Unexpected enhancement of FVIII immunogenicity by endothelial expression in lentivirus-transduced and transgenic mice

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Key Points

- EC-targeted FVIII expression is more immunogenic than platelet-target expression.

- ECs can present antigen FVIII to Tfh cells, promoting cell proliferation.

Factor VIII (FVIII) replacement therapy for hemophilia A is complicated by development of inhibitory antibodies (inhibitors) in ~30% of patients. Because endothelial cells (ECs) are the primary physiologic expression site, we probed the therapeutic potential of genetically restoring FVIII expression selectively in ECs in hemophilia A mice (FVIII[null]). Expression of FVIII was driven by the Tie2 promoter in the context of lentivirus (LV)-mediated in situ transduction (T2F8LV) or embryonic stem cell–mediated transgenesis (T2F8[Tg]). Both endothelial expression approaches were associated with a strikingly robust immune response. Following in situ T2F8LV-mediated EC transduction, all FVIII[null] mice developed inhibitors but had no detectable plasma FVIII. In the transgenic approach, the T2F8[Tg] mice had normalized plasma FVIII levels, but showed strong sensitivity to developing an FVIII immune response upon FVIII immunization. A single injection of FVIII with incomplete Freund adjuvant led to high titers of inhibitors and reduction of plasma FVIII to undetectable levels. Because ECs are putative major histocompatibility complex class II (MHCII)-expressing nonhematopoietic, "semiprofessional" antigen-presenting cells (APCs), we asked whether they might directly influence the FVIII immune responses. Imaging and flow cytometric studies confirmed that both murine and human ECs express MHCII and efficiently bind and take up FVIII protein in vitro. Moreover, microvascular ECs preconditioned ex vivo with inflammatory cytokines could functionally present exogenously taken-up FVIII to previously primed CD4^{+}/CXCR5^{+} T follicular helper (Tfh) cells to drive FVIII-specific proliferation. Our results show an unanticipated immunogenicity of EC-expressed FVIII and suggest a context-dependent role for ECs in the regulation of inhibitors as auxiliary APCs for Tfh cells.

Introduction

Hemophilia A (HA) is a genetic bleeding disorder, resulting from a deficiency of factor VIII (FVIII). Although FVIII protein-replacement therapy is effective, 30% of severe HA patients will develop anti-FVIII inhibitory antibodies (inhibitors), rendering routine FVIII protein-replacement therapy useless. In such patients, immune-tolerance induction, infusing high quantities of FVIII, is the standard care. However, this approach costs over $1 million per patient-year and remains incompletely effective for 30% of inhibitor patients.[1-5]

Data-sharing requests may be e-mailed to the corresponding author, Qizhen Shi (qshi@versiti.org).

The full-text version of this article contains a data supplement.

Gene therapy is an attractive alternative for the treatment of HA. However, the potential to develop inhibitors to the neoprotein remains.[6-15] Though not a natural site for FVIII expression, hepatocyte-targeted FVIII gene therapy has shown promising efficacy.[16-18] Because platelets naturally expressed the FVIII carrier protein von Willebrand Factor (VWF), we previously engineered a lentiviral platelet-specific gene-therapy approach for on-demand delivery of FVIII at the site of injury.[19] This partially restored hemostasis in HA mice and was associated with a degree of immunologic tolerance to infused FVIII protein.[20-24] Recent studies confirm that the major physiologic site for FVIII synthesis is endothelial cells (ECs).[25-30] Thus, we explored the potential for use of ECs as a target for gene therapy of HA.

Here, we investigated the efficacy of a lentivirus-based vector controlled by the EC-specific Tie2 promoter (T2F8) to restore FVIII expression in HA mice. We compared results of EC-targeted vs platelet-targeted FVIII expression using transgenic models. In both systems, we found endothelial-expressed FVIII to be surprisingly immunogenic. Our in vivo and ex vivo studies suggest that ECs can serve as auxiliary major histocompatibility complex class II (MHCII)–expressing antigen (Ag)-presenting cells (APCs) that mediate FVIII-specific stimulation of CD4+/CXCR5+ follicular T helper (Tfh) cells. These somewhat counterintuitive findings, together with previous studies, provide cautionary insight about endothelial-targeted gene therapy and support a putative site- and context-specific role for ECs as modulators of FVIII immunogenicity.

Materials and methods

Additional details on the reagents, methods, and statistics used in this study are provided in supplemental Material and methods.

Mice

Animal studies were approved by the institutional animal care and use committee of Medical College of Wisconsin or Harvard. Mouse models, as summarized in Table 1, included: (1) an FVIII-deficient (FVIIInull) model; (2) a platelet-specific FVIII expression model, 2bF8 transgenic (2bF8Tg) mice, in which human B-domain–deleted FVIII (hBDDFVIII) was expressed via the platelet-specific αIIb promotor[31]; (3) an EC-specific expression model, T2F8 transgenic (T2F8Tg) mice, in which hBDDFVIII was driven by the EC-specific Tie2 promoter[32]; (4) a CIITA−/− (MHCIInull) model; and (5) wild-type (WT) C57BL/6J mice.

In situ transduction of ECs in FVIIInull mice

We engineered a T2F8 lentiviral (LV) expression vector–harboring hBDDFVIII expression cassette under control of the Tie2 promoter (T2F8LV). T2F8LV was administered into FVIIInull mouse via tail-vein injection. Blood was collected for assessment of plasma FVIII levels and inhibitor titers.

Mouse immunization to human FVIII

FVIII immunization protocols were implemented as described.[31-33] Briefly, for the T2F8Tg and 2bF8Tg groups, mice were immunized with recombinant hBDDFVIII (rhF8) at a dose of 600 U/kg plus incomplete Freund adjuvant (IFA) by intraperitoneal injection. WT mice were immunized with recombinant human full-length FVIII (rhfF8) at a dose of 600 U/kg with IFA via intraperitoneal injection. For FVIIInull mouse immunization, rhF8 was administered at a dose of 200 U/kg without IFA via IV injection.

Assays for measuring plasma CXCL13 in mice

WT C57BL/6 mice were immunized as in the previous section. Blood from immunized and unimmunized controls was collected 7 days after the last immunization and plasma CXCL13 was measured by enzyme-linked immunosorbent assay (ELISA).

Analysis of constitutive MHCII expression on ECs in vivo

Organs were harvested from untreated WT and CIITA−/− mice, minced, and collagenase digested. Cells were stained for CD31, CD105, CD11b, CD45, and MHCII and analyzed by flow cytometry as described.[34,35] ECs were identified by gating on the CD31+/CD105+/CD11b−/CD45− cell population. Comparison of MHCII signal on CIITA−/− strain vs WT C57BL/6 was used to determine the specific MHCII signal.

In vitro study of CXCL13 expression and Tfh cell binding to ECs

Human dermal microvascular ECs (MVECs; DMVECs; hDMVECs) were cultured in the absence or presence of tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), interleukin 1β (IL1β), lipopolysaccharide (LPS), or recombinant human CXCL13 (rhCXCL13) and then washed and stained with anti-CXCL13 antibody. To study T-cell adhesion, LPS-pretreated hDMVECs were incubated with equal numbers of flow-sorted, human peripheral blood CD4+/CXCR5− or CD4+/CXCR5+ T cells for 1 hour in the absence and presence of function-blocking anti-CXCL13 antibody, followed by staining for CD4.

In vitro EC Ag-uptake and -processing studies

hDMVECs and murine DMVECs (mDMVECs) were treated with IFN-γ and TNF-α as described.[36] To assess FVIII uptake, ECs incubated with rhfF8–Alexa 488 (10 U/mL) with human VWF (1 U/mL) and fibrinogen (FIB; 150 μg/mL) for 12 hours and with LysotrackerRed for 1 hour. To assess Ag processing by ECs, cells were incubated with the model Ag/APC-processing reporter DQ-OVA (full-length ovalbumin that exhibits proteolysis-dependent 488 nm[37]) and Lysotracker Red. Cells were washed and imaged live. As a more sensitive FVIII detection, rhfF8 was coated onto GMA-8021 (anti-FVIII monoclonal antibody [MAb])–conjugated carboxylate-modified fluorescent nanoparticles. Such "rhfF8 fluorospheres" were incubated with 150 μg/mL FIB (±1 U/mL VWF) and ECs for 1.5 hours followed by washing and imaging.

In vivo EC Ag-uptake and -processing studies

To investigate the ability of ECs to proteolytically process whole proteins in vivo, we immunized C57BL/6 mice subcutaneously in both flanks with 10 μg of DQ-OVA with complete Freund adjuvant. Four days later, ECs isolated from the skins around the immunization sites were analyzed for DQ-OVA.

