SUPPLEMENTARY APPENDIX

Extrahepatic sources of factor VIII potentially contribute to the coagulation cascade correcting the bleeding phenotype of mice with hemophilia A

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 doi:10.3324/haematol.2014.123117
Manuscript received on December 29, 2014. Manuscript accepted on April 22, 2015.
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Extra-hepatic sources of FVIII potentially contribute to coagulation cascade to correct the bleeding phenotype in Hemophilia A mice

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Supplemental Results

Characterization of the immune sera produced against FVIII

To produce polyclonal antibody able to recognize FVIII in cultured cells and tissue sections of human and mouse origins, two rabbits were immunized with recombinant FVIII (Kogenate, Bayer) and bled at different times to follow and characterize their immune responses. The sera were recovered and tested at different concentrations on recombinant FVIII in a solid phase ELISA. The serum titers vs the immunogen increased up to the fourth bleeding and then stabilized. Supplemental Figure 1A reports a typical titration curve of the pooled bleedings, which displays a titer of about 1/10,000. The response was specific, since no binding to BSA was detected; the preimmune sera were negative at all tested dilutions. No significant difference was found between the immune responses of the two immunized animals.

The pooled sera were then analyzed directly by western blot (WB) on purified FVIII. The antibodies recognized a protein of about 200 kDa, the expected molecular weight of FVIII heavy chain (Supplemental Figure 1B). Moreover, a molecular species with the same mass was detectable in immunoprecipitates obtained with these antibodies and decorated in WB (Supplemental Figure 1B). Other lower molecular weight species are also recognized when high amounts of FVIII were loaded for direct WB or possibly due to proteolytic degradation of FVIII, occurring during the immunoprecipitation incubation time. All together these experiments show that the rabbit sera contain specific antibodies, which recognize the immunogen, both in its native and denatured forms. Sera were then tested on human tissues in immunohistochemistry. Supplemental Figure 1C and D shows that the antibodies were able to recognize the FVIII present in human sections of a cirrhotic liver. Finally, the sera were tested for their reactivity against murine and human FVIII. Supplemental Figure 1 E-F shows that, indeed, the sera reacted positively in immunofluorescence on LV-FVIII transduced CHO cells expressing the murine and human B-domain deleted FVIII, respectively. A comparison was also performed with commercial anti-FVIII monoclonal antibody (Supplemental Figure 1G). It can be concluded on the basis of the above experiments it can be concluded that the antisera produced contain high titer specific antibodies reacting with mouse and human FVIII.

Supplemental Methods

Polyclonal antibodies production

Polyclonal antibodies (pAb) against human factor VIII were produced by immunizing two 6 weeks old New Zealand White rabbits (Specific Patogen Free) with recombinant human Factor VIII (KOGENATE®, Bayer HealthCare AG, Leverkusen, Germany) at three week-intervals, for a total of 5 inocula; two more injections were performed in the following six months. Animals were injected at multiple sites intra-muscularly and subcutaneously with 1.5 mg protein in 0.5 ml injectable sterile saline (B. Braun, Melsungen AG) emulsified with 0.5 ml of the Complete Freund's Adjuvant (CFA, Sigma, St Louis, USA) the first time, and with the Incomplete Freund's Adjuvant (IFA, Sigma Aldrich) for all the following boosters. Animals were bled one week after each injection starting from the second one and sera were prepared and stored at -20°C in aliquots. Each bleeding was tested in a solid phase ELISA, in which KOGENATE® (10 µg/ml) or bovine serum albumin (BSA) used as specificity control were adsorbed on PVC microwells, followed by saturation with 0.2% BSA. Serum reactivity was detected with horseradish peroxidase-coupled secondary goat anti-rabbit Ig antibodies and the addition of the chromogen 3,3',5,5'- tetramethylbenzidine (TMB; Sigma Aldrich). Microwelladsorbance was read at 450 nm at a Spectra Count^{TM} Packard.

Sera reactivity for FVIII was tested in western blot either directly or after immunoprecipitation carried on by incubation with KOGENATE® (1 µg) and Sepharose Protein A. After protein separation on 8% SDS-PAGE and transfer on PVDF membranes (Hybond-P, Amersham, GE Healthcare), reactivity was visualized by ECL (Enhanced Chemiluminescence, Amersham, LIFE SCIENCE).

