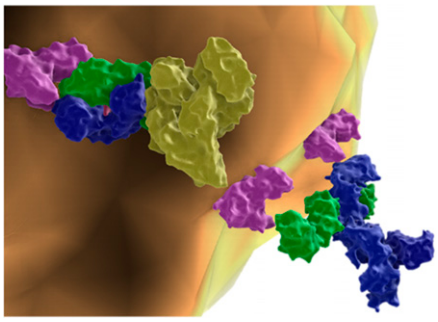


Fc receptors (pink; Protein Data Bank [PDB] ID 3SGJ) expressed on phagocytes can be blocked antagonistically by multivalent full-length anti-Fc receptor antibody (green and blue, bottom right; PDB ID 1HZH). However, such multivalent blockade could lead to undesired, crosslinking-induced, receptor activation. In this report, the authors developed a strategy for Fc receptor blockade without triggering unwanted receptor signaling by using a fusion protein comprising a monovalent Fc receptor-binding domain (green and blue, upper left; PDB ID 4LAR) recombinantly fused to serum albumin (yellow; PDB ID 1GNJ) as a means of half-life extension. Figure courtesy of Dr Ben Yu.

in which Fc function was inhibited by deglycosylation. A pilot study in 2009 confirmed the activity of 3G8 and GMA161 in ITP; however, both antibodies were associated with a similar toxicity profile.[6] These studies provided proof of principle that FcγRIII contributed to clearance of antibody-coated platelets in ITP. However, toxicity from FcγRIII activation halted their further consideration as ITP therapeutics.

Humans express several Fcγ receptors in a cell-specific manner. FcγR1, FcγRIIa, FCγRIIc, and FcγRIIIa are "activating" receptors, whereas FcγRIIb is inhibitory.[7] FcγRI and FcγRIIIa contain a ligand-binding α chain, but signal through the associated γ chain dimer, which contains an immunoreceptor tyrosine-based activation motif. FcγRIIIa is a low-affinity receptor that preferentially binds immune complexes; ligation of FcγRIIIa leads to phosphorylation of the immunoreceptor tyrosine-based activation motif, recruitment of SYK, and activation of downstream targets including SOS, RAS, and phosphatidylinositol 3-kinase, causing cellular activation, phagocytosis, and cytokine release.

Though it had been assumed that the toxicity of 3G8 was a consequence of FcγR activation by the Fc region, Yu et al reasoned that the parallel toxicity of GMA161 suggested that these responses were due instead to ligation of FcγRIIIA by the bivalent F(ab′)2 region. To test this hypothesis, they produced a monovalent 3G8 single chain variable region (scFv) fused to human serum albumin (HSA) (see figure). This fusion protein specifically bound and blocked binding of human immunoglobulin G to the extracellular domain of human FcγRIIIa. The investigators then created a murine counterpart of the 3G8 scFv-HSA fusion protein using an scFv from monoclonal antibody 2.4G2, which targets murine FcγRIII/IIb, and murine serum albumin (MSA). This construct specifically bound its target and inhibited development of thrombocytopenia in mice treated with the antiplatelet antibody MWReg30, which induces thrombocytopenia by stimulating platelet clearance through FcγRIII.[1] In contrast, 2.4G2 scFv-MSA did not impair platelet clearance in response to 6A6, a murine antiplatelet antibody that mediates clearance through FcγRIV.[1] Importantly, the 2.4G2 scFv-MSA fusion protein had an extended half-life and did not cause the systemic drop in temperature or activation of basophils seen with the bivalent parental antibody, which resulted from activation of FcγRIII.

This study extends previous work demonstrating the role of FcγRIII in ITP and suggests the feasibility of a monovalent FcγRIII scFv-fusion protein as an ITP therapeutic. However, many questions remain. For example, human ITP is a complex disorder with a heterogeneous array of antiplatelet antibodies that may cause platelet clearance through different Fcγ receptors. Moreover, a recent report suggests a role for an entirely different receptor, the hepatocyte Ashwell-Morell receptor, in clearance of platelets bound by antibodies to GP1bα that cause Fcγ-independent activation and desialylation.[8] Finally, it is likely that inhibition of platelet production is of primary importance in at least some cases of ITP.