ECs as APCs for T-cell proliferation

To test whether ECs can functionally present FVIII Ag, we performed a T-cell proliferation assay. Primary mDMVECs were cultured under conditions that recapitulate in vivo APC properties as established.[36] Briefly, IFN-γ– and TNF-α–pretreated mDMVECs were cultured with CellTrace Violet–labeled CD4+ T cells isolated from rhF8-primed FVIIInull mouse spleens in media containing rhCXCL13 and rhF8 for 7 days. Cells were stained with anti-CD4,

Table 1. Expression models/strains used in this study

Model/strain name	Description	Deletions	Lentiviral expression	Transgenic expression	Genetic background
WT	Wild type				C57BL/6
FVIII[null]	Total mF8 deletion	mF8			C57BL/6
2bF8LV	Total mF8 deletion, platelet-specific LV hF8 expression	mF8	2bF8LV via the αIIb promotor		B6/129S
T2F8LV	Total mF8 deletion, endothelial-specific LV hF8 expression	mF8	T2F8LV via the Tie-2 promotor		B6/129S
2bF8[tg+/−]	Total mF8 deletion, platelet-specific Tg hF8 expression	mF8		hF8 via αIIb promotor	B6/129S
T2F8[tg+/−] (line: ES#5)	Total mF8 deletion, EC-specific Tg hF8 expression (1 copy)	mF8		hF8 via Tie-2 promotor	B6/129S
T2F8[tg+/+] (line: ES#5)	Total mF8 deletion, EC-specific Tg hF8 expression (2 copies)	mF8		hF8 via Tie-2 promotor	B6/129S
T2F8[tg+/+] (line: ES #65)	Total mF8 deletion, EC-specific Tg hF8 expression (5 copies)	mF8		hF8 via Tie-2 promotor	B6/129S
T2F8[Tg]F8^{−/−}VWF^{−/−}	Total mF8 and VWF deletions, EC-specific Tg hF8 expression	mF8, mVWF		hF8 via Tie-2 promotor	B6/129S
CIITA^{−/−}	Total CIITA deletion (MHCII null)	CIITA (MHCII transactivator)			C57BL/6

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anti−T-cell receptor β (TCRβ), and anti-CXCR5 antibodies, and analyzed by flow cytometry.

Results

FVIII[null] mice developed inhibitory antibodies after in situ lentivirus-mediated EC-specific transduction

Our previous studies showed that lentivirus-mediated platelet-specific gene delivery of FVIII (via αIIb promotor; 2bF8) can introduce sustained therapeutic levels of platelet FVIII expression and induce FVIII-specific immune tolerance in FVIII[null] mice.[20-22] However, levels of platelet FVIII in transduced recipients are low (∼5% to 10%) compared with healthy plasma.[23] As an alternate approach, we performed lentivirus-mediated FVIII gene transfer with targeted expression in ECs via the panendothelial Tie2 promotor (T2F8LV). We confirmed that T2F8LV could introduce FVIII expression in ECs in vitro (supplemental Figure 1). Next, we introduced FVIII expression into ECs in vivo/in situ through systemic transduction of ECs in FVIII[null] mice (Figure 1A). To our disappointment, no plasma FVIII was detected in T2F8LV-transduced animals (Figure 1B). However, all animals developed anti-FVIII inhibitors (Figure 1C), indicating that FVIII was expressed, but undetectable because of these antibodies. Total anti-FVIII immunoglobulin G (IgG) detected by ELISA was substantial and increased over 8 weeks (Figure 1D). These results suggest that in situ panendothelial transduction by T2F8LV with FVIII triggers strong anti-FVIII immune responses that sharply contrast the tolerance produced by lentivirus-mediated transduction of platelets,[22-24] hepatocytes,[16-18] and specific EC subsets.[38,39]

Immune responses are influenced by endothelial transgenic FVIII expression

To further evaluate the influence of EC-FVIII expression in immune responses, we prepared transgenic mice. Three groups of T2F8[Tg] mice from 2 lines generated by embryonic stem (ES) cell−mediated transgenesis[32] were used for the FVIII immune response study (Table 1). One was the T2F8[tg+/+] (ES#5) group, in which animals were homozygous T2F8[Tg] from line #5, with 100% normal levels of plasma FVIII (1.35 ± 0.12 U/mL). The second group of T2F8[Tg] mice was composed of heterozygous animals from line #5 (T2F8[tg+/−]; ES#5) with plasma FVIII coagulant activity (FVIII:C) of 0.58 ± 0.06 U/mL. In the third group of T2F8[Tg] mice were homozygous animals from another line (#65) (T2F8[tg+/+]; ES#65), with a FVIII:C level of 0.34 ± 0.14 U/mL.

We used an active immunization protocol[31,32] to evaluate whether FVIII immunogenicity is diminished by endothelial expression. Surprisingly, we found that FVIII:C in the plasma dropped to undetectable levels in all 3 groups of T2F8[Tg] mice after a single immunization dose of rhF8 and IFA (Figure 2A-B). The plasma FVIII remained undetectable 4 weeks after this single immunization (not shown). In contrast, control WT C57BL/6 mice retained 69% of plasma FVIII after 1 immunization and still had detectable plasma FVIII even after a second immunization at week 4 (Figure 2A-B). These data suggest that targeted endothelial FVIII expression promotes strong immunogenicity, even if the expression is via germ line genetic modification.

After a single rhF8/IFA immunization, there were no statistically significant differences in inhibitor titers between the different groups of T2F8 mice in the FVIII[null] background (supplemental Figure 2A). This indicates that FVIII-inhibitor formation in mice with endothelial expression is not substantially influenced by the level of FVIII expression over this range. We asked whether VWF might influence inhibitor development (supplemental Figure 2B). The inhibitor titer in the T2F8[tg+/+]VWF^{−/−} group (252 ± 144 BU/mL) was significantly lower than the T2F8[tg+/+] group (810 ± 508 BU/mL; supplemental Figure 2B), although plasma FVIII:C in the T2F8[tg+/+]VWF^{−/−} group was not detectable (not shown). After the second FVIII immunization, the inhibitor titers in the T2F8[tg+/+] group (8560 ± 7074 BU/mL) were significantly higher
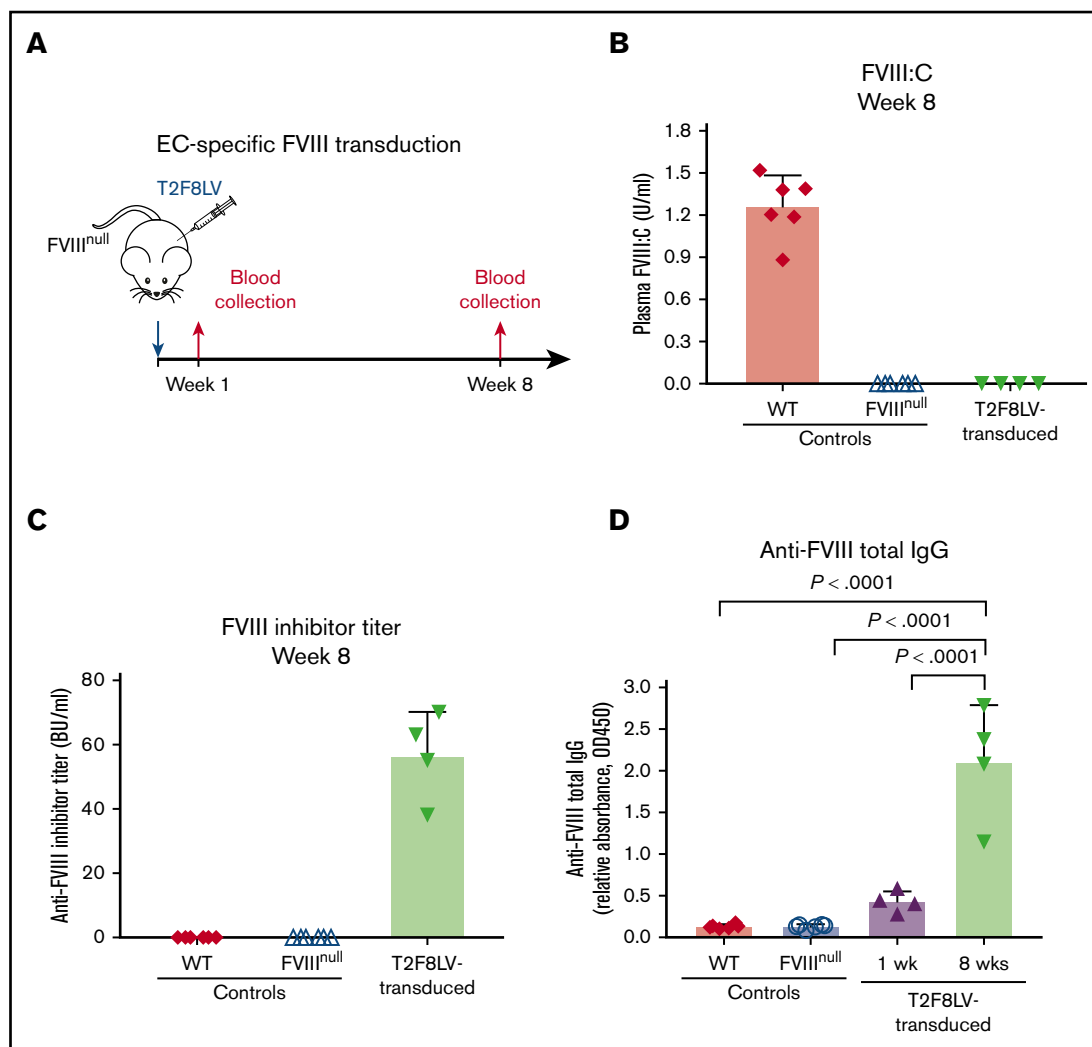
Figure 1. T2F8 LV-mediated in situ transduction of ECs in FVIII^null mice. (A) Schematic diagram of EC-specific FVIII transduction. Approximately 1 × 10^8 IU of T2F8LV in 300 μL of X-VIVO 10 media were administered into each 6-week-old FVIII^null mouse via tail-vein injection to transduce ECs in situ. Blood samples were collected following transduction, and plasmas were isolated for assays. Blood plasma from nontransduced WT C57BL/6 and naive FVIII^null mice served as controls. (B) Functional FVIII activity (FVIII:C) in plasma. Plasma FVIII:C levels in T2F8LV-transduced mice as determined by chromogenic assay. (C) Anti-FVIII inhibitor titers. Anti-FVIII inhibitor titers in WT, FVIII^null, and FVIII^null T2F8LV-transduced mice were determined by Bethesda assay. (D) Anti-FVIII total IgG in plasmas from T2F8LV-transduced mice as determined by ELISA. Data were analyzed using the 1-way analysis of variance followed by the Tukey test.

than in the WT control group (2576 ± 4224 BU/mL) (supplemental Figure 2C).