Human cells isolation and differentiation

Human liver samples were perfused as previously described [1]. After liver dissociation with a cell scraper, cells were passed through Dacron fabric with 80-µm pores and centrifuged twice at 50 g for 5 minutes to separate hepatocytes from non-parenchymal cells (NPCs). NPCs were washed and pelleted at 350 g for 10 minutes. Human liver sinusoidal endothelial cells (LSECs) were selected from the NPC fraction by immunomagnetic sorting, incubating NPCs with anti-human CD31 that was biotin-conjugated (eBioscience) for 20 min at 4°C followed by incubation with Streptavidin MicroBeads (MiltenyiBiotec) for 15 min at 4°C and finally isolated by MS Separation Columns (Miltenyi Biotec), according to the manufacturer's protocol. Isolated LSECs were plated at a density of 1.5-2x10^5 cells/cm^2 and cultured on collagen-coated tissue culture dishes in EGM-2 medium (Lonza). Human Kupffer cells were isolated by plastic adhesion from NPCs after LSEC isolation. Briefly, CD31 negative fraction of NPCs were resuspended in serum-free RPMI and plated on plastic tissue culture dishes. After 45 minutes of incubation at 37°C, the supernatant was

removed, plates were washed twice in PBS and finally fresh RPMI containing 10% fetal bovine serum (FBS) and 10 ng/ml rhM-CSF was added. KC were cultured for up to 3 weeks under these conditions. Peripheral blood lymphocytes were obtained from PBMC as non adherent cell fraction after 2 step of monocytes adhesion. Granulocytes were obtained after erythrocytes lysis from basal layer of peripheral blood after density gradient centrifugation.

To obtain macrophage differentiation from hematopoietic stem cells 10^5 CD34+ cells/ml were plated in STEM-SPAM medium (STEMCELL Technologies Inc.) containing 20% FBS, 2mM glutamine, 50 U/ml penicillin,50 µg/ml streptomycin, 30 ng/ml interleukin 3, 30 ng/ml M-CSF, 30 ng/ml Flt-3 ligand, 25 ng/ml SCF, as previously described [2]. Medium was changed every 2 days. After 14 days cells acquired macrophage morphology.

To obtain megakaryocytes (MK) from human hematopoietic stem cells $1.5x10^6$ CD34+ cells/ml were plated in STEM-SPAM medium containing 2mM glutamine, 50 U/ml penicillin,50 µg/ml streptomycin, 10 ng/ml interleukin 6, 10 ng/ml interleukin 11 and 20 ng/ml thrombopoietin (TPO). The medium was changed at day 3, 7 and 10 [3]. After 13 days megakaryocytes were harvested and used for experiments.

Monocytes for transplantation in NOD-SCID hemophilia A mice were isolated from cord blood by immunomagnetic selection using the anti-CD11b biotin conjugated antibody (clone M1/70; eBioscence) followed by streptavidin microbeads separation as described above.

Murine cells isolation and differentiation

Murine monocytes were obtained from total peripheral blood. After removal of red blood cells using red blood lysis buffer (RBLB) (150mM NH_4Cl,10mM $NaHCO_3$, 1 mM disodium EDTA) total white cells were plated at the density of $1.5\text{-}2x10^6$/ml in DMEM containing 10% FBS. After 24 hour monocytes were attached.

BM cells were flushed from tibias and femurs of 8-9 weeks-old wild-type or HA mice with DMEM containing 5% FBS. After RBC lysis $0.8\text{-}1x10^6$ of total BM cells/cm^2 plastic were differentiated in macrophages (BM-DM, bone marrow-derived macrophages) by culturing cells in IMDM containing 10% FBS and 5 ng/ml recombinant mM-CSF in vitro. For these studies, BMDM were released 5-7 days later by Versene (Gibco).

For mouse MK differentiation, c-Kit+ cells were isolated using the mouse CD117 MicroBeads kit (Miltenyi Biotech) from total BM cells obtained as described above. Isolated cells were cultured for 2 days in Stem Span containing 20 ng/ml SCF. Then 10^6 cells/ml were cultured for 3-4 days in Stem Span containing 100 ng/ml TPO, 10 ng/ml IL-6 and 10 ng/ml IL-11. All cytokines were purchased from Immunotools.

