

mean corpuscular hemoglobin within 2 to 4 months after bone marrow transplantation.

These responses, which characterize adaptation to iron deficiency, were associated with enhanced terminal erythropoiesis due to reduced apoptosis of late erythroid progenitor cells (basophilic, polychromatic, and orthochromatic erythroblasts), reticulocytes, and RBCs. However, they were not accompanied by increased plasma Epo levels, a hallmark of true iron deficiency. Notably, under conditions of mild dietary iron restriction, erythroid differentiation of control mice was similar to that of iron-replete *Tfr2<sup>BMKO</sup>* mice, and as expected, plasma Epo was increased. In iron-poor *Tfr2<sup>BMKO</sup>* mice, erythropoiesis was not further modified, and plasma Epo levels remained unchanged, whereas EpoR messenger RNA (mRNA) downregulation during erythroid cell differentiation was delayed. Taken together, these data suggest that the lack of *Tfr2* confers enhanced Epo sensitivity to erythroid progenitor cells, which is further supported by the induction of Epo target genes (*Bcl-xL*, *FasI*, *Serpina3g*, *Cng2*, *Epor*, and *Erfe*). Future studies are expected to clarify whether the lack of erythroid *Tfr2* directly stimulates EpoR signaling. It is also conceivable that *Tfr2* may modulate the capacity of EpoR to form productive signaling complexes and/or affect its expression (if EpoR protein levels correspond to mRNA).

Among the Epo downstream targets that are induced in *Tfr2*-deficient erythroid progenitor cells, erythroferrone (Erfe) is of particular importance. This erythroid hormonal regulator suppresses hepcidin expression during stress erythropoiesis, as a homeostatic adaptation to augment iron supply.<sup>9</sup> Thus, the induction of Erfe presumably accounts for the reduced hepcidin mRNA levels that are observed in livers of *Tfr2<sup>BMKO</sup>* mice. These findings highlight the interconnection between the hepatic and erythroid functions of *Tfr2* in regulating iron metabolism and erythropoiesis.

A unifying model is depicted in the figure. In an iron-replete state (panel A), *Tfr2* is stabilized in response to high Tf saturation. Hepatic *Tfr2* promotes iron signaling to hepcidin to inhibit further iron fluxes to the bloodstream. Erythroid *Tfr2* restricts Epo sensitivity to limit excessive erythropoiesis. Conversely, in an iron-deficient state (panel B), *Tfr2* is unstable. Downregulation of hepatic

*Tfr2* inhibits iron signaling to hepcidin to stimulate iron efflux from cells and thereby increase iron supply to erythroblasts. Downregulation of erythroid *Tfr2* enhances Epo sensitivity to stimulate erythropoiesis; at the same time, it contributes to suppression of hepcidin via Erfe. Thus, the sensor of circulating iron *Tfr2* couples systemic iron traffic and erythroid utilization. The importance of this network is also emphasized by the existence of an analogous link: the sensor of cellular iron, iron regulatory protein 1, likewise couples systemic iron traffic and erythroid utilization by controlling renal and hepatic Epo production as an upstream regulator of hypoxia inducible factor 2 $\alpha$ ,<sup>10</sup> a major transcriptional inducer of Epo during hypoxaemia or iron deficiency.

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

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## ● ● ● THROMBOSIS & HEMOSTASIS