We then compared FVIII immune responses in the endothelial-expressed, T2F8^Tg, and platelet-expressed 2bF8^Tg models (Figure 2C). The 2bF8^Tg mice used in this study were also generated by ES cell–mediated transgenesis.[31] In 2bF8^Tg mice, FVIII expression is targeted to platelet α-granules with a level of ~0.75 mU/10^8 platelets, which corresponds to 1.2% of FVIII in WT mouse whole blood (though no plasma FVIII:C was detectable[31]). When the same immunization protocol was applied to our platelet-specific FVIII (2bF8^Tg) transgenic mice, 15 of 18 2bF8^{Tg+/−} mice developed inhibitors with a titer of 54 ± 49 BU/mL. All T2F8^Tg mice developed inhibitors with the average titer of 1811 ± 1953 BU/mL, which was significantly higher than obtained in 2bF8^Tg mice (Figure 2C). Thus, expression in ECs greatly enhances immunogenicity of FVIII, whereas

expression in megakaryocytes/platelets induces a degree of immune tolerance.

ECs exhibit functional properties of APCs

B-cell and antibody responses are tightly regulated by the CD4^+/CXCR5^+ lymph node Tfh cell subset, which in turn is regulated by MHCII-expressing APCs.[40-46] Because ECs have emerged as auxiliary MHCII-expressing APCs,[47,48] we hypothesized that they might directly influence FVIII immunogenicity by presenting MHCII/Ag to Tfh cells. Human ECs are well established to constitutively express MHCII in a response to tonic levels of IFN-γ.[47,49] In mice the situation remains less studied and less clear.[50,51] Therefore, we first examined whether murine ECs express MHCII at steady state. We conducted flow cytometric analysis of ECs freshly isolated from a range of tissues in healthy C57BL/6 WT or CIITA^{−/−} (MHCII^null) mice. EC-specific MHCII
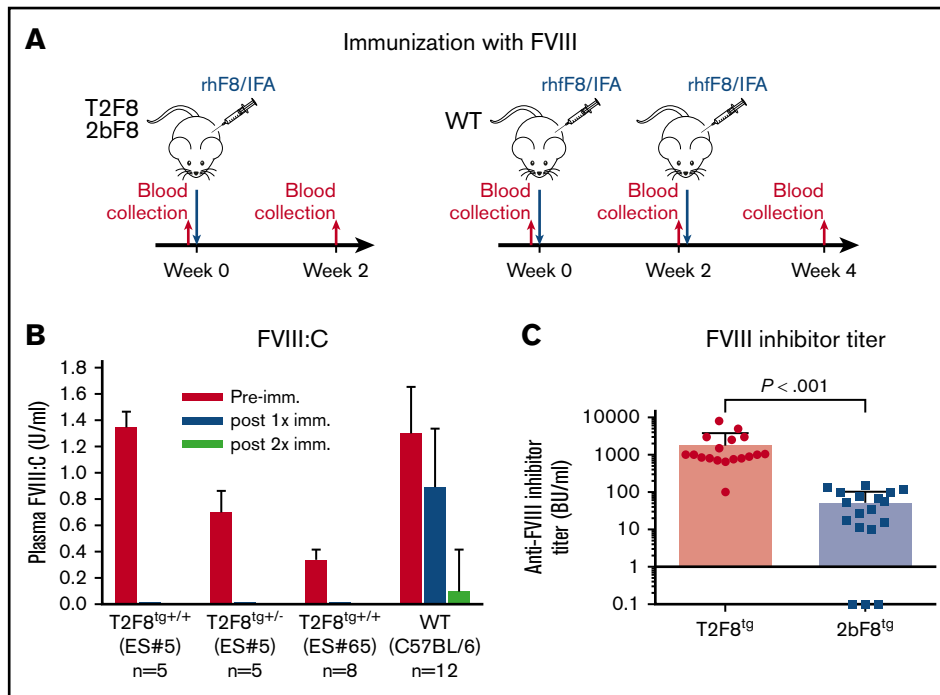
Figure 2. FVIII activity levels and anti-FVIII inhibitor titers in plasma of FVIII transgenic following FVIII immunization in the presence of IFA. (A) Immunization schemes for T2F8 and 2bF8 transgenic (left) and WT (control; right) mice. Preimmune blood was collected for all strains for FVIII:C assay. For T2F8 and 2bF8 transgenic mice that express hBDDFVIII, animals were immunized with rhF8 at 600 U/kg in the presence of IFA. Blood samples were collected 2 weeks after immunization. Because WT have full-length endogenous murine FVIII, mice received rhfF8 at 600 U/kg plus IFA and further blood collection was made the following week (week 3 overall). (B) Functional FVIII activity (FVIII:C) levels in T2F8 transgenic mice before and after immunization. FVIII:C levels were determined by chromogenic assay. Two lines of the endothelial-specific hBDDFVIII-expressing T2F8 transgenic mice with differing numbers of gene insertions (T2F8[Tg][ES#5] and T2F8[Tg][ES#65]) were used. WT mice were used as a control. (C) Comparison of inhibitor titers in T2F8 (EC-specific FVIII expression) and 2bF8 (platelet-specific FVIII expression) transgenic mice after a single dose of rhF8 immunization (week 2). The unpaired 2-tailed Student t test was used to analyze data.

was measured by gating on the CD31^{+}/CD105^{+}/CD11b^{−}/CD45^{−} cell population (supplemental Figure 3A). As shown in supplemental Figure 3B, ECs of heart, lung, kidney, liver, and skin all showed strong constitutive levels of MHCII in WT mice as compared with CIITA^{−/−} mice. These results suggest that, like human ECs, murine ECs exhibit broad constitutive IFN-γ–/CIITA-dependent MHCII expression and have potential to function as auxiliary class II APCs in vivo. Of note, all cultured ECs require addition of exogenous IFN-γ to support in vitro MHCII expression[36,47,51] (as we confirm in supplemental Figure 4A-B).

CXCL13 supports interaction of ECs with circulating Tfh cells

A defining feature of Tfh is expression of the CXCL13-selective chemokine receptor CXCR5, which orchestrates its functions within lymphoid organs.[40-46] Circulating CD4^{+}/CXCR5^{+} Tfh cells with memory-like properties (cTfh cells), as well as CXCL13, are also found in the blood with elevated levels seen during humoral and inflammatory responses.[52-60] We hypothesized that cTfh cells might interact with vascular ECs in a CXCL13-promoted manner. We first examined blood plasma levels of CXCL13 in mice following rhfF8/IFA immunization during inhibitor formation, and found a substantial, approximately fivefold, increase in the immunized vs control mice. (Figure 3A). Because ECs can bind chemokines via cell-surface heparan

sulfates,[61,62] we tested whether exogenous CXCL13 could be "captured" by ECs. Brief incubation of resting ECs with rhCXCL13 followed by staining and imaging revealed robust levels of cell-surface–captured CXCL13 (Figure 3B-C). ECs also have potential to express CXCL13 under inflammatory conditions,[63-67] and LPS is known to promote CXCL13 expression by professional APCs.[68,69] Thus, we treated hDMVECs with LPS or several inflammatory cytokines (ie, TNF-α, IFN-γ, IL1β). We found that the former induced substantial expression and surface presentation of endogenous CXCL13 (Figure 3B-C). In parallel studies, we also confirmed previous studies indicating that LPS, TNF-α, and IL1β (but not IFN-γ) upregulated ICAM-1, a representative proinflammatory immune-adhesion molecule (supplemental Figure 4C-D).

To assess the function of EC-presented CXCL13 on cTfh cell adhesion, we incubated LPS-pretreated hDMVECs with sorted human peripheral blood mononuclear cell (PBMC)–derived CD4^{+}/CXCR5^{−} (conventional) and CD4^{+}/CXCR5^{+} (cTfh) T cells in the absence and presence of a CXCL13 function-blocking antibody (Figure 4A). Under these conditions, the level of CD4^{+}/CXCR5^{+} cTfh binding to ECs was approximately threefold greater than that of the conventional CD4^{+}/CXCR5^{−} T cells (Figure 4B). Anti-CXCL13 antibody largely blocked binding of CXCR5^{+}CD4^{+}, but not the CD4^{+}/CXCR5^{−}, T cells. These data indicate that binding of CD4^{+}/CXCR5^{+} cTfh cells to ECs can be promoted by CXCL13.