The late baseball legend, Yogi Berra, was famous for his "Yogi-isms," the most well-known of which is "It ain't over 'til it's over," meaning that a baseball game was not over until the last out, and there was always a chance for a comeback. In this report, Yu et al show that this also applies to treatment of ITP through inhibition of FcγRIII. Future studies of the 3G8 scFv-HSA fusion protein or its derivatives in human ITP will be of great interest.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

REFERENCES

1. Yu X, Menard M, Prechl J, Bhakta V, Sheffield WP, Lazarus AH. Monovalent Fc receptor blockade by an anti-Fcγ receptor/albumin fusion protein ameliorates murine ITP with abrogated toxicity. *Blood.* 2016;127(1):132-138.
2. Kistangari G, McCrae KR. Immune thrombocytopenia. *Hematol Oncol Clin North Am.* 2013;27(3):495-520.
3. Fehr J, Hofmann V, Kappeler U. Transient reversal of thrombocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous gamma globulin. *N Engl J Med.* 1982;306(21):1254-1258.
4. Psaila B, Bussel JB. Fc receptors in immune thrombocytopenias: a target for immunomodulation? *J Clin Invest.* 2008;118(8):2677-2681.
5. Clarkson SB, Bussel JB, Kimberly RP, Valinsky JE, Nachman RL, Unkeless JC. Treatment of refractory immune thrombocytopenic purpura with an anti-Fc gamma-receptor antibody. *N Engl J Med.* 1986;314(19):1236-1239.
6. Nakar CT, Bussel JB. 3G8 and GMA161, anti FcγRIII inhibitory monoclonal antibodies in the treatment of chronic refractory ITP (summary of two pilot studies) [abstract]. *Blood.* 2009;114(22). Abstract 2404.
7. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol.* 2008;8(1):34-47.
8. Li J, van der Wal DE, Zhu G, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat Commun.* 2015;6:7737.