Comment on Hofbauer et al, page 1180

# Factor VIII inhibitor: affinity is the clue

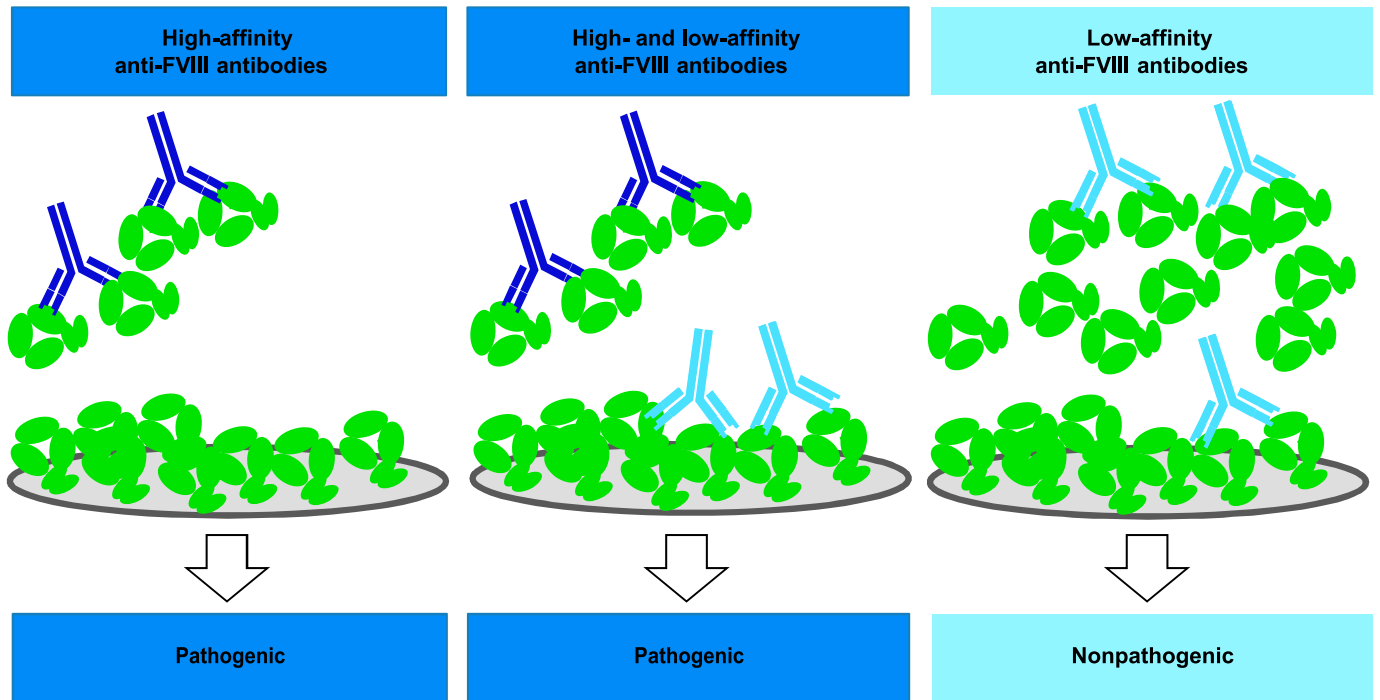
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In this issue of *Blood*, Hofbauer et al<sup>1</sup> provide an explanation to the puzzling observation that, in the plasma of healthy subjects and hemophilia A patients treated with factor VIII (FVIII) concentrates, the presence of anti-FVIII antibodies is not consistently associated with a reduction in FVIII activity. On the basis of these findings, they describe a novel sensitive method for the identification of the clinically relevant high-affinity anti-FVIII antibodies.<sup>1</sup>

**H**emophilia A is a hereditary bleeding disorder characterized by deficient or dysfunctional FVIII. Treatment includes the transfusion of plasma-derived or recombinant FVIII concentrates, which can result in the induction of anti-FVIII antibody production in up to 30% of patients with severe hemophilia.<sup>2</sup> Anti-FVIII antibodies are also responsible for the autoimmune disorder acquired hemophilia A.

