To the editor:

CD20 antibodies induce production and release of reactive oxygen species by neutrophils

In a recent study, Golay and coworkers address the commonly over-looked role of neutrophils in therapy of chronic lymphocytic leukemia (CLL) with monoclonal antibodies (mAbs).1 Convincingly, they demonstrate that both rituximab (RTX) and, to a greater extent, glycoengineered obinutuzumab, trigger neutrophil effector functions via the Fc receptors CD16b and CD32. Moreover, utilizing a 2′,7′-dichlorofluorescein diacetate (H2DCFDA)–based assay, the authors claim that neutrophil activation occurs without concomitant production of reactive oxygen species (ROS; oxygen radicals).

However, the experimental approach used to assess ROS production has serious limitations: first, H2DCFDA assays are relatively insensitive and unspecific2 and second, in the study, neutrophils were stained with a fluorescein-conjugated antibody preventing the ability to correctly assess the ROS formation in response to CD20 antibodies with a fluorescein-based assay. Thus, in a series of experiments, we used a sensitive isoluminol-enhanced chemiluminescence method to monitor ROS responses in neutrophils exposed to CD20 mAbs or αCD20-opsonized leukemic cells. These experiments showed that malignant CLL cells in the presence of either RTX or the second-generation agent ofatumumab (OFA), triggered a robust extracellular release of oxygen radicals from neutrophils. ROS production was readily blocked by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenyleneiodonium (DPI). Similar results were obtained in experiments where we exposed CLL patient-derived neutrophils to immobilized CD20 mAbs (Figure 1A-D).

A significant part of the benefit of CD20 mAbs in therapy of CLL is attributed to antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells.3,4 However, cytotoxic NK cells are also highly sensitive to oxygen radical-mediated inactivation.5,6 Thus, we investigated whether αCD20-induced neutrophil ROS production had an impact on NK-cell viability.5 Indeed, we found that NK cells...
displayed significant cell death after exposure to neutrophils in the presence of either RTX or OFA, but not to either agent alone. The addition of DPI rescued NK cells, strongly suggesting NADPH oxidase– and ROS-dependent NK cell death (Figure 1E). During the course of these experiments we did not have access to the glycoengineered antibody obinutuzumab, but given its profound capacity to stimulate neutrophils, it is likely to share the ROS-triggering characteristics of RTX and OFA.

Collectively, our findings raise the question of whether oxygen radical release from αCD20-exposed neutrophils may inactivate NK cells also in vivo and thus limit the efficacy of therapeutic mAbs in CLL. More studies are warranted to investigate whether neutrophils or neutrophil-derived ROS are important effector arms in antibody treatment of CLL, and whether it may be beneficial to supplement αCD20 therapy with antioxidative strategies to unravel the full effector function of NK cells in CLL.

Approval was obtained from the Ethical Review Board of Gothenburg for these experiments. Informed consent was provided according to the Declaration of Helsinki.

Olle Werlenius
Sahlgrenska Cancer Center and Department of Hematology,
The Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

Rebecca E. Riise
Sahlgrenska Cancer Center and Department of Infectious Diseases,
The Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

Maria Simpanen
Sahlgrenska Cancer Center and Department of Hematology,
The Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

Johan Aurelius
Sahlgrenska Cancer Center and Department of Infectious Diseases,
The Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

To the editor:

**Novel severe hemophilia A and moyamoya (SHAM) syndrome caused by Xq28 deletions encompassing F8 and BRCC3 genes**

A 10-year-old boy with severe hemophilia A and no other obvious morbidity arrived at the hospital with focal neurological signs and a suspected intracranial hemorrhage. Surprisingly, radiological studies demonstrated an ischemic stroke. Neither active thromboembolism nor genetic predisposition to thrombosis was found. Neuroimaging demonstrated severe narrowing of internal carotid arteries and their branches and development of a collateral vascular network, diagnostic of moyamoya syndrome (Figure 1). Further clinical workup revealed mild facial dysmorphia, hypertension, osteopenia, and duplication of the right renal artery, a phenotype likely caused by a genetic aberration. Next-generation sequencing followed by long-range polymerase chain reaction (Figure 1 and supplemental Materials) demonstrated a large Xq28 deletion of ~150 kbp encompassing exons 1 to 6 of F8, as well as the FUNDC2, MTCP1NB, MTCP1, and BRCC3 genes. BRCC3 was recently identified as a familial moyamoya gene. We demonstrate that both centromeric and telomeric breakage sites of the deletion are located in nearly identical repetitive Alu sequences that could be mutational hotspots. The patient’s sister and mother are heterozygous for the same deletion. At the age of 18, the sister presented a mild phenotype including low levels of factor VIII (22%), aortic coarctation, and hypertension, but she has no signs of moyamoya angiopathy.

A review of the literature yields 3 more likely individuals/families with this novel severe hemophilia and moyamoya (SHAM) syndrome: 1 clinical description in a Japanese patient and 2 descriptions of Xq28 rearrangements in hemophilia A patients that disrupt BRCC3 and bear striking clinical similarity to Xq28-linked familial moyamoya, although no neuroimaging data are available to confirm the diagnosis. There is also a genetic report of BRCC3 deletion in a hemophilia patient without phenotype data. The ratio of BRCC3 inactivation in hemophilia A is unknown because the regions telomeric to F8 are rarely subjected to genetic diagnostics. We accessed the Centers for Disease Control Hemophilia A mutation project database that contains >2000 pathological F8 mutations.

Fredrik B. Thorén
Sahlgrenska Cancer Center and Department of Infectious Diseases,
The Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

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**Correspondence:** Olle Werlenius, Sahlgrenska Cancer Center, University of Gothenburg, Box 425, 405 30 Gothenburg, Sweden; e-mail: olle.werlenius@g.uu.se

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