DOI 10.1182/blood-2015-11-678680

● ● ● RED CELLS, IRON, AND ERYTHROPOIESIS

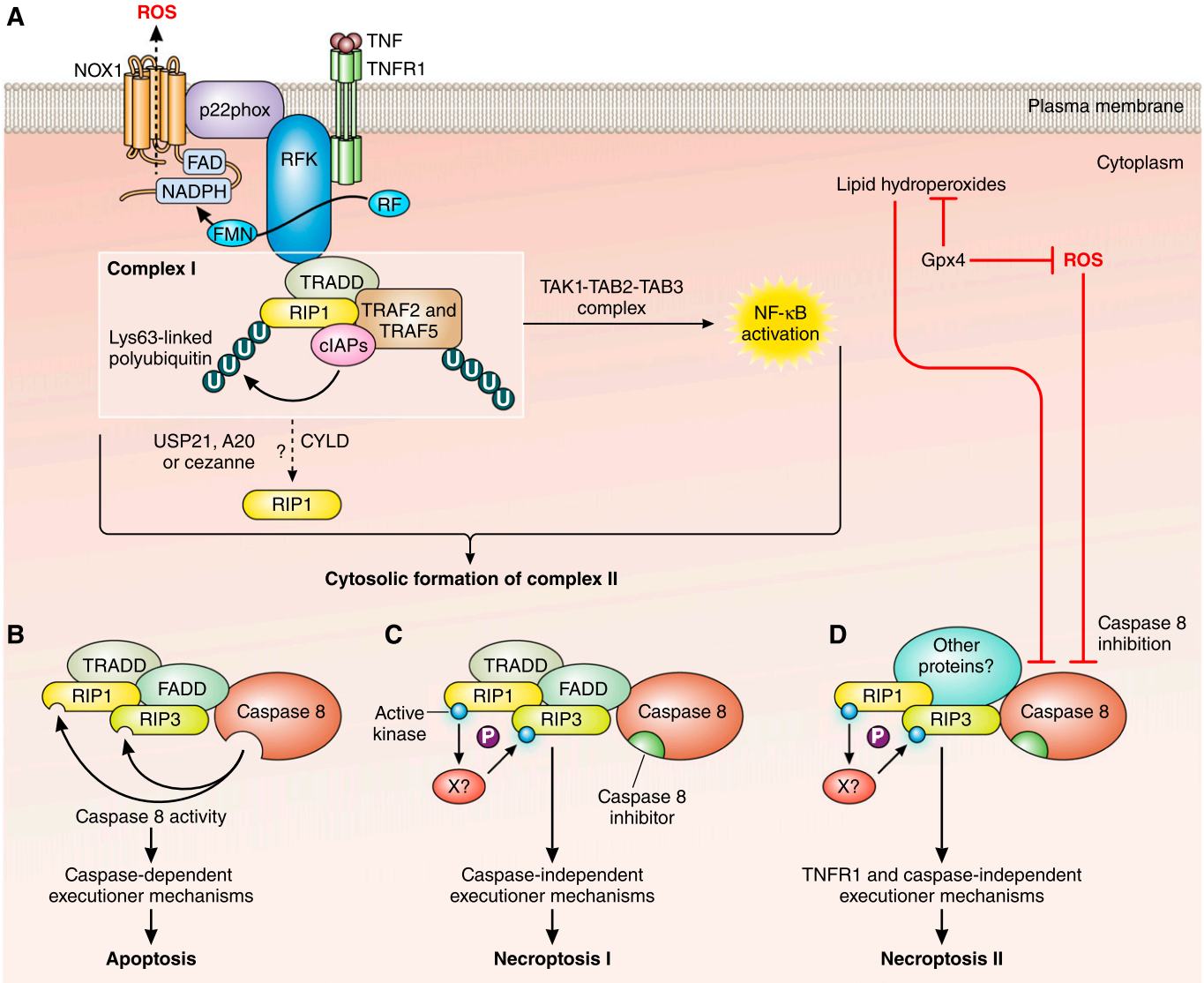
Comment on Canli et al, page 139

Unconventional cell death in erythroid cells

Lesley J. Bruce and David J. Anstee BRISTOL INSTITUTE FOR TRANSFUSION SCIENCES

In this issue of *Blood*, Canli et al demonstrate that reactive oxygen species (ROS) and lipid hydroperoxides can function as unconventional upstream signaling activators of receptor-interacting protein 3 (RIP3) kinase-dependent necroptosis, causing anemia in mice lacking erythroid glutathione peroxidase 4 (Gpx4).[1]

In vertebrates, the most common forms of programmed cell death are apoptosis and necroptosis, and their activation pathways overlap.[2] Both may be triggered by ligation of cell death receptors, such as tumor necrosis factor (TNF) receptor 1 (TNFR1). The ligation of the receptor results in the formation of a membrane-associated complex (complex I) consisting of adaptor proteins required for downstream signaling, such as TNFR-associated death domain (TRADD) protein, TNFR-associated factor (TRAF), RIP kinases, and Fas-associated protein with death domain (FADD) (see figure, panel A). Internalization of the TNFR complex and deubiquitination of RIP1 promotes the conversion of complex I to the cytosolic complex II often comprising RIP1, RIP3, TRADD, FADD, and caspase 8. Caspase functions as a molecular switch between apoptosis and necroptosis; when caspase is inhibited the necrosome is activated.[4] Activation of caspase 8 inactivates the RIP proteins and initiates apoptosis (see figure, panel B). Inhibition of caspase 8 results in activation of the RIP proteins and initiation of necroptosis (see figure, panel C). What Canli et al[1] have shown is that, in erythroid cells, a different cytoplasmic necroptosis complex may be formed that is initiated



Activation pathways of cell death. (A) Binding of TNF to TNFR1 causes a conformational change and the intracellular assembly of TNFR complex I. TNFR complex I includes TRADD, RIP1, cellular inhibitor of apoptosis proteins (cIAPs), TRAF2, and TRAF5. Ubiquitylation of RIP1 results in recruitment of transforming growth factor-β–activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), and TAB3, which initiate the nuclear factor-κB (NF-κB) activation pathway. Riboflavin kinase (RFK) links the TNFR1 death domain to p22phox, a subunit of NADPH oxidase 1 (NOX1), which contributes to TNFα-induced necroptosis by generating ROS. Deubiquitylation of RIP1 results in 2 distinct types of cell death. (B) The internalization of TNFR1 and the cytosolic assembly of TNFR complex II, which often contain TRADD, FADD, caspase 8, RIP1, and RIP3. Caspase 8 triggers apoptosis by activating the classical caspase cascade, and cleaves and inactivates RIP1 and RIP3. (C) When caspase 8 is inhibited, RIP1 and RIP3 become phosphorylated, triggering necroptosis. (D) ROS and lipid hydroperoxides are normally kept in check by Gpx4 but in oxidative conditions may increase and inhibit caspase 8. Loss of caspase 8 activity activates RIP1 and RIP3, resulting in a caspase-independent, TNFR-independent form of necroptosis. CYLD, cylindromatosis (turban tumor syndrome); FAD, flavin adenine nucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; P, phosphate; RF, riboflavin; U, ubiquitin; USP, ubiquitin-specific peptidase; X, unidentified kinase. Adapted from Figure 1 of Vandenabeele et al[3] with permission. Professional illustration by Patrick Lane, ScEYEnce Studios.

by an increase in ROS and/or lipid hydroperoxides and activated by RIP3 (see figure, panel D).