Figure 3. Circulating CXCL13 in vivo and CXCL13 expression and presentation by ECs in vitro. (A) WT C57BL/6 mice were immunized with rhfF8 by intraperitoneal injection with IFA. Blood samples from immunized and paired unimmunized controls were collected 7 days later and plasma CXCL13 was measured by ELISA. (B-C) hDMVECs were cultures in the absence (control) or presence of 100 ng/mL recombinant human inflammatory cytokines (TNF-α, IFN-γ, IL1-β) or 10 ng/mL LPS for 24 hours. Alternatively, untreated control hDMVECs were incubated with 100 ng/mL recombinant human CXCL13 for 1 hour. Cells were then rinsed, fixed, and stained (without permeabilization) with anti-CXCL13 antibody. (B) Representative images (for select conditions) with anti-CXCL13 antibody staining shown in green. Scale bar, 10 μm. (C) For each condition, mean fluorescence intensity (MFI) of CXCL13 staining (representing endogenous expression and cell-surface presentation of CXCL13 or capture/presentation of exogenous CXCL13) was quantified per field of view for a total of 10 fields of view for each of 2 separate experiments and averaged.
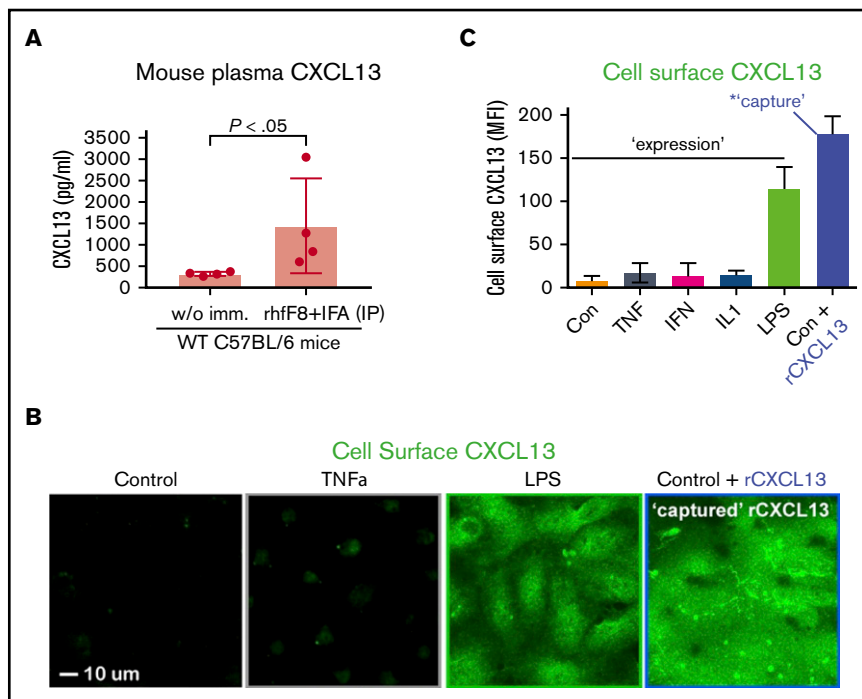
ECs take up and process protein Ag, including FVIII, in vitro and in vivo

ECs might process endogenously expressed FVIII that is diverted from the secretory pathway as well as exogenous FVIII that is actively taken up by endocytosis.[70-77] To evaluate the latter, we incubated rhfF8–Alexa 488 with conditioned human DMVECs in the presence of VWF and FIB. rhfF8–Alexa 488 was readily taken up into perinuclear clusters that colocalized with late endosomes and lysosomes, features consistent with class II processing (Figure 5A). Similar observations were made with conditioned mDMVECs (not shown). Complementary experiments were performed using rhfF8-labeled with 40-nm fluorospheres that provide an enhanced signal-to-noise ratio. Upon incubation with conditioned mDMVECs in the presence or absence of VWF, these "rhfF8 fluorospheres" bound to membrane surface patches and
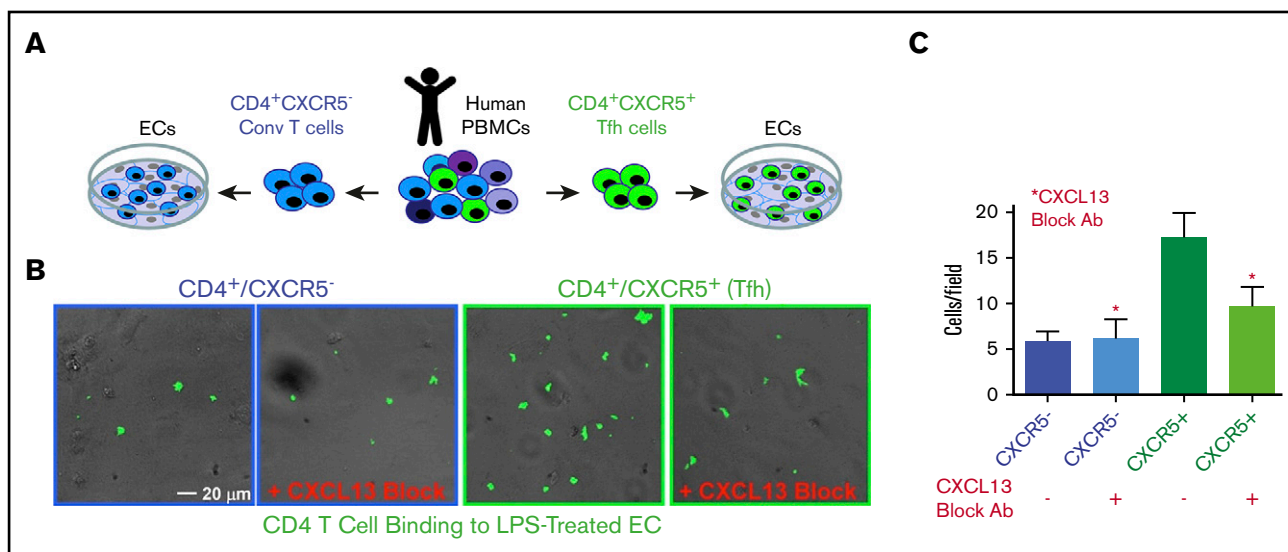

Figure 4. Adhesion of conventional and CXCR5⁺ CD4⁺ T cells to LPS-activated hDMVECs. (A) Human peripheral blood CD4⁺/CXCR5⁻ (conventional) or CD4⁺/CXCR5⁺ (cTf) T cells flow sorted. Equal numbers of CD4⁺/CXCR5⁻ and CD4⁺/CXCR5⁺ T cells were then incubated for 1 hour on hDMVECs that were pretreated for 24 hours with LPS (10 ng/mL) in the absence and presence of 20 μg/mL function-blocking anti-CXCL13 antibody. Samples were then subjected to brief washing with warm media, fixation, staining for CD4, and imaging. (B) Representative fields of view showing phase and fluorescence images overlaid. T cells were labeled with anti-CD4-Alexa488 antibody, shown in green. Scale bar, 20 μm. (C) Quantitation of average number of cells per field of view (10 images per condition for 2 separate experiments).
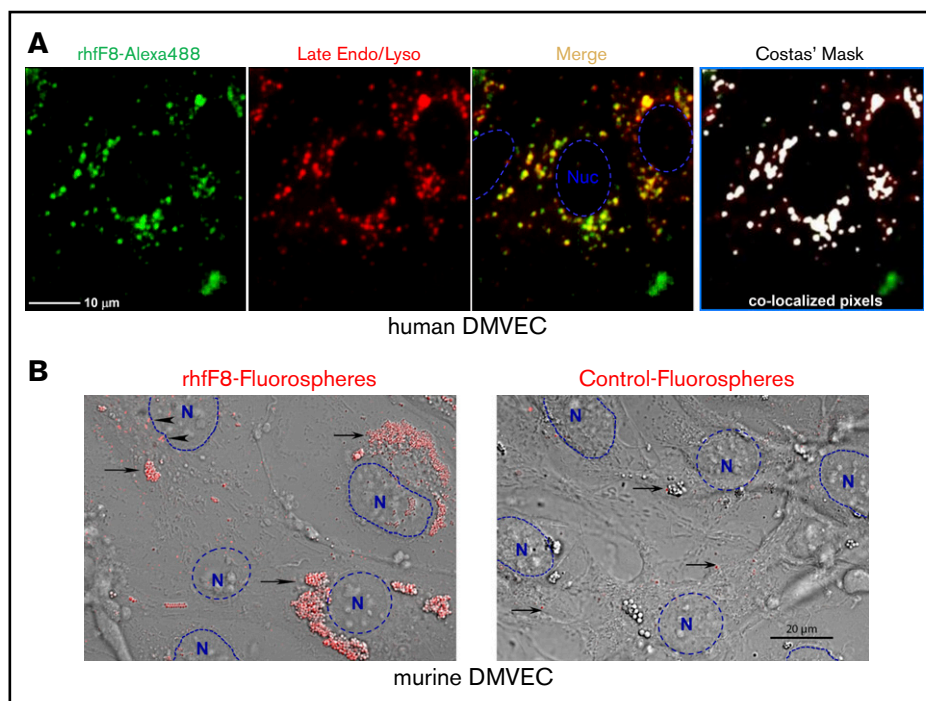
Figure 5. Binding and uptake of FVIII by conditioned DMVECs. (A) Confluent hDMVECs were pretreated with 100 nM IFN-γ (48 hours) and 100 nM TNF-α (24 hours) prior to incubation for 12 hours rhfF8–Alexa 488 (10 U/mL; in the presence of 1 U/mL VWF and 150 μg/mL FIB) together with Lysotracker Red (late endosome/lysosome marker; 50 nM). Cells were then rinsed and imaged live. Fluorescence microscopy showed significant rhfF8–Alexa 488 (green) accumulated into perinuclear punctae, which partially colocalized with late endosomes/lysosomes (red) as seen by the yellow signal in the merged image. This was validated by computational image analysis, which also identified substantial areas of rhfF8-488 and Lysotracker Red coincidence, as depicted by white pixels in the Costes mask (right panel) and reflected in an average Pearson correlation coefficient of 0.680 ± 0.033 ($r^2 > 0.98$; Costes $P = 100\%$). Scale bar, 25 μm. (B) As a complementary and more sensitive approach to detect FVIII binding and uptake, rhfF8 was coated onto GMA-8021 (anti-FVIII MAb)-conjugated carboxylate-modified red fluorescent nanoparticles (rhfF8 fluorospheres). The same fluorescent particles were subjected to blocking of the carboxylate modification with 2% BSA to demonstrate the level of nonspecific binding and cellular uptake (Control fluorospheres). Fluorospheres were then incubated together with 150 μg/mL fibrinogen in the presence (not shown) or absence of 1 U/mL VWF for 1.5 hours on IFN-γ– and TNF-α–conditioned mDMVECs. Differential interference contrast (DIC) and confocal fluorescence images were superimposed. Arrowheads indicate patches of putative membrane-bound rhfF8. Arrows indicate putatively internalized perinuclear clusters of rhfF8 that presumably represent endosomes and lysosomes (left panel). The same pattern was observed in the presence of VWF. In the absence of rhfF8 coating, control fluorospheres showed very little EC binding or uptake (right panel, arrows). Scale bar, 20 μm.

accumulated in putative intracellular perinuclear puncta (presumptive endosomes/lysosomes; Figure 5B left panel). VWF did not alter the pattern of binding or uptake (not shown). Little binding or uptake was visible on ECs incubated with control, non–rhfF8-coupled fluorospheres (Figure 5B right panel). Both methods indicated that FVIII is taken up by a majority of ECs into a perinuclear compartment.

