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von Willebrand factor binds to the surface of dendritic cells and modulates peptide presentation of factor VIII

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ABSTRACT

It has been proposed that von Willebrand factor might affect factor VIII immunogenicity by reducing factor VIII uptake by antigen presenting cells. Here we investigate the interaction of recombinant von Willebrand factor with immature monocyte-derived dendritic cells using flow cytometry and confocal microscopy. Surprisingly, von Willebrand factor was not internalized by immature dendritic cells, but remained bound to the cell surface. As von Willebrand factor reduces the uptake of factor VIII, we investigated the repertoire of factor VIII presented peptides when in complex with von Willebrand factor. Interestingly, factor VIII-derived peptides were still abundantly presented on major histocompatibility complex class II molecules, even though a reduction of factor VIII uptake by immature dendritic cells was observed. Inspection of peptide profiles from 5 different donors showed that different core factor VIII peptide sequences were presented upon incubation with factor VIII/von Willebrand factor complex when compared to factor VIII alone. No von Willebrand factor peptides were detected when immature dendritic cells were pulsed with different concentrations of von Willebrand factor, confirming lack of von Willebrand factor endocytosis. Several von Willebrand factor derived peptides were recovered when cells were pulsed with von Willebrand factor/factor VIII complex, suggesting that factor VIII promotes endocytosis of small amounts of von Willebrand factor by immature dendritic cells. Taken together, our results establish that von Willebrand factor is poorly internalized by immature dendritic cells. We also show that von Willebrand factor modulates the internalization and presentation of factor VIII-derived peptides on major histocompatibility complex class II.

Introduction

Hemophilia A is an X-linked bleeding disorder caused by reduced levels of functional human coagulation factor VIII (FVIII). Patients are treated with regular intravenous injections of FVIII concentrates.[1] Approximately 25% of the severe hemophilia A patients [defined as <1 IU/dL (<1% FVIII activity)] develop inhibitory antibodies against FVIII. Both genetic and non-genetic risk factors for inhibitor formation have been identified.[2-4] Genetic risk factors include F8 gene mutation[5] and poly-

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morphisms in IL10, TNFA, FCGR2A and CTLA4.[6,7] Moreover, large epidemiological studies have shown that treatment intensity of hemophilia A patients is also linked to inhibitor development.[8]

The initial step in FVIII inhibitor formation is the endocytosis of FVIII by professional antigen presenting cells such as dendritic cells (DCs). Once endocytosed, FVIII is cleaved in endo-lysosomal compartments into discrete peptides that are loaded on MHC class II.[9,10] The FVIII peptide-MHC class II complexes are then transported to the cell surface for recognition by antigen-specific $CD4^+$ T-helper cells.

Until now, most studies have focused on unravelling the mechanism of endocytosis and presentation of FVIII itself. However, the majority of FVIII circulates in complex with its carrier protein von Willebrand factor (VWF), a multimeric glycoprotein with two critical functions in hemostasis.[11] Besides its role in platelet binding in primary hemostasis, VWF prevents premature activation of FVIII and increases FVIII half-life by preventing its degradation and clearance.[12] Recently, VWF has also been shown to play an important role in FVIII inhibitor formation. It has been shown that VWF reduces the uptake of FVIII by DCs.[13] The exact mechanism of interaction of VWF with DCs is still unknown. Here, the interaction and processing of VWF by DCs, alone or in complex with FVIII, was explored. Surprisingly, no endocytosis of VWF was observed when iDCs were treated with VWF alone or in complex with FVIII. Prolonged incubation times did not lead to internalization of VWF by iDCs; instead, VWF remained tightly bound to the cell surface. To determine the effect of VWF on FVIII peptide presentation, the repertoire of naturally presented FVIII-derived peptides by DCs on MHC class II molecules was analyzed by pulsing DCs with FVIII or FVIII/VWF complex. Interestingly, our findings show that although FVIII endocytosis is reduced in the presence of VWF, FVIII-derived peptides are still efficiently presented on MHC class II. In agreement with its lack of internalization, no VWF derived peptides could be detected when cells were treated with VWF alone, whereas a small number of VWF-derived peptides were presented on MHC class II when cells were pulsed with FVIII/VWF complex. Taken together these data suggest that VWF alone or in complex with FVIII binds to the cell surface, thereby modulating the internalization and peptide presentation of FVIII by DCs.