Anti-FVIII antibodies are currently detected *in vitro* by their capacity to

inhibit FVIII activity in functional assays.<sup>3</sup> However, these assays cannot detect antibodies that are directed toward the nonfunctional sites of FVIII, even though they may accelerate the clearance rate of FVIII<sup>4-6</sup> and are therefore of pathological relevance. Alternative techniques such as the use of immunoblotting, enzyme-linked immunosorbent assay (ELISA), and immunoprecipitation have therefore been developed to detect a broader range of anti-FVIII antibodies. Recently, very



Competition-based ELISA discriminates between pathogenic high-affinity and nonpathogenic low-affinity anti-FVIII antibodies. High-affinity pathogenic antibodies (dark blue) interact with a low concentration of FVIII (green) in the fluid phase and therefore do not bind to FVIII insolubilized on a solid surface. On the contrary, low-affinity nonpathogenic antibodies (light blue) do not make complexes with a low concentration of FVIII in solution but bind to insolubilized FVIII, possibly because each antibody can interact with 2 FVIII molecules that are held in close proximity at the solid surface. High- and low-affinity antibodies bind to FVIII when it is present at a high concentration in solution. Only a fraction of low-affinity antibodies are then still available to interact with insolubilized FVIII. High-affinity antibodies are found only in hemophilia A patients treated with FVIII concentrates who develop FVIII inhibitors or in patients with acquired hemophilia A. The low-affinity anti-FVIII antibodies are found in hemophilia A patients with a normal response to FVIII concentrates and in healthy controls.

sensitive techniques based on immunofluorescence have also been implemented.<sup>6,7</sup> Such assays can also identify the isotypes and the immunoglobulin(Ig)G subclasses of the antibodies,<sup>7</sup> notably, the IgG4 subclass, which predominates in the immune response to FVIII.<sup>8</sup> Despite these advances, the usefulness of nonfunctional assays has remained questionable because they do not establish a clear distinction between pathogenic and nonpathogenic anti-FVIII antibodies. Nonpathogenic antibodies can be found in healthy donors and in some hemophilia A patients treated with FVIII concentrates and do not elicit the clinical manifestations associated with inhibitor development.

To better discriminate between the different types of anti-FVIII antibodies, Hofbauer et al<sup>1</sup> have taken into account antibody affinities. Such studies have previously been attempted with monoclonal antibodies. For example, the affinity of the human monoclonal antibody BO2C11, which was produced by the immortalization of B lymphocytes from a patient with a high titer inhibitor, was determined by Scatchard analysis of FVIII inhibition and surface

plasmon resonance.<sup>9</sup> The high affinity of this antibody ( $K_A \geq 10^{11}$  M) correlated with the congruence observed in crystallography between the antibody binding site and the phospholipid binding site of the FVIII C2 domain.<sup>10</sup> Unfortunately, these techniques for evaluating the affinity of monoclonal antibodies cannot be used for polyclonal antibodies such as anti-FVIII antibodies in the plasma of patients.

Hofbauer et al<sup>1</sup> have therefore developed a sensitive ELISA platform that can discriminate between high- and low-affinity anti-FVIII antibodies in plasma. In this assay, both types of antibodies are able to bind to FVIII insolubilized on ELISA plates, possibly because in these circumstances, the antibodies can bind to 2 FVIII molecules that are held in close proximity at the solid surface (see figure). However, the activity of low- vs high-affinity antibodies differs when FVIII is added in the fluid phase. According to the law of mass action, only high-affinity antibodies make complexes with FVIII when FVIII is at a low concentration in solution. This prevents the high-affinity antibodies from being available to interact with insolubilized

FVIII (see figure). In contrast, a high concentration of FVIII in solution allows low-affinity antibodies to make complexes with FVIII and reduces the binding of low-affinity antibodies to insolubilized FVIII (see figure).

Using this principle, Hofbauer et al<sup>1</sup> have characterized anti-FVIII antibodies from different cohorts of patients with congenital and acquired hemophilia A and compared them to healthy individuals. They analyzed the apparent affinities of FVIII-specific antibodies found in patients with FVIII inhibitors detected through the Nijmegen-Bethesda assay.<sup>3</sup> This revealed that antibody affinities from these patients were up to 100-fold higher than the affinities of antibodies found in hemophilia A patients without inhibitors and in healthy individuals. This novel method also appears to be much more sensitive for the detection of inhibitor than functional assays, because high-affinity anti-FVIII antibodies were detected in the plasma of one patient more than a year before the first detection of FVIII inhibitors.