Several studies support the ability of ECs to mediate functional class II Ag processing.[70-74] To extend this idea, we implement DQ-OVA, a common Ag-processing reporter.[37,78] We incubated hDMVECs (supplemental Figure 5A) and mouse heart MVECs (not shown) with DQ-OVA together with a LysoTracker Red. DQ-OVA was readily taken up into ECs and converted to fluorescent peptides that partially colocalized with late endosomes/lysosomes (supplemental Figure 5A). We extended these studies into the in vivo setting by subcutaneous immunization of DQ-OVA in C57BL/6. Injection-site skin was later harvested and analyzed by flow cytometry. This showed substantial levels of processed DQ-OVA signal in ECs (supplemental Figure 5B). Together, these studies show that whole protein Ag is readily taken up and

proteolytically processed by human and murine ECs both in vitro and in vivo.

ECs can mediate functional FVIII-dependent stimulation of Tfh

To determine whether murine ECs can promote Ag-specific immune responses in $CD4^+/CXRC5^+$ Tfh cells, we performed a T-cell proliferation assay (Figure 6B). $CD4^+$ T cells isolated from FVIII-primed mice were cocultured ex vivo with ECs and CXCL13, with or without rhF8. Proliferating T-cell clusters/clones were seen only in cocultures that contained rhF8 Ag (not shown). Flow cytometry showed that ~7% of the FVIII-primed $CD4^+$ T cells were proliferating (daughter) when restimulated with ECs with either 0.2 μg/mL or 2 μg/mL rhF8 (Figure 6B). In contrast, only 1% of T cells proliferated when cocultured in the absence of rhF8 or cocultured with an unrelated Ag rhF9 (2 μg/mL; Figure 6B). When the $CD4^+/CD5^+$ Tfh cells were selectively analyzed, there were 18% and 12.7% Tfh proliferating daughter cells in cocultures containing 0.2 and 2 μg/mL rhF8, respectively. In contrast, there were only 5% Tfh daughter cells when cocultured without rhF8 or
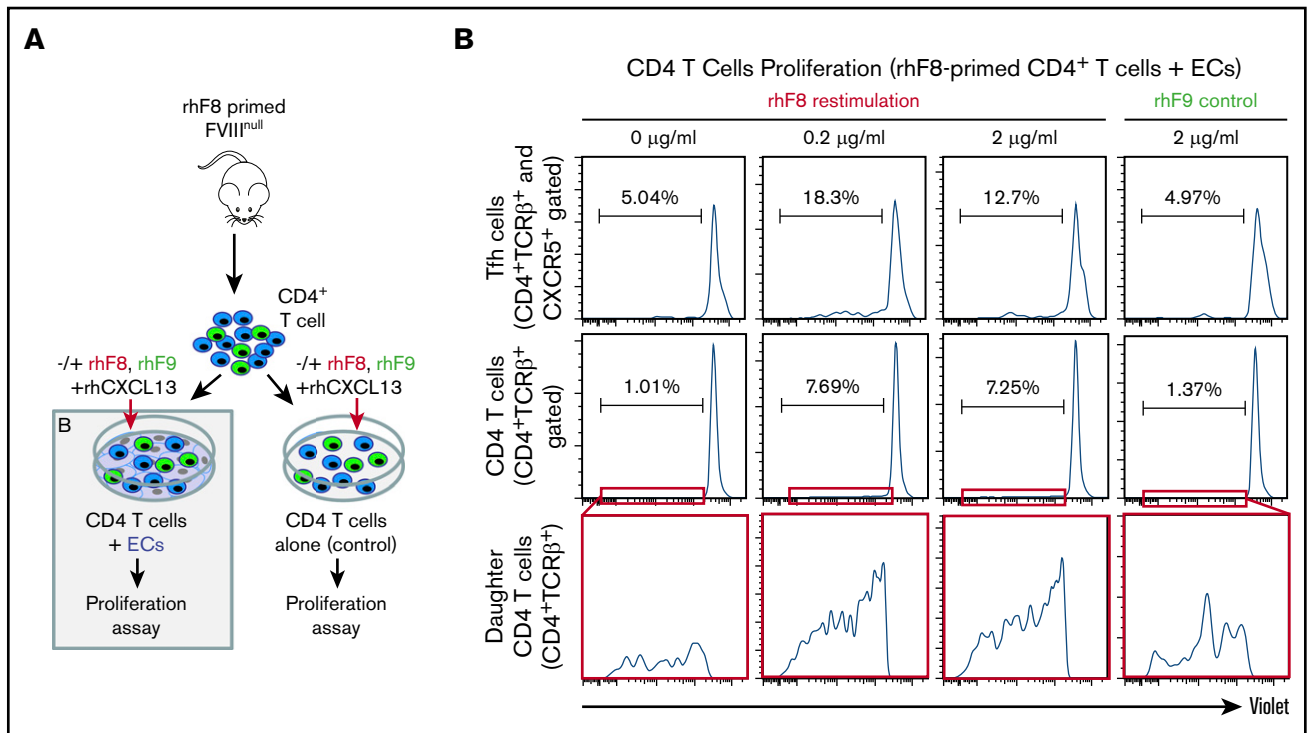
Figure 6. Ag-specific CD4 T-cell proliferation assay. (A) Schematic diagram of T-cell proliferation assay. CD4^{+} T cells isolated from rhF8-primed FVIII[null] mouse were labeled with CellTrace Violet and cocultured with DMVECs that were pretreated with IFN-γ/TNF-α in the presence of CXCL13 and the absence and presence of rhF8 at 37°C in 5% CO_2 for 1 week. rhF9 was used as an unrelated Ag control. CD4 T cells plated in the absence of endothelium served as an additional control (supplemental Figure 6). (B) Flow cytometry analysis daughter (proliferated) cells. Cells were stained for CD4, TCRβ, and CXCR5, and analyzed by flow cytometry. Dead cells were excluded by the 7-AAD staining. CD4 T-cell divisions were quantified as the dilution of CellTrace Violet signal in daughter cells away from the initially labeled peak. Results for both CD4 Tfh (top row) and total CD4 (middle row) are shown. Bottom row shows expanded view of daughter cell in the red, boxed regions from the middle row. These data demonstrate that ECs can present FVIII to Tfh cells, promoting Ag-specific cell proliferation.

with 2 μg/mL rhF9 control Ag (Figure 6B). To confirm that the isolated FVIII-primed CD4^{+} T-cell population did not contain APCs, we cultured isolated CD4^{+} T cells with rhF8 in the absence of ECs and saw no detectable proliferation in either the total CD4^{+} T or Tfh cell populations (supplemental Figure 6). These data confirm that ECs can take up FVIII and functionally present FVIII Ag to FVIII-primed CD4^{+} T cells, leading to Ag-selective Tfh cell proliferation.

Discussion

Our experiments demonstrate that ECs have an unexpected capacity to enhance anti-FVIII antibody formation in HA mice. This was true whether FVIII gene expression was via LV-mediated gene therapy or by germ line expression in transgenic mice. Our supporting ex vivo/in vitro studies led us to hypothesize a direct pathway through which inflammatory-conditioned ECs can act as auxiliary APCs in FVIII immune responses. These findings have relevance for gene therapy and possibly for understanding more generally the unusual immunogenicity of FVIII.

ECs are known as the major physiologic site of FVIII expression[25-30] and anti-FVIII antibodies are rare in healthy subjects.[79] As such, our observation of strong anti-FVIII immunological responses to EC-targeted therapeutic expression of FVIII seems rather counter-intuitive. We view this as an apparent, rather than an actual, conflict. Our data bear directly on engineered FVIII expression, whereby synthesis was driven in all ECs via the panendothelial Tie2

promotor. Native FVIII production was recently shown to be restricted to a smaller subset of ECs that primarily include liver sinusoidal (LSEC), lymphatic (LEC), and possibly splenic sinusoidal (SSEC) ECs.[25,26,29,30] It follows that the ectopic expression of FVIII within nonnative ECs (ie, ECs other than LSEC, LEC, and SSEC) is responsible for the observed immunogenicity, and this seems to be specific for ECs, as ectopic nonnative expression of FVIII in hepatocytes[16-18] and platelets[20-24,80] are both tolerated.