Methods

Materials

Spray dried ethylenediaminetetraacetic acid (EDTA) vacutainers (Greiner Bio-One, Kremsmuenster, Austria) were used for blood collection from healthy HLA class II-typed volunteers after giving informed consent in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Monocytes were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), CD14 microbeads (Miltenyi Biotech, Auburn, CA, USA) and cultured in Cellgro medium supplemented with human recombinant GM-CSF and IL-4 (CellGenix, Freiburg, Germany) in 6-well plates (Falcon, n. 353046, Corning, Amsterdam, the Netherlands). Human serum albumin was supplied by Sanquin Blood Supply, Amsterdam, the Netherlands. B-domain-deleted FVIII[14] and recombinant VWF[15] were cultured in 6320 cm^2 factories (Nunc, Roskilde, Denmark) in DMEM/F12 (Lonza, Walkerville, MD, USA) supplemented with 10% heat inactivated fetal calf serum (Bodinco, Alkmaar, the Netherlands), which is stepwise reduced to 8% FCS. Medium was concentrated using the hemoflow F5 HPS system (Fresenius Medical Care, Bad Homburg vor der Höhe, Germany) before protein purification. CNBr activated sepharose 4B and Q sepharose fast flow were purchased from GE Healthcare (Wauwatosa, WI, USA). Halt EDTA free protease inhibitor cocktail (100x) was used from Thermo Scientific (Bremen, Germany). Human collagen (VitroCol) used for quantification of VWF activity was obtained from Advanced BioMatrix (Carlsbad, CA, USA). Monoclonal antibodies against different FVIII domains CLB-CAg12, CLB-CAg117 and CLB-EL14 have been described previously.[16-19] Monoclonal antibody CLB-RAg20 against VWF has been described previously.[14,20] DC-SIGN and EEA1 antibodies were from AbD Serotec (Bio-Rad, Veenendaal, the Netherlands) and BD Biosciences (San Jose, CA, USA), respectively. Polyclonal antibody against human VWF from DAKO (Heverlee, Belgium) was used. Antibodies were labeled employing Alexa Fluor micro scale protein labeling kits (Molecular probes, Invitrogen, Breda, the Netherlands). Paraformaldehyde 20% EM grade was supplied by Electron Microscopy Sciences (Hatfield, PA, USA). FVIII activity was determined using the Chromogenix FVIII activity assay (Chromogenix Technologies, Llanelli, UK). For confocal analysis, cells were mounted using MOWIOL (Calbiochem, EMD Millipore, Billerica, MA, USA) supplemented with 2.5% triethylenediamine (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/mL Hoechst 33342 Fluorescent Stain (Life Technologies, Carlsbad, CA, USA).

Endocytosis/binding of VWF and FVIII

Immature dendritic cells were harvested at day 5 and $2x10^5$ cells were incubated with different concentrations of recombinant VWF ranging from 10 nM to 200 nM for 30 min at 37°C in 100 µL serum free medium (Cellgro). Next, cells were washed with Tris buffered saline (TBS) and fixed with 1% paraformaldehyde in TBS at room temperature for 15 min. Samples were incubated with quench buffer (50 mM NH4Cl with or without 0.2% saponine in TBS) for 15 min at room temperature and stained with a specific monoclonal antibody against VWF, CLB-RAg20, in staining buffer (TBS supplemented with 0.5% human serum albumin), with or without 0.05% saponine, and subsequently with rabbit anti-mouse Alexa-568 conjugated secondary antibody. For FVIII uptake experiments, 25 nM of recombinant FVIII was used and samples were stained with Alexa Fluor 488-labeled human monoclonal antibody CLB-EL14. FVIII/VWF complex was obtained by incubation of 25 nM FVIII or 50 nM of FVIII with 250 nM of VWF (ratio 1:10 or 1:5) in serum free medium for 30 min at 37°C. The ability of FVIII to bind to VWF under these conditions was confirmed by ELISA (*Online Supplementary Figure S1*). Uptake and binding of FVIII/VWF complex was performed as described above. Uptake and binding was analyzed by flow cytometry (LSR Fortessa, BD Biosciences, San Jose, CA, USA). Histograms were processed using FlowJo V10 software (Tree Star Inc., Ashland, OR, USA). Data are expressed as percentage of mean fluorescent intensity (MFI) where 100% of uptake/binding corresponds to the maximal fluorescent signal obtained. For each experiment, a sample which was stained for VWF or FVIII without adding VWF or FVIII was used as a negative control to determine background levels.