Cell lines

Liver hepatocellular carcinoma cell lines HepG2 and Huh7 were cultured in DMEM (Lonza) supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Chinese hamster ovary (CHO) cells were cultured in HAM-F12 supplemented with 10% FBS, 2mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

RNA isolation and RT-PCR

All the PCRs were performed with GoTaq® Flexi DNA Polymerase (Promega). PCR protocol were as follow: initial denaturation at 95°C for 5 min followed by 30 cycles (25 cycles for β-actin) of denaturation at 94°C for 30", annealing at 54-62°C for 30-45", extension at 72°C for 60", and final extension at 72°C for 7 minutes. Primers, annealing temperatures and product sizes are listed in the table. PCR products were resolved in 2% agarose gels

Table 1: Primers used in RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	T annealing	Product size (bp)
hβ-actin	GAGAAAATCTGGCACCACACC	CGACGTAGCACAGCTTCTC	56°C	412
mβ-actin	GTGGGGCGCCCCAGGCACCA	CTTCCTTATTGTCACGCACGATTTC	56°C	540
hFVIII	GGAGAGTAAAGCAATATCAGATGC	GGTGAATTCGAAGGTAGCGAC	56°C	389
mFVIII	GGTATCAAAGTGACAATGTACC	CCAATTAATCCCGAGTGCATATC	54°C	394
mGPαIIβ	CAGGGCCAAGTGCTGATATT	TTGAAGCAGCTGACTGGTGT	54°C	302
mvWF	ACAGACGCCATCTCCAGATTCA	TGTTCATCAAATGGTGGGCAGC	62°C	272

qPCR

qPCR used GoTaq® qPCR Master Mix (Promega). Primers were as follow : hFVIII; reverse, 5'-caatggctacataatggatacactacct-3'; forward, 5'-tgtccactgaaatgaatagaatggat-3'; hβ-actin; reverse, 5'-cgccgccagctcaccatg-3'; forward, 5'-cacgatggaggggaagacgg-3'. PCR cycles were as follows: denaturation at 95°C x 2 min followed by 40 cycles of denaturation at 95°C x 15" and annealing/extension at 60°C x 60" according to the manufacturer's protocol.

Immunostaining

5- to 6-µm thick paraffin-embedded human organs sections were treated in boiling 50 mM EDTA pH 8 for antigen retrieval using a microwave oven and then blocked in a buffer containing 5% goat serum, 1% BSA, 0.1% Triton X-100 in PBS. Five-6 µm thick sections of mouse tissues were directly incubated in blocking buffer. For cell staining both human and mouse cells were plated on 12 mm Ø dish glass at concentration of $2x10^4$, the same number of megakaryocytes were

prepared by cyto-spin at 1000 rpm for 5 min. Cells were fixed in 4% PFA for 10 min at 4°C, permeabilized with ice-cold 0.5% Triton X-100 in PBS for 10 min. For FVIII detection samples were stained with rabbit anti-FVIII (1:200 for cells or 1:1000 for tissue speciments), or mouse anti-FVIII (1:200, GM 8015, Green Mountain Antibodies) for 2 h at room temperature. For cell type specific markers the staining was performed with mouse anti-hCD14 (1:150; eBioscience), rat anti-mF4/80 (1:300; AB serotec), mouse anti-hCD11b (1:250; ImmunoTools), rabbit anti-vWF (1:100; SIGMA), mouse anti-hCD61 (1:250; Santa Cruz Biotecnology), mouse anti-hCD31 (1:100; BD Pharmingen) and mouse anti-hCD146 (1:200; Miltenyi Biotec) for 2 h at RT.

After washing in PBS, Alexa Fluor®488- or 546-conjugated goat anti-rabbit, anti-rat or anti-mouse IgGs (1:500, Molecular Probes) were added for 1 h. Nuclei were stained with DAPI-Antifade (Molecular Probes). As control for FVIII staining, samples were incubated using rabbit serum collected before FVIII immunization (as primary antibody).

Immunohistochemical reactions were performed by a standard procedure, as previously reported [4]. Immunostaining was performed by Dako Cytomation Envision plus system (DAKO Cytomation), using diaminobenzidine as chromogen. Sections were counterstained by hema lume (Merck, Germany).