Previous studies are consistent with this dichotomy among EC types. We reported that transgenic panendothelial expression of hFVIII (eg, confirmed in lung and heart ECs) in HA mice had heightened responses to hFVIII immunization compared with mice with platelet expression of FVIII.[32] Similarly, use of the "sleeping beauty transposon" to express FVIII specifically in lung ECs was immunogenic,[81] whereas use of the same FVIII transposon system targeted to LSEC promoted tolerance.[82] Finally, 2 recent reports described transduction strategies that were similar to our T2F8LV, but used either the native FVIII[38] or the "vascular endothelial cadherin"[39] promoter. FVIII expression in both cases was restricted largely to liver ECs (ie, LSEC) and was not found in ECs of the lung or kidney.[38,39] In these studies, FVIII was restored to ~25% and ~5%, respectively, of normal and was associated with degree of immune tolerance.[38,39] Taken together, these reports suggest that ectopic expression of FVIII in hepatocytes and platelets seems relatively nonimmunogenic and partially tolerogenic. In contrast,

endothelial expression of FVIII leads to either enhanced immunogenicity or tolerance, depending on the specific endothelial location or subsets that are targeted.

We hypothesized that the mechanism lies in the ability of ECs to function directly as APCs with site- and context-specific properties.[47,48] Contrasting with hepatocytes and platelets, all ECs in both humans and mice (eg, shown herein and in Choo et al[50] and in Kreisel et al[51]) constitutively express MHCII and can mediate class II presentation of both endogenous and exogenous Ags.[47-49,73] LSEC, LEC, where most FVIII is normally expressed, exhibit unique constitutive protolerogenic properties (eg, expression of programmed death ligand 1 [PD-L1], transforming growth factor [TGF-β]), which would be expected to promote Ag-specific tolerance to FVIII.[83-89] Alternatively, the other nonnative ECs tend to stimulate Ag-specific responses, particularly in inflammatory settings.[48,72,90-93] Our findings with engineered panendothelial expression of FVIII in both native and nonnative ECs suggest that the proimmunogenic potential of the latter can exert a dominant stimulatory regulation on FVIII inhibitors (ie, broken immunological tolerance).

Development of FVIII inhibitors requires professional APCs, such as lymphoid dendritic cells, to prime naive CD4$^+$ T cells and initiate formation of Ag-specific memory CD4$^+$/CXCR5$^+$ Tfh cells.[94-104] Our data do not conflict with this model. Rather, they indicate that ECs can act as complementary APCs that modulate response of memory-like cTfh cells after they have been primed by lymphoid dendritic cells entering the blood. Studies in other contexts suggest that cTfh cells receive distinct regulatory cues within the circulation.[53,55-57,105-107] cTfh cells ultimately traffic back to lymph nodes and spleen, with robust cytokine responsiveness and presumably altered capacity for directing antibody formation.[53,55-57] Thus, we propose that inflammatory-conditioned, particularly nonnative, ECs displaying FVIII Ag can promote the maturation and function of FVIII-specific cTfh cells.

Our experiments support this hypothesis by showing that panendothelial, but not platelet, expression upregulates the immunogenicity of FVIII in mice under inflammatory settings. We show that ECs express and bind CXCL13 in response to LPS and that this promotes adhesion of Tfh cells. We show that inflammatory-conditioned ECs take up and traffic FVIII consistently with class II Ag processing. Finally, we show that CD4$^+$/CXCR5$^+$ Tfh cells from FVIII-immunized mice proliferate in response to ex vivo presentation of FVIII by ECs. These observations support the plausibility of our hypothesis, though more work is needed to establish the extent to which this pathway may influence development of anti-FVIII antibodies.

In mice, immunogenicity was highest for the T2F8LV-mediated in situ EC transduction. We speculate that ECs sense proinflammatory danger signals[108] and/or endoplasmic reticulum stress generated by the viral vector delivering of T2F8, which upregulates their immunogenic APC functions and promotes FVIII humoral response. Inhibitors were detected in T2F8LV-transduced FVIII^{null} mice even when plasma FVIII:C was undetectable. Immune responses in the transgenic and WT controls required an active immunization with FVIII together with IFA. Response of WT control mice to rhF8/IFA is likely to be explained by species differences in FVIII epitopes. However, this does not explain the response of T2F8^{Tg} mice, which are born with normal levels of rhF8 and should be tolerized to rhF8. In vivo Ag presentation by ECs has been tested using a Tie2-driven EC-specific β-gal transgenic mouse model.[73] Studies with this model suggest that ECs can present intracellular self-Ag to the immune system without completely deleting Ag-specific T cells.[73] Apparently, EC-expressed FVIII results in immunological ignorance rather than tolerance, implying that EC presentation during T-cell development allows escape of FVIII-specific CD4$^+$ T cells in T2F8^{Tg} mice.

Our data suggest the potential immunogenic roles of FVIII Ag presentation by ECs in the context of engineered gene therapy. It is attractive to speculate that ECs could also modulate FVIII immune responses to exogenous FVIII (eg, HA therapy). LSEC, LEC, and SSEC constitutively express FVIII/VWF-scavenging receptors/activity. Under inflammatory conditions, many other ECs can upregulate expression of MHCII and costimulatory molecules, as well as scavenging properties such as stabilin-2.[75-77,109-113] Moreover, in contrast to dendritic cells, ECs are exposed to circulating VWF and are positioned to distinguish between multimer size and conformation. Because these factors appear to influence immunogenicity of FVIII, it is tempting to speculate that endothelial Ag presentation plays a broader role in FVIII immunogenicity.[114,115] Further investigation of the role of EC subsets in modulating FVIII immune responses is warranted.

We report binding, uptake, and presentation of FVIII by inflammatory conditioned DMVECs. Although binding of FVIII to ECs has previously been reported, the mechanism(s) remain poorly characterized. FVIII may bind directly to ECs, presumably binding through exposed phosphatidylserine,[116] enabling assembly of the intrinsic FXa complex. FVIII may bind indirectly through VWF and possibly fibrin(ogen)[117] via stabilin-2.[118] VWF also binds ECs via P-selectin and $\alpha_v\beta_3$ integrin,[119,120] as well as other scavenger receptors aimed at molecular clearance and recycling.[118] FVIII has been shown to bind to lipoprotein-related protein and other scavenger receptors shared by ECs and other cell types.[121] In addition, there are other candidate FVIII-binding molecules, particularly SREC-1, a scavenger receptor that is partially restricted to ECs.[122] Further studies will be needed to identify the receptor and the uptake pathway responsible for FVIII uptake in the MVECs.

In summary, our data demonstrate that panendothelial targeting of FVIII is more immunogenic than expression by megakaryocytes (or hepatocytes), implying a novel role of ECs in modulation of the humoral immune response to FVIII. We showed for the first time the FVIII uptake by MVECs and functional FVIII presentation by MVECs to FVIII-primed Tfh cells, leading to cell proliferation. Together, our results indicate that ECs may play an important role in FVIII immune responses.

Acknowledgments

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References

1. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost*. 2009;7(9):1446-1456.

2. Astermark J. Treatment of the bleeding inhibitor patient. *Semin Thromb Hemost*. 2003;29(1):77-86.

3. Wight J, Paisley S, Knight C. Immune tolerance induction in patients with haemophilia A with inhibitors: a systematic review. *Haemophilia*. 2003;9(4):436-463.

4. Knight C, Paisley S, Wight J, Jones ML. Economic modelling of different treatment strategies for haemophilia A with high-responding inhibitors. *Haemophilia*. 2003;9(4):521-540.

5. Bohn RL, Aledort LM, Putnam KG, Ewenstein BM, Mogun H, Avorn J. The economic impact of factor VIII inhibitors in patients with haemophilia. *Haemophilia*. 2004;10(1):63-68.

6. Andrews JL, Kadan MJ, Gorziglia MI, Kaleko M, Connelly S. Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. *Mol Ther*. 2001;3(3):329-336.

7. Monahan PE, White GC II. Hemophilia gene therapy: update. *Curr Opin Hematol*. 2002;9(5):430-436.

8. VandenDriessche T, Collen D, Chuah MK. Gene therapy for the hemophilias. *J Thromb Haemost*. 2003;1(7):1550-1558.

9. Kootstra NA, Matsumura R, Verma IM. Efficient production of human FVIII in hemophilic mice using lentiviral vectors. *Mol Ther*. 2003;7(5 pt 1):623-631.

10. Saenko EL, Ananyeva NM, Moayeri M, Ramezani A, Hawley RG. Development of improved factor VIII molecules and new gene transfer approaches for hemophilia A. *Curr Gene Ther*. 2003;3(1):27-41.

11. Brown BD, Shi CX, Rawle FE, et al. Factors influencing therapeutic efficacy and the host immune response to helper-dependent adenoviral gene therapy in hemophilia A mice. *J Thromb Haemost*. 2004;2(1):111-118.

12. Rawle FE, Shi CX, Brown B, et al. Heterogeneity of the immune response to adenovirus-mediated factor VIII gene therapy in different inbred hemophilic mouse strains. *J Gene Med*. 2004;6(12):1358-1368.

13. Xu L, Mei M, Ma X, Ponder KP. High expression reduces an antibody response after neonatal gene therapy with B domain-deleted human factor VIII in mice. *J Thromb Haemost*. 2007;5(9):1805-1812.

14. Ponder KP. Gene therapy for hemophilia. *Curr Opin Hematol*. 2006;13(5):301-307.

15. High KA. Update on progress and hurdles in novel genetic therapies for hemophilia. *Hematology Am Soc Hematol Educ Program*. 2007;2007:466-472.