To monitor uptake/binding by confocal microscopy, $5x10^5$ cells were incubated with 50 nM of VWF for 30 min and/or 5 h at 37°C. Next, cells were washed with TBS and fixed with 4%

PFA for 15 min at room temperature. Subsequently, samples were quenched and stained for VWF, as described above. Cells were also stained with antibodies against DC-SIGN (CD209) and early endosomal marker (EEA1), and subsequently with Alexa Fluor 488 labeled secondary antibody in TBS supplemented with 0.5 % HSA and 0.05% saponine. Stained cover slips were mounted with MOWIOL containing 2.5% triethylenediamine, imaged using 63x objective on a Leica TCS SP8 confocal microscope, and analyzed using Leica Application Suite X (Leica Microsystems, Wetzlar, Germany).

Purification and mass spectrometry analysis of HLA-DR-bound peptides

HLA-DR/peptide complexes were purified and analyzed by mass spectrometry, as described previously; further details are available in the *Online Supplementary Methods*.[9,21,22]

Characterization of peptides

Peptides were identified using Proteome Discoverer 1.4 (Thermo Scientific, Bremen, Germany) and core peptides were predicted using NetMHCpan 2.8.[23] The core peptide with the highest predicted binding affinity was used to indicate the location of that specific group of identified peptides. Further details are available in the *Online Supplementary Methods*.

Results

VWF interacts with human monocyte-derived dendritic cells

It is well established that FVIII and VWF circulate in plasma in a non-covalent complex.[24] *In vitro* experiments suggest that the presence of VWF reduces interaction of FVIII with immature monocyte-derived dendritic cells (iDCs) and subsequent uptake by iDCs, leading to reduced immunogenicity.[13] However, until now, it has not been established how VWF interacts with iDCs. IDCs were incubated with 25 nM of FVIII alone or in complex with VWF (ratio 1:10) for 30 min at 37°C and subsequently analyzed by flow cytometry. FVIII/VWF complex formation was confirmed by ELISA (*Online Supplementary Figure S1*). As expected, an increase in mean fluorescent intensity was observed when cells were incubated with FVIII alone, indicative for FVIII binding and/or uptake, while the presence of VWF reduced the FVIII signal (Figure 1A). These findings are in agreement with previous observations.[13,18] Interestingly, an increase in mean fluorescent intensity was also observed when samples were stained with monoclonal antibody CLB-RAg20 directed against VWF (Figure 1B), indicating that VWF also interacts with iDCs. To