During erythropoiesis, erythroid precursor cells undergo transformation, including complicated membrane and cytoskeletal rearrangements, massive protein production, accumulation of hemoglobin, and, finally, enucleation and reticulocyte maturation. This is a highly oxidative environment and the accumulation of hemoglobin in erythroid precursor cells leads to increased expression of ROS scavenging proteins and enzymes, such as catalase, peroxiredoxin 2, superoxide dismutase, glutathione peroxidase, and glutathione reductase.[5] Here, Canli et al show for the first time that Gpx4 plays a key role in the homeostasis of ROS and lipid hydroperoxides in erythroid precursors. Gpx4 is essential for cell survival and embryonic development so Canli et al had to make a conditional erythroid mouse knockout.[1] These Gpx4Δ mice were anemic with increased reticulocytes and increased erythroid precursor cell death. Inhibition experiments showed that the cell death did not involve TNFR or caspases. To confirm that the cell death occurred through a necroptosis pathway, the authors generated Gpx4Δ/RIP3$^{-/-}$ mice.[1] Levels of cell death in these mice returned to normal even in the presence of high levels of ROS showing the pathway to be completely RIP3 dependent. In immunoprecipitation assays, Canli et al found that the novel necroptosis complex included RIP1, RIP3, and caspase 8 but not FADD. However, caspase 8 was inactive; absence of Gpx4 leads to functional inactivation of caspase 8 by glutathionylation. The novel necroptosis complex may also include other, as-yet-unidentified, components (see figure, panel D).

Apoptosis and necroptosis are important mechanisms for the removal of diseased and damaged cells. Autophagy, which shares some features with apoptosis, is required to remove damaged or defunct proteins from cells and plays an essential role in reticulocyte maturation.[6] In contemporary culture systems for the generation of red cells the yield of mature reticulocytes is suboptimal because prolonging the cultures to maximize reticulocyte yield is accompanied by significant cell death. As the cells die, they likely release factors into the medium which exacerbate cell death. Similarly, in donor red cell storage, the cells are exposed to oxidative stress; by day 35 of refrigerated storage, up to 20% of cells may be effectively "dead" and these cells are removed by the spleen in the first 24 hours posttransfusion.[7] A greater understanding of the processes of regulated erythroid cell death through necroptosis and apoptosis may increase yields and stability of manufactured red cells for transfusion therapy and may lead to improvements in the quality of stored donor red cells.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

REFERENCES

1. Canli Ö, Alankuş YB, Grootjans S, et al. Glutathione peroxidase 4 prevents necroptosis in mouse erythroid precursors. *Blood.* 2016;127(1):139-148.
2. Inoue H, Tani K. Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments. *Cell Death Differ.* 2014;21(1):39-49.
3. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol.* 2010; 11(10):700-714.
4. Salvesen GS, Walsh CM. Functions of caspase 8: the identified and the mysterious. *Semin Immunol.* 2014;26(3): 246-252.
5. Marinkovic D, Zhang X, Yalcin S, et al. Foxo3 is required for the regulation of oxidative stress in erythropoiesis. *J Clin Invest.* 2007;117(8): 2133-2144.
6. Mankelow TJ, Griffiths RE, Trompeter S, et al. Autophagic vesicles on mature human reticulocytes explain phosphatidylserine-positive red cells in sickle cell disease. *Blood.* 2015;126(15): 1831-1834.
7. Hess JR, Hill HR, Oliver CK, et al. Twelve-week RBC storage. *Transfusion.* 2003;43(7):867-872.

DOI 10.1182/blood-2015-11-677229

● ● ● TRANSPLANTATION

Comment on Palmer et al, page 160

Predicting chronic GVHD outcomes: are we there yet?

David A. Jacobsohn CHILDREN'S NATIONAL HEALTH SYSTEM; GEORGE WASHINGTON UNIVERSITY SCHOOL OF MEDICINE AND HEALTH SCIENCES

In this issue of *Blood*, Palmer et al provide encouragement that important chronic graft-versus-host disease (GVHD) patient outcomes (such as overall survival [OS] and failure-free survival [FFS]) are predicted by clinician-assessed response, patient-reported outcomes, and 2014 National Institutes of Health (NIH)-response criteria.[1]

FFS has advantages for studies in chronic illness where reaching end points of OS and nonrelapse mortality (NRM) may take too long. The FFS end point takes a very global approach and incorporates objective end points (absence of systemic treatment change, NRM, and recurrent malignancy) into 1 composite end point.[2-4] FFS is simple and takes into account addition of immunosuppression as well as significant (ie, life-threatening) toxicity from it. What the FFS end point does not tell us, however, is how chronic GVHD is impacting a particular patient, what manifestations are affecting them, and exactly in what order the manifestations are responding.

Because FFS does not address the symptoms and burden to the individual patient, it is very important to have shorter-term measures of disease that help predict FFS and OS. These measures are crucial to understand how immunosuppression is working on different patient groups (eg, patients with steroid-refractory disease or patients starting therapy at a particular stage in an organ), whether clinically or in a research study. These measures give us a snapshot at specific intervals on the overall burden of chronic GVHD in a specific patient.

What is so exciting from the work by Palmer et al is that criteria such as clinician-reported response, patient-reported response, and the 2014 NIH criteria are predictive of important long-term outcomes. For example, patients with a clinician-reported response had higher likelihood of FFS than patients with stable disease (SD) or progressive disease (PD)