16. Doshi BS, Arruda VR. Gene therapy for hemophilia: what does the future hold? *Ther Adv Hematol*. 2018;9(9):273-293.

17. Sherman A, Biswas M, Herzog RW. Tolerance induction in hemophilia: innovation and accomplishments. *Curr Opin Hematol*. 2018;25(5):365-372.

18. Peyvandi F, Garagiola I. Clinical advances in gene therapy updates on clinical trials of gene therapy in haemophilia. *Haemophilia*. 2019;25(5):738-746.

19. Shi Q. Platelet-targeted gene therapy for hemophilia. *Mol Ther Methods Clin Dev*. 2018;9:100-108.

20. Shi Q, Wilcox DA, Fahs SA, et al. Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost*. 2007;5(2):352-361.

21. Kuether EL, Schroeder JA, Fahs SA, et al. Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. *J Thromb Haemost*. 2012;10(8):1570-1580.

22. Schroeder JA, Chen Y, Fang J, Wilcox DA, Shi Q. In vivo enrichment of genetically manipulated platelets corrects the murine hemophilic phenotype and induces immune tolerance even using a low multiplicity of infection. *J Thromb Haemost*. 2014;12(8):1283-1293.

23. Chen Y, Luo X, Schroeder JA, et al. Immune tolerance induced by platelet-targeted factor VIII gene therapy in hemophilia A mice is CD4 T cell mediated. *J Thromb Haemost*. 2017;15(10):1994-2004.

24. Chen J, Schroeder JA, Luo X, Montgomery RR, Shi Q. The impact of GPIbα on platelet-targeted FVIII gene therapy in hemophilia A mice with pre-existing anti-FVIII immunity. *J Thromb Haemost*. 2019;17(3):449-459.

25. Hollestelle MJ, Thinnes T, Crain K, et al. Tissue distribution of factor VIII gene expression in vivo–a closer look. *Thromb Haemost*. 2001;86(3):855-861.

26. Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem*. 1999;274(28):19587-19592.

27. Fahs SA, Hille MT, Shi Q, Weiler H, Montgomery RR. A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. *Blood*. 2014;123(24):3706-3713.

28. Everett LA, Cleuren AC, Khoriaty RN, Ginsburg D. Murine coagulation factor VIII is synthesized in endothelial cells. *Blood*. 2014;123(24):3697-3705.

29. Shahani T, Covens K, Lavend'homme R, et al. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. *J Thromb Haemost*. 2014; 12(1):36-42.

30. Pan J, Dinh TT, Rajaraman A, et al. Patterns of expression of factor VIII and von Willebrand factor by endothelial cell subsets in vivo. *Blood*. 2016;128(1): 104-109.

31. Shi Q, Wilcox DA, Fahs SA, et al. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J Clin Invest*. 2006;116(7):1974-1982.

32. Shi Q, Fahs SA, Kuether EL, Cooley BC, Weiler H, Montgomery RR. Targeting FVIII expression to endothelial cells regenerates a releasable pool of FVIII and restores hemostasis in a mouse model of hemophilia A. *Blood*. 2010;116(16):3049-3057.

33. Shi Q, Fahs SA, Wilcox DA, et al. Syngeneic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. *Blood*. 2008;112(7):2713-2721.

34. Singer BD, Mock JR, D'Alessio FR, et al. Flow-cytometric method for simultaneous analysis of mouse lung epithelial, endothelial, and hematopoietic lineage cells. *Am J Physiol Lung Cell Mol Physiol*. 2016;310(9):L796-L801.

35. Czupalla CJ, Yousef H, Wyss-Coray T, Butcher EC. Collagenase-based single cell isolation of primary murine brain endothelial cells using flow cytometry. *Bio Protoc*. 2018;8(22):8.

36. Sage PT, Varghese LM, Martinelli R, et al. Antigen recognition is facilitated by invadosome-like protrusions formed by memory/effector T cells. *J Immunol*. 2012;188(8):3686-3699.

37. Daro E, Pulendran B, Brasel K, et al. Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. *J Immunol*. 2000;165(1):49-58.

38. Merlin S, Famà R, Borroni E, et al. FVIII expression by its native promoter sustains long-term correction avoiding immune response in hemophilic mice. *Blood Adv*. 2019;3(5):825-838.

39. Merlin S, Cannizzo ES, Borroni E, et al. A novel platform for immune tolerance induction in hemophilia A mice. *Mol Ther*. 2017;25(8):1815-1830.

40. Webb LMC, Linterman MA. Signals that drive T follicular helper cell formation. *Immunology*. 2017;152(2):185-194.

41. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol*. 2013;13(6): 412-426.

42. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular Helper T Cells. *Annu Rev Immunol*. 2016;34:335-368.

43. Mackay CR. Follicular homing T helper (Th) cells and the Th1/Th2 paradigm. *J Exp Med*. 2000;192(11):F31-F34.

44. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med*. 2000;192(11):1553-1562.

45. Breitfeld D, Ohl L, Kremmer E, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med*. 2000;192(11):1545-1552.

46. Jing W, Chen J, Cai Y, et al. Induction of activated T follicular helper cells is critical for anti-FVIII inhibitor development in hemophilia A mice. *Blood Adv*. 2019;3(20):3099-3110.

47. Choi J, Enis DR, Koh KP, Shiao SL, Pober JS. T lymphocyte-endothelial cell interactions. *Annu Rev Immunol*. 2004;22:683-709.

48. Carman CV, Martinelli R. T lymphocyte-endothelial interactions: emerging understanding of trafficking and antigen-specific immunity. *Front Immunol*. 2015;6:603.

49. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of MHC class II antigens in normal human organs. *Transplantation*. 1984; 38(3):293-298.

50. Choo JK, Seebach JD, Nickeleit V, et al. Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium: implications for cell-mediated xenoreactivity. *Transplantation*. 1997;64(9):1315-1322.

51. Kreisel D, Richardson SB, Li W, et al. Cutting edge: MHC class II expression by pulmonary nonhematopoietic cells plays a critical role in controlling local inflammatory responses. *J Immunol*. 2010;185(7):3809-3813.

52. Qi H. T follicular helper cells in space-time. *Nat Rev Immunol*. 2016;16(10):612-625.

53. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest*. 2014;124(12):5191-5204.

54. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev*. 2016;271(1):246-259.

55. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity*. 2011;34(1):108-121.

56. Ueno H. Human circulating T follicular helper cell subsets in health and disease. *J Clin Immunol*. 2016;36(suppl 1):34-39.

57. Vella LA, Buggert M, Manne S, et al. T follicular helper cells in human efferent lymph retain lymphoid characteristics. *J Clin Invest*. 2019;129(8):3185-3200.

58. Schiffer L, Worthmann K, Haller H, Schiffer M. CXCL13 as a new biomarker of systemic lupus erythematosus and lupus nephritis - from bench to bedside? *Clin Exp Immunol*. 2015;179(1):85-89.

59. Havenar-Daughton C, Lindqvist M, Heit A, et al; IAVI Protocol C Principal Investigators. CXCL13 is a plasma biomarker of germinal center activity. *Proc Natl Acad Sci USA*. 2016;113(10):2702-2707.

60. Mehraj V, Ramendra R, Isnard S, et al. CXCL13 as a biomarker of immune activation during early and chronic HIV infection. *Front Immunol*. 2019;10: 289.

61. Proudfoot AE, Handel TM, Johnson Z, et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci USA*. 2003;100(4):1885-1890.

62. Monneau YR, Luo L, Sankaranarayanan NV, et al. Solution structure of CXCL13 and heparan sulfate binding show that GAG binding site and cellular signalling rely on distinct domains. *Open Biol*. 2017;7(10):

63. Smith JR, Braziel RM, Paoletti S, Lipp M, Uguccioni M, Rosenbaum JT. Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma. *Blood*. 2003;101(3):815-821.

64. Amft N, Curnow SJ, Scheel-Toellner D, et al. Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjögren's syndrome. *Arthritis Rheum*. 2001;44(11): 2633-2641.

65. Martinet L, Le Guellec S, Filleron T, et al. High endothelial venules (HEVs) in human melanoma lesions: major gateways for tumor-infiltrating lymphocytes. *OncoImmunology*. 2012;1(6):829-839.

66. Corey DM, Rinkevich Y, Weissman IL. Dynamic patterns of clonal evolution in tumor vasculature underlie alterations in lymphocyte-endothelial recognition to foster tumor immune escape. *Cancer Res*. 2016;76(6):1348-1353.

67. Kitaya K, Yasuo T. Aberrant expression of selectin E, CXCL1, and CXCL13 in chronic endometritis. *Mod Pathol*. 2010;23(8):1136-1146.

68. Perrier P, Martinez FO, Locati M, et al. Distinct transcriptional programs activated by interleukin-10 with or without lipopolysaccharide in dendritic cells: induction of the B cell-activating chemokine, CXC chemokine ligand 13. *J Immunol*. 2004;172(11):7031-7042.

69. Bandow K, Kusuyama J, Shamoto M, Kakimoto K, Ohnishi T, Matsuguchi T. LPS-induced chemokine expression in both MyD88-dependent and -independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. *FEBS Lett*. 2012;586(10):1540-1546.

70. Vora M, Yssel H, de Vries JE, Karasek MA. Antigen presentation by human dermal microvascular endothelial cells. Immunoregulatory effect of IFN-gamma and IL-10. *J Immunol*. 1994;152(12):5734-5741.