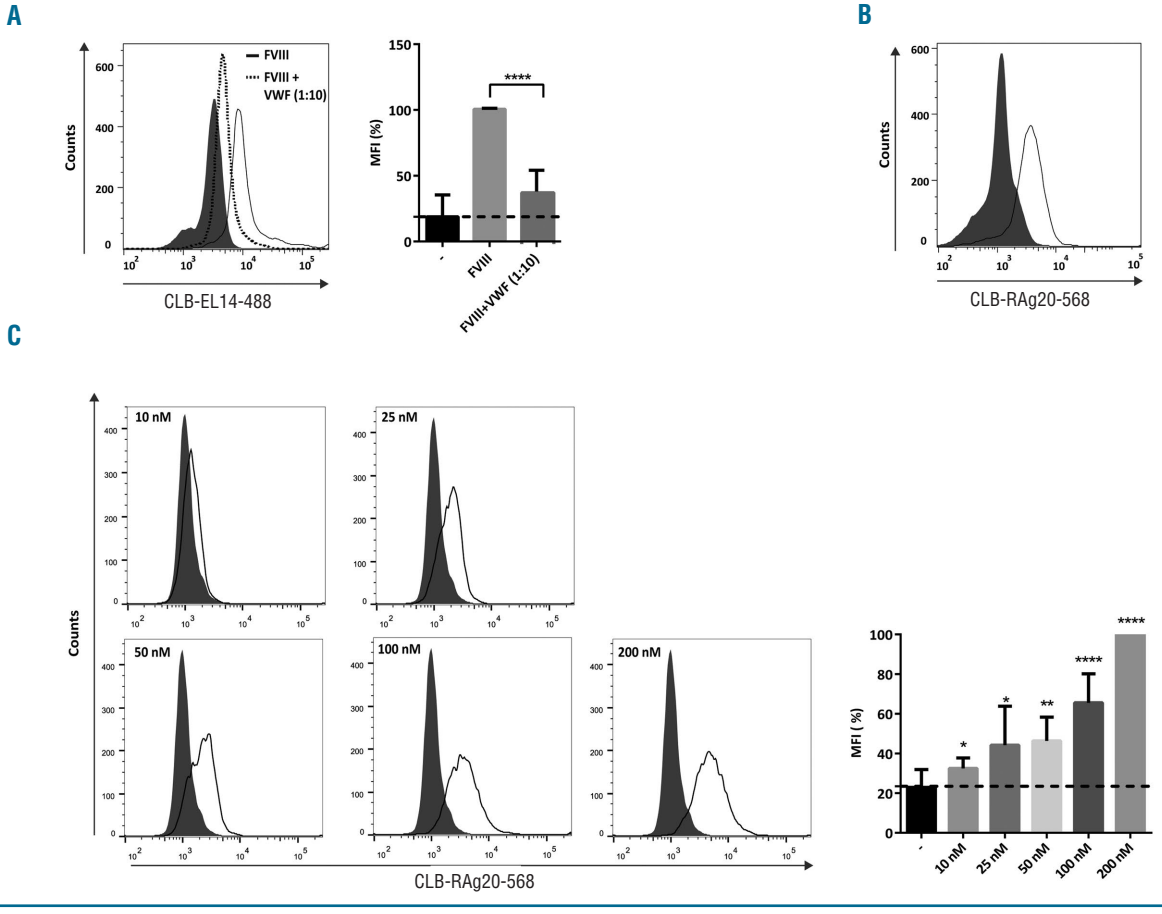

Figure 1. Interaction of von Willebrand factor (VWF) with monocyte-derived dendritic cells. (A) 25 nM of FVIII alone or in complex with VWF (1:10) was incubated with iDCs for 30 min at 37°C. Cells were analyzed by flow cytometry. Gray histograms represent control cells not pulsed with FVIII or VWF. (B) iDCs pulsed with FVIII/VWF complex (1:10) were stained with an antibody against VWF. (C) Uptake/binding was performed with increased concentrations of VWF (10-200 nM). Graphs represent data of 3 independent experiments ± SD. Uptake/binding is represented as percentage of mean fluorescent intensity (MFI) where 100% corresponds to the highest fluorescent signal for each individual experiment such as FVIII alone (A) and 200 nM of VWF (B).