Flow cytometric analysis

Cultured human CD34-derived macrophages, LSEC and KC were characterized by flow cytometric analysis. To evaluate human cells engraftment in transplanted mice by FACS, peripheral blood was collected by intracardiac or retro-orbital puncture, BM cells were flushed as decribed above and total cells from spleen were obtained by mechanical disruption of the organ. Antibodies used and incubation conditions are reported in Table 2

For each sample, $1x10^5$ events were acquired by FACS calibur. Data were analyzed by Windows Multiple Document Interface for Flow Cytometry (winMDI, v. 2.9; Joseph Trotter, The Scripps Institute).

Table 2: Antibodies used for FACS

Antibody	Reactivity	Manufacturer	Format	Incubation condition
CD45	human	Miltenyi Biotec	PE	30 min on ice
CD19	human	Miltenyi Biotec	PE	30 min on ice
CD14	human	Immunotools	FITC	30 min on ice
CD3	human	Immunotools	FITC	30 min on ice
CD33	human	Immunotools	FITC	30 min on ice
CD11b	human	Immunotools	PE	30 min on ice
Tie-2	human	Miltenyi Biotec	PE	30 min on ice
CD31	human	Immunotools	APC	30 min on ice

Mice

For hematopoietic stem cells xenotransplantation studies, we used the NOD/SCID hemophilia A (NSHA) mice previously generated at Albert Einstein College of Medicine, NY and presently reside in the lab [1]. Immunocompromised NOD/SCID-γNull Hemophilia A mice (NSG-HA) were generated by crossing NSHA mice with NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (γNull) purchased by Jackson lab [5]. Since both F8 and IL2rg gene are located on chromosome X the double knockout was obtained after several attempts of crossing-over. Homologous recombination was initially achieved in a male mouse, which was then crossed with γNull in order to obtain homozygous mice. For xenotransplantation studies, double knockout homozygous mice were used. Mice genotyping was assessed at each generation by PCR using mutations specific primers. Primers were as follow: γNULL forward GTGGGGAGCCAGCTCTTCAG, γNULL reverse CCTGGAGCTGGACAACAAAT, γNULL reverse mut GCCAGAGGCCACTTGTGTAG, HA forward TGCAAGGCCTGGGCTTATTT, HA reverse GAGCAAATTCCTGTACTGAC, HA forward mut TGTGTCCCGCCCCTTCCTTT. Hemophilic phenotype was evaluated by activated partial thromboplastin time (aPTT) assay that showed no FVIII activity in the plasma of generated mice. Both NSHA and NSGHA were kept in autoclaved microisolator cages and fed with sterile food and water at the animal facilities of the Università del Piemonte Orientale. Human hepatocytes and NPC were transplanted in the peritoneal cavity of NSG-HA as previously described [6]. Briefly, 10^7 isolated hepatocytes or NPC were mixed with cytodex 3 micro-carriers (GE healthcare Life Science) and then transplanted intraperitoneally in 1 ml of volume. We injected 3 control mice (beads only), 9 mice with hepatocytes (from 3 different donors) and 5 mice with NPC (from 2 different donors).

Supplementary Reference

1. Follenzi A, Benten D, Novikoff P, Faulkner L, Raut S, Gupta S. Transplanted endothelial cells repopulate the liver endothelium and correct the phenotype of hemophilia A mice. J Clin Invest. 2008;118(3):935-945.
2. Stec M, Weglarczyk K, Baran J, et al. Expansion and differentiation of CD14+CD16(-) and CD14+ +CD16+ human monocyte subsets from cord blood CD34+ hematopoietic progenitors. J Leukoc Biol. 2007;82(3):594-602.
3. Di Vito C, Bergante S, Balduini A, et al. The oestrogen receptor GPER is expressed in human haematopoietic stem cells but not in mature megakaryocytes. Br J Haematol. 2010;149(1):150-152.
4. Valente G, Mamo C, Bena A, et al. Prognostic significance of microvessel density and vascular endothelial growth factor expression in sinonasal carcinomas. Hum Pathol. 2006;37(4):391-400.
5. Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood. 2005;106(5):1565-1573.
6. Kumaran V, Benten D, Follenzi A, Joseph B, Sarkar R, Gupta S. Transplantation of endothelial cells corrects the phenotype in hemophilia A mice. J Thromb Haemost. 2005;3(9):2022-2031.