71. Savage CO, Brooks CJ. Human vascular endothelial cells do not induce anergy in allogeneic CD4+ T cells unless costimulation is prevented. *Transplantation*. 1995;60(7):734-740.

72. Greening JE, Tree TI, Kotowicz KT, et al. Processing and presentation of the islet autoantigen GAD by vascular endothelial cells promotes transmigration of autoreactive T-cells. *Diabetes*. 2003;52(3):717-725.

73. Rothermel AL, Wang Y, Schechner J, et al. Endothelial cells present antigens in vivo. *BMC Immunol*. 2004;5:5.

74. Schurich A, Böttcher JP, Burgdorf S, et al. Distinct kinetics and dynamics of cross-presentation in liver sinusoidal endothelial cells compared to dendritic cells. *Hepatology*. 2009;50(3):909-919.

75. Sørensen KK, McCourt P, Berg T, et al. The scavenger endothelial cell: a new player in homeostasis and immunity. *Am J Physiol Regul Integr Comp Physiol*. 2012;303(12):R1217-R1230.

76. Sørensen KK, Simon-Santamaria J, McCuskey RS, Smedsrød B. Liver sinusoidal endothelial cells. *Compr Physiol*. 2015;5(4):1751-1774.

77. Santambrogio L, Berendam SJ, Engelhard VH. The antigen processing and presentation machinery in lymphatic endothelial cells. *Front Immunol*. 2019; 10:1033.

78. Santambrogio L, Sato AK, Carven GJ, Belyanskaya SL, Strominger JL, Stern LJ. Extracellular antigen processing and presentation by immature dendritic cells. *Proc Natl Acad Sci USA*. 1999;96(26):15056-15061.

79. Franchini M, Lippi G. Acquired factor VIII inhibitors [published correction appears in *Blood*. 2009;113(21):5368]. *Blood*. 2008;112(2):250-255.

80. Gao C, Schroeder JA, Xue F, et al. Nongenotoxic antibody-drug conjugate conditioning enables safe and effective platelet gene therapy of hemophilia A mice. *Blood Adv*. 2019;3(18):2700-2711.

81. Liu L, Mah C, Fletcher BS. Sustained FVIII expression and phenotypic correction of hemophilia A in neonatal mice using an endothelial-targeted sleeping beauty transposon. *Mol Ther*. 2006;13(5):1006-1015.

82. Kren BT, Unger GM, Sjeklocha L, et al. Nanocapsule-delivered Sleeping Beauty mediates therapeutic factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice. *J Clin Invest*. 2009;119(7):2086-2099.

83. Limmer A, Ohl J, Kurts C, et al. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med*. 2000;6(12):1348-1354.

84. Knolle PA, Wohlleber D. Immunological functions of liver sinusoidal endothelial cells. *Cell Mol Immunol*. 2016;13(3):347-353.

85. Shetty S, Lalor PF, Adams DH. Liver sinusoidal endothelial cells - gatekeepers of hepatic immunity. *Nat Rev Gastroenterol Hepatol*. 2018;15(9):555-567.

86. Doherty DG. Antigen-specific immune tolerance in the liver. *Nat Biomed Eng*. 2019;3(10):763-765.

87. Tewalt EF, Cohen JN, Rouhani SJ, Engelhard VH. Lymphatic endothelial cells - key players in regulation of tolerance and immunity. *Front Immunol*. 2012; 3:305.

88. Dubrot J, Duraes FV, Harlé G, et al. Absence of MHC-II expression by lymph node stromal cells results in autoimmunity. *Life Sci Alliance*. 2018;1(6): e201800164.

89. Humbert M, Hugues S, Dubrot J. Shaping of peripheral T cell responses by lymphatic endothelial cells. *Front Immunol*. 2017;7:684.

90. Savinov AY, Wong FS, Stonebraker AC, Chervonsky AV. Presentation of antigen by endothelial cells and chemoattraction are required for homing of insulin-specific CD8+ T cells. *J Exp Med*. 2003;197(5):643-656.

91. Galea I, Bernardes-Silva M, Forse PA, van Rooijen N, Liblau RS, Perry VH. An antigen-specific pathway for CD8 T cells across the blood-brain barrier. *J Exp Med*. 2007;204(9):2023-2030.

92. Wheway J, Obeid S, Couraud PO, Combes V, Grau GE. The brain microvascular endothelium supports T cell proliferation and has potential for alloantigen presentation [published correction appears in *PLoS One*. 2013;8(4)]. *PLoS One*. 2013;8(1):e52586.

93. Howland SW, Gun SY, Claser C, Poh CM, Rénia L. Measuring antigen presentation in mouse brain endothelial cells ex vivo and in vitro. *Nat Protoc*. 2015; 10(12):2016-2026.

94. Qadura M, Othman M, Waters B, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost*. 2008;6(12):2095-2104.

95. Pfistershammer K, Stöckl J, Siekmann J, Turecek PL, Schwarz HP, Reipert BM. Recombinant factor VIII and factor VIII-von Willebrand factor complex do not present danger signals for human dendritic cells. *Thromb Haemost*. 2006;96(3):309-316.

96. Qadura M, Waters B, Burnett E, et al. Recombinant and plasma-derived factor VIII products induce distinct splenic cytokine microenvironments in hemophilia A mice. *Blood*. 2009;114(4):871-880.

97. Hartholt RB, Wroblewska A, Herczenik E, et al. Enhanced uptake of blood coagulation factor VIII containing immune complexes by antigen presenting cells. *J Thromb Haemost*. 2017;15(2):329-340.

98. Herczenik E, van Haren SD, Wroblewska A, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2012;129(2):501-509.

99. Gangadharan B, Ing M, Delignat S, et al. The C1 and C2 domains of blood coagulation factor VIII mediate its endocytosis by dendritic cells. *Haematologica*. 2017;102(2):271-281.

100. Dasgupta S, Navarrete AM, André S, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica*. 2008;93(1):83-89.

101. Sorvillo N, Hartholt RB, Bloem E, et al. von Willebrand factor binds to the surface of dendritic cells and modulates peptide presentation of factor VIII. *Haematologica*. 2016;101(3):309-318.

102. Ragni MV, Wu W, Liang X, Hsieh CC, Cortese-Hassett A, Lu L. Factor VIII-pulsed dendritic cells reduce anti-factor VIII antibody formation in the hemophilia A mouse model. *Exp Hematol*. 2009;37(6):744-754.

103. Su RJ, Epp A, Feng J, et al. Suppression of the immune response to FVIII in hemophilia A mice by transgene modified tolerogenic dendritic cells. *Mol Ther*. 2011;19(10):1896-1904.

104. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen-presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009;7(11):1816-1823.

105. Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol*. 2013;14(2):152-161.

106. Sardella C, Russo D, Raggi F, et al. Ectopic expression of FSH receptor isoforms in neoplastic but not in endothelial cells from pancreatic neuroendocrine tumors. *J Endocrinol Invest*. 2013;36(3):174-179.

107. He J, Tsai LM, Leong YA, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5+ CD4+ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity*. 2013;39(4):770-781.

108. Mai J, Virtue A, Shen J, Wang H, Yang XF. An evolving new paradigm: endothelial cells–conditional innate immune cells. *J Hematol Oncol*. 2013;6:61.

109. Rubinstein D, Roska AK, Lipsky PE. Liver sinusoidal lining cells express class II major histocompatibility antigens but are poor stimulators of fresh allogeneic T lymphocytes. *J Immunol*. 1986;137(6):1803-1810.

110. Lohse AW, Knolle PA, Bilo K, et al. Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology*. 1996;110(4):1175-1181.

111. Uhrig A, Banafsche R, Kremer M, et al. Development and functional consequences of LPS tolerance in sinusoidal endothelial cells of the liver. *J Leukoc Biol*. 2005;77(5):626-633.

112. Patten DA, Shetty S. More than just a removal service: scavenger receptors in leukocyte trafficking. *Front Immunol*. 2018;9:2904.

113. Anderson CL. The liver sinusoidal endothelium reappears after being eclipsed by the Kupffer cell: a 20th century biological delusion corrected. *J Leukoc Biol*. 2015;98(6):875-876.

114. Peyvandi F, Mannucci PM, Garagiola I, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. *N Engl J Med*. 2016;374(21): 2054-2064.

115. Goudemand J, Peyvandi F, Lacroix-Desmazes S. Key insights to understand the immunogenicity of FVIII products. *Thromb Haemost*. 2016;116(suppl 1): S2-S9.

116. Brinkman HJ, Mertens K, van Mourik JA. Phospholipid-binding domain of factor VIII is involved in endothelial cell-mediated activation of factor X by factor IXa. *Arterioscler Thromb Vasc Biol*. 2002;22(3):511-516.

117. Gilbert GE, Novakovic VA, Shi J, Rasmussen J, Pipe SW. Platelet binding sites for factor VIII in relation to fibrin and phosphatidylserine. *Blood*. 2015; 126(10):1237-1244.

118. Swystun LL, Lai JD, Notley C, et al. The endothelial cell receptor stabilin-2 regulates VWF-FVIII complex half-life and immunogenicity. *J Clin Invest*. 2018; 128(9):4057-4073.

119. Chow TW, Hellums JD, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood*. 1992;80(1):113-120.

120. Pendu R, Terraube V, Christophe OD, et al. P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. *Blood*. 2006;108(12):3746-3752.

121. Schwarz HP, Lenting PJ, Binder B, et al. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood*. 2000;95(5):1703-1708.

122. Tamura Y, Osuga J, Adachi H, et al. Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide. *J Biol Chem*. 2004;279(30):30938-30944.