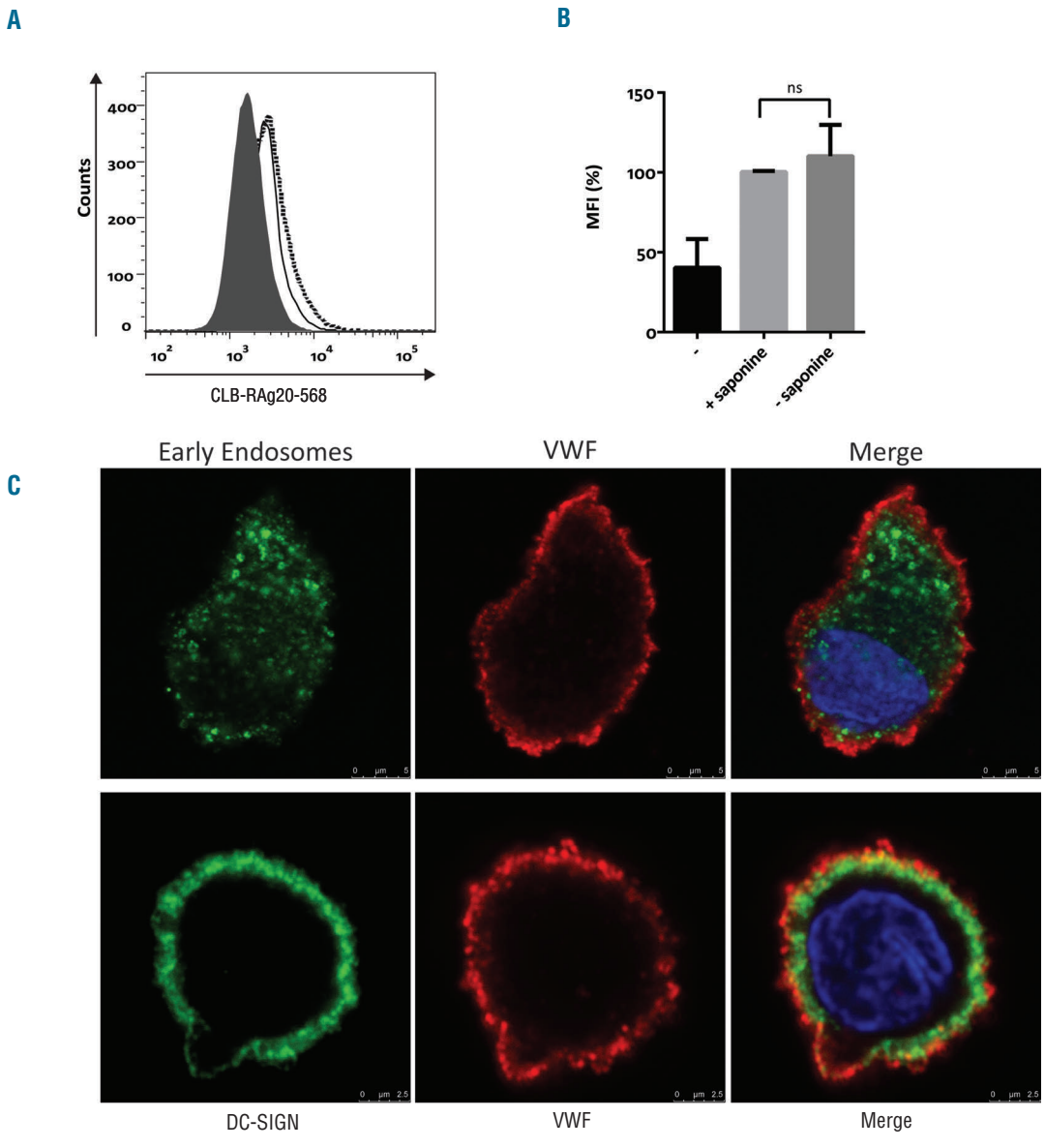

Figure 2. Binding of von Willebrand factor (VWF) to iDCs. (A) iDCs were pulsed with 50 nM VWF in the presence of saponin (continuous line) or without saponin (dotted line) for 30 min. Binding/uptake was analyzed by flow cytometry. (A) Representative histograms where the gray histrogram represents control cells not pulsed with VWF. The solid line and dotted line represent cells treated with or without saponine, respectively. (B) Uptake/binding is represented as percentage of mean fluorescent intensity (MFI) where condition with saponin is set at 100%. Graph represents data of 3 independent experiments ± SD. (C) Binding of VWF to iDCs assessed by confocal microscopy. Cells were incubated with 50 nM VWF for 30 min, subsequently cells were fixed, permeabilized and stained with VWF polyclonal antibody (DAKO) followed by a secondary antibody Alexa Fluor-568 (VWF staining in red) and EEA1 antibody or anti-DC-SIGN antibody followed by secondary antibody Alexa Fluor-488 (EEA1 and DC-SIGN staining in green; upper and lower panel, respectively) with nuclear staining in blue.

determine if the interaction of VWF with iDCs is dependent on FVIII, increasing concentrations of recombinant human VWF were incubated with iDCs. A dose-dependent increase in signal was observed (Figure 1C), further suggesting that VWF interacts with iDCs even when not in complex with FVIII.

VWF is not internalized by dendritic cells

To establish whether VWF is endocytosed or bound to the cell surface, iDCs were incubated with 50 nM VWF for 30 min at 37°C and subsequently stained with anti-VWF antibody CLB-RAg20 in presence or absence of 0.05% saponine. Surprisingly, a similar increase in mean fluorescent intensity was observed in both permeabilized and non-permeabilized cells. This suggests that VWF is predominantly bound and not internalized by iDCs (Figure 2A and B). To further assess the mechanism of VWF interaction, iDCs were incubated with VWF for 30 min at 37°C, and analyzed by confocal microscopy. Confocal microscopy revealed that VWF only binds to the cell surface and is not internalized (Figure 2C). In fact, no co-localization with early endosome marker EEA1 was observed (Figure 2C, upper panel), while VWF was detected in close proximity to the surface marker DC-SIGN. We next ana-