These results suggest that the appearance of high-affinity antibodies against FVIII is a suitable marker for the early detection of

a clinically relevant immune response to FVIII. This competition-based ELISA may therefore be of interest for evaluating the immunogenicity of novel FVIII concentrates in a well-standardized and sensitive manner. It may also help in identifying the poorly characterized noninhibitory antibodies suspected of accelerating the clearance of FVIII. Given that FVIII is at a low concentration in plasma (0.1–0.2 nM), such antibodies should have a high affinity for making complexes with FVIII.

The ability of this novel assay to detect early anti-FVIII antibody development may also have useful clinical applications for optimizing inhibitor eradication because the success rate of immune tolerance induction is higher when treatment is started early after inhibitor detection and when inhibitor titer is low. Similarly, this novel assay may be useful for the follow-up of patients with mild/moderate hemophilia A, in whom the spreading of an immune response from the allogeneic FVIII to the patient's self FVIII can transform the patients' bleeding phenotype into that of severe hemophilia A. In such patients, the detection of an initial immune response to allogeneic FVIII may allow their therapy to be adapted to prevent the development of an autoimmune response to self FVIII.

Prospective clinical studies will thus be needed to investigate the opportunities provided by this novel approach of inhibitor detection.

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

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## ● ● ● TRANSPLANTATION

Comment on Morishima et al, page 1189

# HLA mismatching in transplantation

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In this issue of *Blood*, Morishima et al report on the risks of acute and chronic graft-versus-host disease (GVHD), relapse, and mortality associated with mismatching for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci after unrelated donor transplantation for Japanese patients.<sup>1</sup>

**H**LA matching between a transplant donor and recipient remains the most robust predictive factor for outcome after hematopoietic cell transplantation from unrelated donors.<sup>2–4</sup> The presence of recipient HLA-A, -B, -C, or -DRB1 differences not shared by the donor is associated with higher risks of GVHD, increased morbidity, and increased mortality compared with complete matching, whereas the presence of donor disparity not shared by the recipient increases the risk of graft failure. For this reason, current pretransplant donor selection strategies include the use of molecular methods to ascertain donor-recipient matching for allelic subtypes at each HLA locus and to limit the total mismatches to the least possible number.

Early on in the unrelated transplant experience, preliminary observations suggested that risks associated with HLA mismatching were not necessarily equivalent across all HLA loci. In other words, depending on the mismatched locus, risks could differ. Early data suggested that HLA mismatching for the class I locus HLA-A, -B, or -C may be associated with different risks than mismatching for the class II locus HLA-DRB1 or -DQB1.<sup>3</sup> As the unrelated donor transplant experience matured, registry data from the United States and Japan confirmed the negative impact of HLA disparity on GVHD

and mortality<sup>4,5</sup> and furthermore suggested that mismatching at HLA-DQB1 was better tolerated in general than mismatching at any other HLA locus.

Until recently, the clinical significance of the third classical class II locus, HLA-DPB1, has remained ill defined. Because of the weak linkage disequilibrium between HLA-DR, -DQ, and -DP, the vast majority of otherwise HLA-matched transplant pairs are mismatched for 1 or both HLA-DPB1 alleles. The limited number of HLA-DPB1-matched transplants precluded definitive retrospective examination of this locus until recently. Like HLA-DRB1 and -DQB1, HLA-DPB1 mismatching is associated with higher incidence of acute GVHD; however, HLA-DPB1 mismatching is also associated with lower recurrence of disease after transplantation, and this favorable graft-versus-leukemia (GVL) effect can in part balance the deleterious effects of severe GVHD on mortality.<sup>6</sup>

It is in this context that the current analysis by the Japan Marrow Donor Program takes on new meaning. Morishima et al<sup>1</sup> mounted a large-scale effort to retype all HLA loci to ensure that modern nomenclature could be applied to each patient's and donor's HLA assignment and to accurately define donor-recipient allele matching. By using an exceedingly large transplant population of