Supplemental figures

Fig. 1 Characterization of anti-hFVIII polyclonal antibody reactivity.

Immune (IS) and pre-immune (NRS) rabbit sera were tested in ELISA on recombinant purified commercial human FVIII (A, pink). Sera reactivity was assessed also by western blot analysis directly on recombinant FVIII (B, lanes 1-2, 40 and 10 ng FVIII, respectively) or after immunoprecipitation with the same protein (B, lane 3) and on IHC on human liver sections (C, D). The antibody detected both human and mouse FVIII, as shown by immunofluorescence on CHO cells transduced with a lentiviral vector expressing either mFVIII (E) or hFVIII (F). Reactivity of a commercial hFVIII antibody on CHO expressing FVIII is shown for comparison (G). No signal was detectable with rabbit pre-immune serum (NRS) (A-C). Scale bar: 25μm.

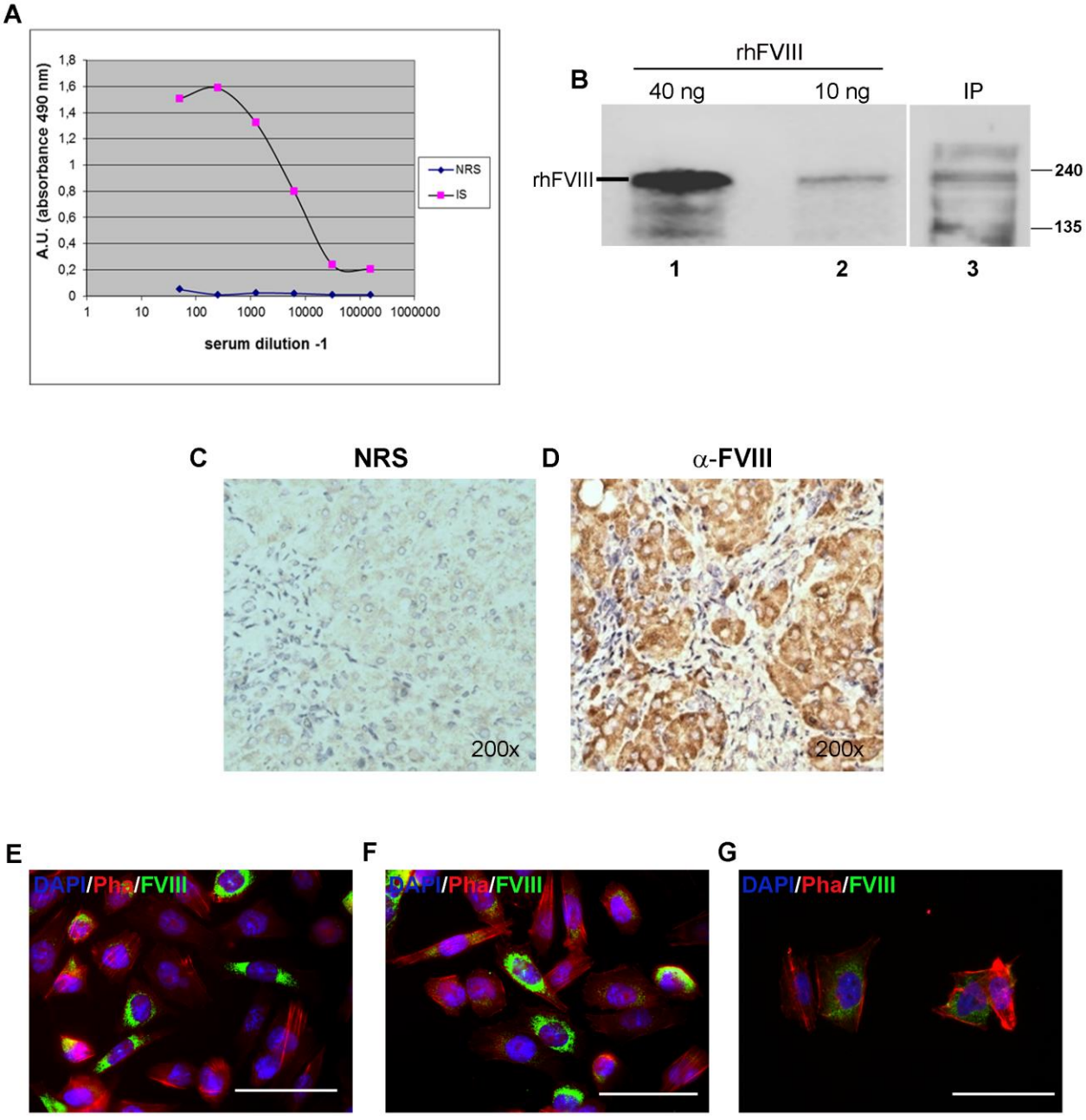
Fig. 2 Expression of FVIII in peripheral mouse blood cells