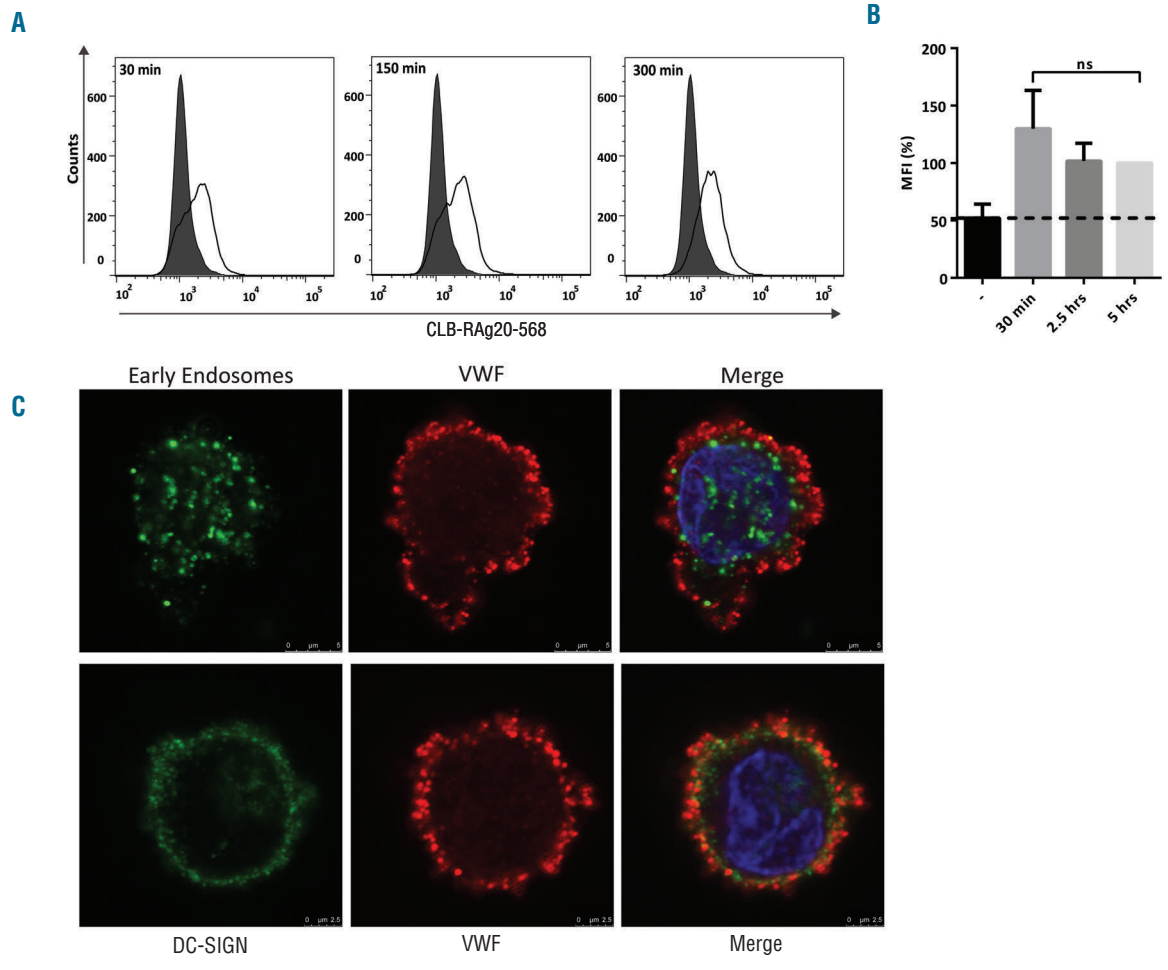

Figure 3. Increased incubation times does not lead to von Willebrand factor (VWF) endocytosis. (A) iDCs were pulsed with 50 nM of VWF for 30, 150 and 300 min, respectively. Binding/uptake was analyzed by flow cytometry. (A) Representative histograms where the gray histograms represent control cells not pulsed with VWF. Black line VWF signal. (B) Quantification of time-dependent binding of VWF to iDCS. Signal is indicated as percentage of MFI and represents data from 3 independent experiments +/- SD. (C) Confocal analysis of VWF binding to iDCs. Cells were incubated with VWF for 6 h. Cells were fixed, permeabilized and stained with VWF polyclonal antibody (DAKO) followed by secondary antibody Alexa Fluor-568 (VWF staining in red) and EEA1 antibody or anti-DC-SIGN antibody followed by secondary antibody Alexa Fluor-488 (EEA1 and DC-SIGN staining in green; upper and lower panel, respectively) with nuclear staining in blue.

