence that TCF/LEF transcription factors directly bind to the *CSF3R* promoter and enhancer in a number of human cell lines, and importantly, that treatment with G-CSF results in reduced downstream JAK/STAT3 signaling and granulocytic differentiation in dnTCF4-expressing HSPCs.

Intriguingly, the fact that mice deficient in the master transcriptional regulator of steady-state hematopoiesis, CCAAT enhancer binding protein α (C/EBPα), completely lack granulocyte differentiation,[7] demonstrates that β-catenin-TCF4–mediated transcription fails to compensate for C/EBPα deficiency, and thus primarily acts by fine-tuning steady-state granulopoiesis. However, the striking similarities between the gene sets regulated by C/EBPα and β-catenin-TCF4 may suggest some extent of redundancy or even cooperation between those 2 transcriptional regulators.

Overall, these data establish a role for the canonical Wnt signaling pathway in steady-state granulopoiesis, albeit with relatively modest effects. However, given the vital importance of adequate neutrophil numbers, especially during times of hematopoietic challenge such as systemic infection, the effect of loss of β-catenin-TCF4–mediated transcription on steady-state granulopoiesis may underestimate the true clinical relevance. Therefore, Danek et al test the functional consequences of impaired β-catenin-TCF4–mediated transcription in various models of emergency hematopoiesis including lipopolysaccharide (LPS) stimulation mimicking systemic gram-negative bacterial infection, *Candida albicans* infection, and 5-fluorouracil (5-FU)–induced BM cell depletion and cytopenia. Collectively, these results reveal not only an impaired emergency granulopoietic response but also a significant survival disadvantage in mice with impaired canonical Wnt signaling challenged with *C albicans* or 5-FU, thereby demonstrating the clinical significance of intact β-catenin-TCF4 transcriptional activity.

The authors also provide preliminary evidence for the conservation of the role of the canonical Wnt signaling pathway in granulopoiesis across species. Genetic and pharmacological inhibition or stimulation of the Wnt pathway in human $CD34^+$ HSPCs leads to a decreased or increased propensity for granulocytic differentiation, respectively.

The study by Danek et al adds important novel insights into the regulation of steady-state and emergency granulopoiesis. However, a number of important questions remain unanswered. An obvious question is the upstream regulation of Wnt signaling. Which of the more than a dozen Wnt ligands stimulate granulocytic differentiation from HSPCs? Which cell types secrete these Wnt ligands, and where are they located? Do these ligands increase during emergency conditions, and if so, how is this secretion being regulated by, for instance, an inflammatory insult such as infection?

Finally, it might be tempting to explore the use of pharmacological stimulation of canonical Wnt signaling[8] to boost granulopoiesis in clinical settings of neutropenia such as congenital neutropenia, inherited BM failure syndromes, or the myelodysplastic syndromes. However, it will be important to gain further insight into the role of canonical Wnt signaling in the regulation of normal HSCs and their

malignant counterparts in myeloid neoplasms[9] to prevent unintended adverse effects.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Perreault et al, page 2588

Catch those antibodies before they fall

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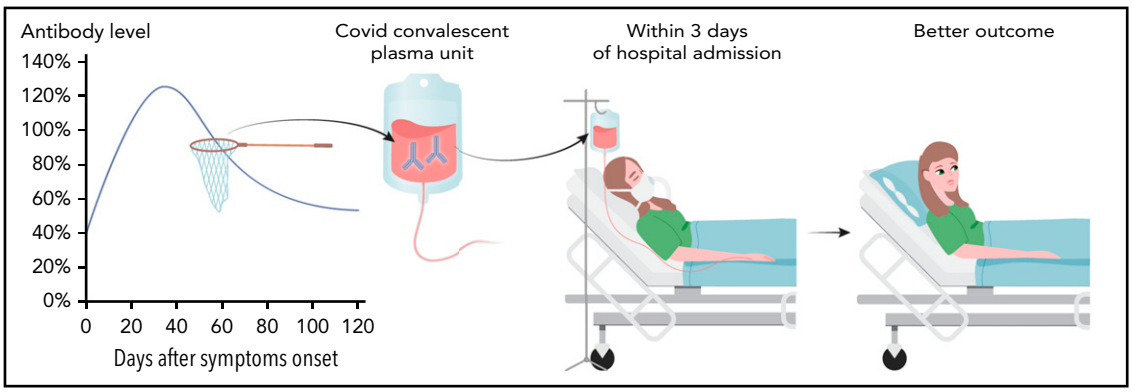
In this issue of *Blood*, Perreault et al showed decreasing total antibody levels over time in 15 repeat COVID-19 convalescent plasma (CCP) donors with at least 4 donations.[1] The decrease was greatest between the final 2 time points reported, from days 70 to 84 to days 85 to 114, after disease onset. This decrease was time dependent and independent of donation number.

Falling antibody levels after recovery disease are well documented, including in COVID-19 patients. Wang et al followed neutralizing antibody titers in hospitalized patients starting shortly after symptom onset and demonstrated antibodies peaked at 4 to 5 weeks after disease onset and then declined.[2] In the Perreault et al study, the earliest antibody levels were obtained 33 to 53 days after disease onset as their study population was plasma donors, who were ≥14 days symptom free and never hospitalized. Muecksch et al used neutralizing antibody assays to show similar antibody titer decline in individuals who were weeks after diagnosis, but this decline was less apparent and highly variable when measuring antibody levels using high-throughput assays.[3] Most recently, Gudbjartsson et al followed antibody levels in 48 hospitalized patients for ~100 days after diagnosis and found that these levels change depending on the antibody tests: levels increasing in total immunoglobulin assays, slightly decreasing in immunoglobulin G (IgG) assays, and greatly decreasing in IgM and IgA assays.[4] Thus antibody level changes during the few months after diagnosis depend on the assay.

The study by Perreault et al has important limitations. In addition to the small study population, the study used 1 enzyme-linked immunosorbent assay (ELISA) to measure severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein receptor binding domain (RBD) total (IgM/IgA/IgG) immunoglobulin from ~30 to 100 days after symptoms. Thus, the rise and potentially continued fall of antibody levels are missing. Other studies end follow-up at a similar time point. Accordingly, studies that continue to follow individuals past a few months are needed. Finally, the assay used measured immunoglobulin titers to qualify donors with a cutoff of ≥100. These CCP donations are part of a multicenter randomized clinical trial, CONCOR-1 (https://clinicaltrials.gov/ct2/show/NCT04348656), which compares adults with COVID-19 respiratory illness treated with 500 mL of CCP to standard of care. Further CCP characterization, including comparison of



Collecting and transfusing high-titer CCP units early in hospitalization improves outcome. Studies have demonstrated that antibody levels are highest when donors are first eligible to donate. The high-titer CCP units when given soon after diagnosis or hospitalization improve patient outcomes.