RT-PCR analysis of the expression of FVIII in peripheral blood mononuclear cells (PBMC), Lymphocytes (Lymph) and Monocytes (Mono) from Hemophilic A (HA) mice and wild type (WT) mice (A). FVIII immunoreactivity on murine monocytes, macrophages and dendritic cells (B). Scale bar: 25μm

Fig. 3 FVIII expression in hematopoietic stem derived macrophages.

Phase contrast microscopy of the morphology of c-Kit+ cells isolated by immunoselection from mouse bone marrow and differentiated in macrophages or in megakaryocytes with a cocktail of cytokines (A,C). PCR (B,E) and immunofluorescence (F,G) analysis of differentiated macrophages and megakaryocytes respectively, in which expression of FVIII is detected both at mRNA and protein levels. RT-PCR analysis of mouse Kit+ cells and megakaryocytes for allβ and vWF-specific markers (D). BM-DM, bone marrow derived macrophages; MK, megakaryocytes. Scale bar: 25 μm.

Supp.Fig.1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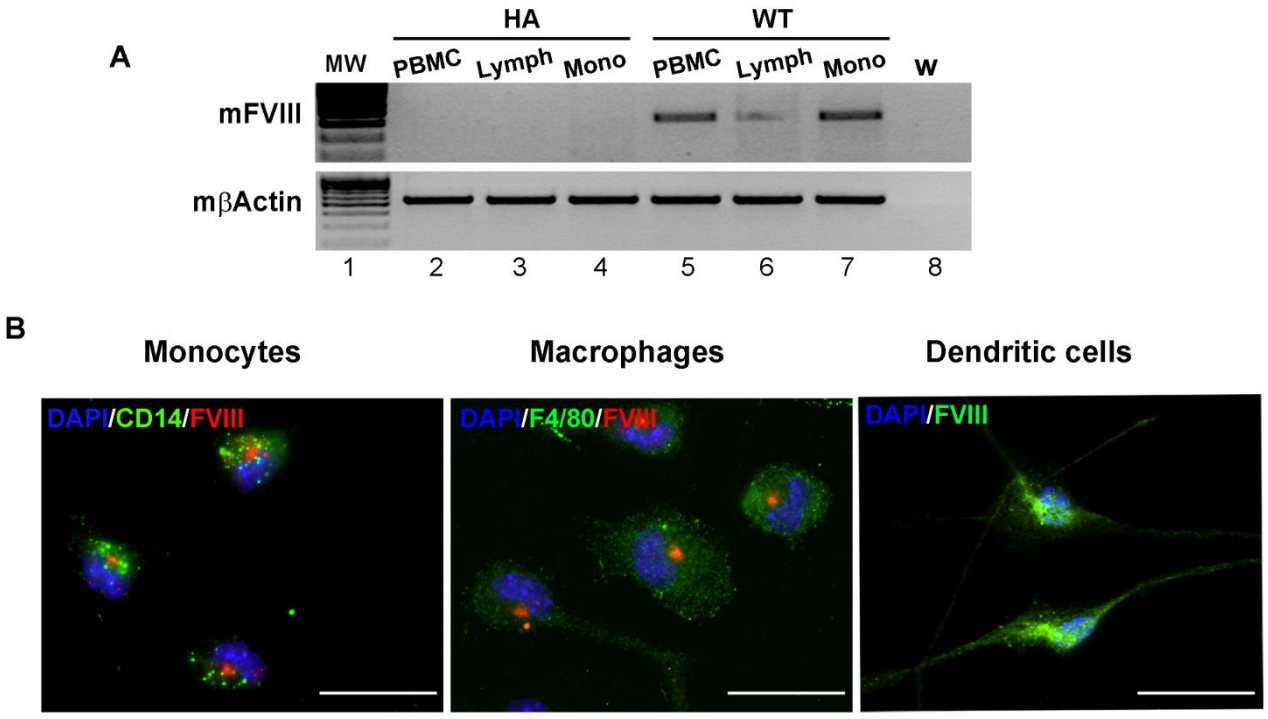
Supp.Fig.2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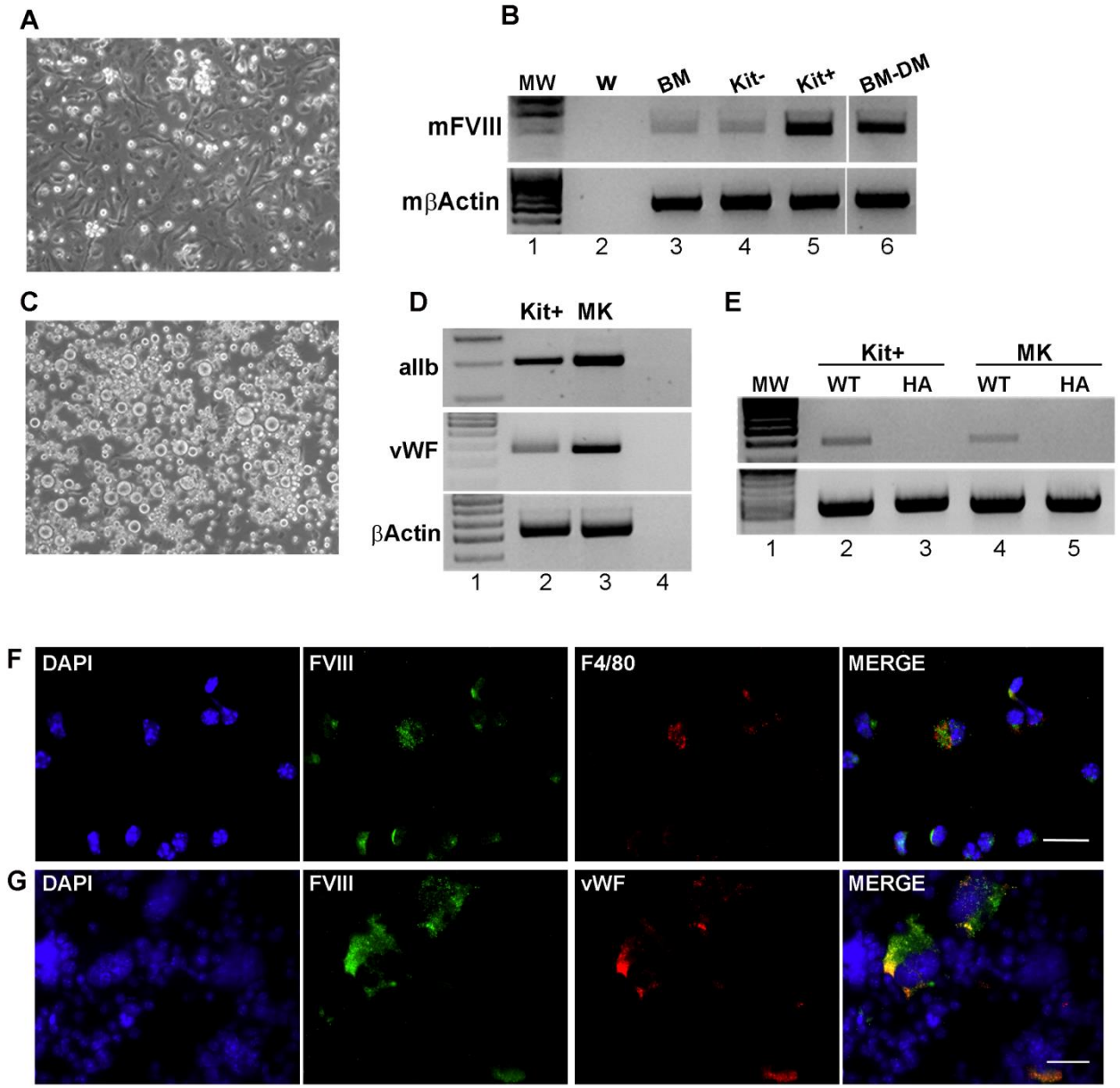
DAPI/F4/80/FVIII
DAPI/FVIII

Supp.Fig.3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