lyzed whether prolonged incubation results in the internalization of VWF by iDCs. Fifty nanomolar of VWF was incubated with iDCs for several time points ranging from 30 min to 5 h at 37°C and analyzed by flow cytometry. No increase in mean fluorescent intensity was observed after longer incubation times (Figure 3A and B). Confocal analysis revealed no co-localization with the early endosome marker EEA1 (Figure 3C, upper panel) whereas even after prolonged incubation times VWF was only detected in close proximity to DC-SIGN (Figure 3C, lower panel). These results suggest that VWF is not internalized by iDCs.

VWF modulates the presentation of FVIII derived peptides on MHC class II

It is well established that after internalization by iDCs FVIII is efficiently presented on MHC class II.[9] Several studies have shown that VWF reduces the uptake of FVIII by iDCs.[13,18] However, whether VWF affects the processing and presentation of FVIII-derived peptides on MHC class II has not been addressed. Immature DCs from 3 healthy HLA-DRB1-typed donors (donors A, B and C) were pulsed with 50 nM of FVIII alone or in complex with VWF (ratio 1:5) for 5 h at 37°C. Subsequently, the repertoire of MHC class II-derived peptides was identified by mass spectrometry. In agreement with previous observations, the majority of peptides presented on MHC class II molecules originate from endogenous proteins expressed by iDCs (*data not shown*). Figure 4 represents the repertoire of naturally presented FVIII peptides obtained from 3 different HLA typed donors, indicated as donor A, B and C. FVIII-derived peptides with the same predicted core MHC class II binding amino acid sequence were grouped. An overview of the complete set of identified peptides is provided in *Online Supplementary Figure S3*. Figure 4 shows that peptides originating from all FVIII domains were detected and differences in domain-specificity of the presented peptides were noted. Donor A predominantly presented peptides from the A2 and A3 domains. Cells from donor B additionally presented peptides from the A1

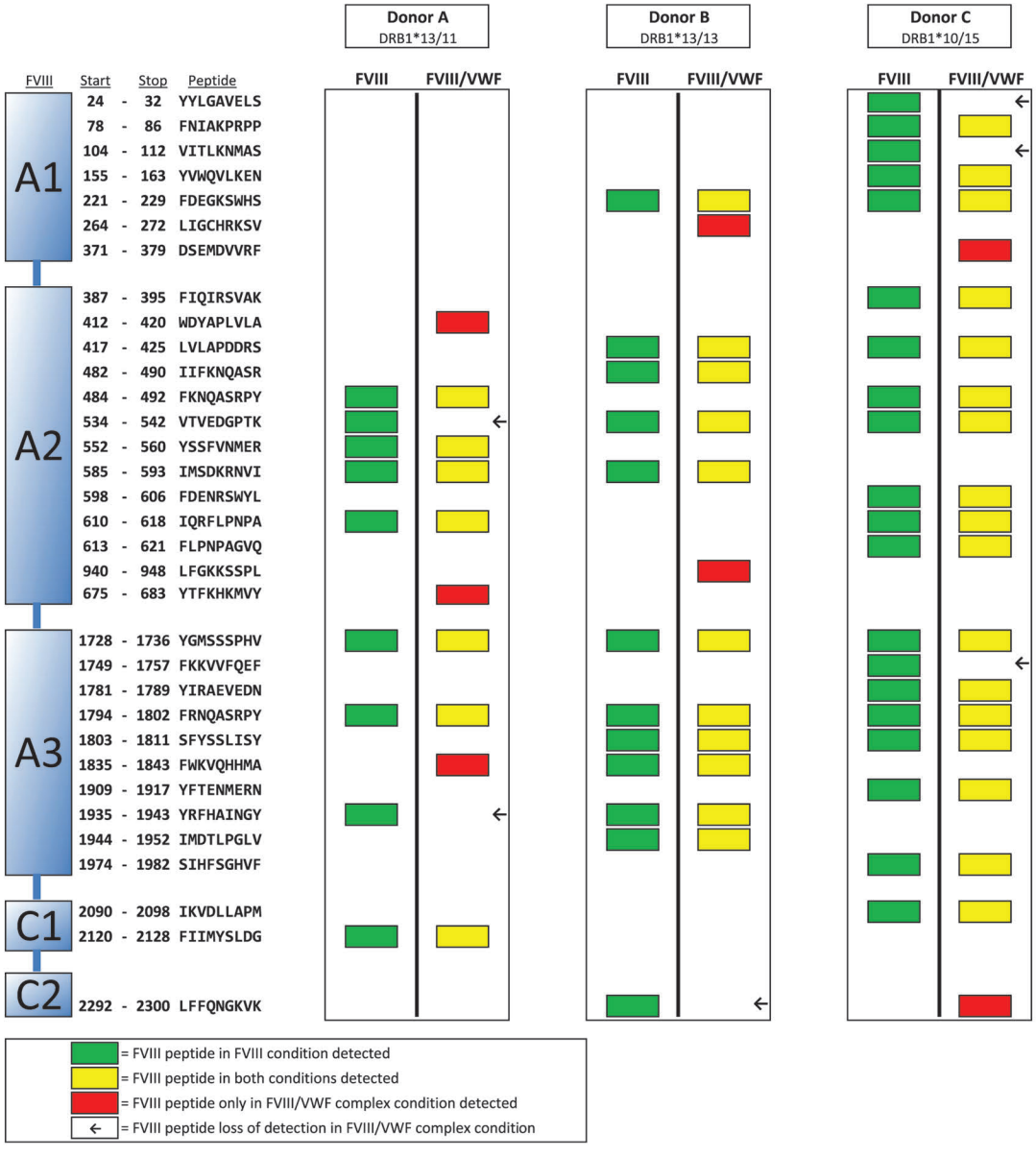

Figure 4. FVIII-derived MHC class II core peptides identified from donors A, B and C. Cells from donors A, B and C were pulsed with 50 nM FVIII or a complex of 50 nM FVIII and 250 nM VWF. After maturation MHC class II ligands were extracted and analyzed using mass spectrometry. FVIII specific peptides were identified using Proteome Discoverer and subsequently HLA-DRB1 genotype specific core peptides for both HLA-DRB1 alleles were predicted using NetMHCpan 2.8. The first column shows predicted core peptides with corresponding residue numbers and corresponding FVIII domain (using HGVS numbering). When multiple core peptides are predicted, the core peptide with the highest predicted affinity for one of the two HLA alleles was used to represent the cluster of peptides identified (see *Online Supplementary Figure S3* for all peptides identified for each donor). Subsequent columns depict which peptides are found for each donor upon incubation with FVIII or FVIII/VWF. Green: FVIII peptide detected following incubation with FVIII; yellow: FVIII peptide detected following incubation with FVIII and FVIII/VWF complex; red: FVIII peptide detection following incubation with FVIII/VWF; arrow: FVIII peptide not detected following incubation with FVIII/VWF.

domain, whereas cells from donor C showed the largest variation in presented peptides spanning most FVIII domains. Interestingly, FVIII-derived peptides were also detected when samples were pulsed with FVIII/VWF complex. We have previously shown that only small variations in peptides were found between duplicate samples using mass spectrometry.[9] This allowed for the comparison of peptide repertories found in FVIII/VWF pulsed DCs with those found in DCs pulsed with FVIII only. Several FVIII-derived peptides were no longer detected in samples pulsed with FVIII/VWF complex (as indicated by the arrows) when compared to samples incubated with FVIII alone. Interestingly, several FVIII-derived peptides were only observed in iDCs pulsed with FVIII/VWF complex (Figure 4, red boxes). The observed changes in peptide repertoire most likely arise from alterations in the levels of presentation of specific peptides. Indeed, analysis of the raw MS spectra confirmed the presence of low levels of "unidentified" peptides based on their position in the ion-chromatogram. We then explored whether increasing the ratio of VWF over FVIII affected peptide presentation of FVIII. Immature DCs were incubated with 25 nM of FVIII alone or in complex with VWF in a ratio of 1:10 instead

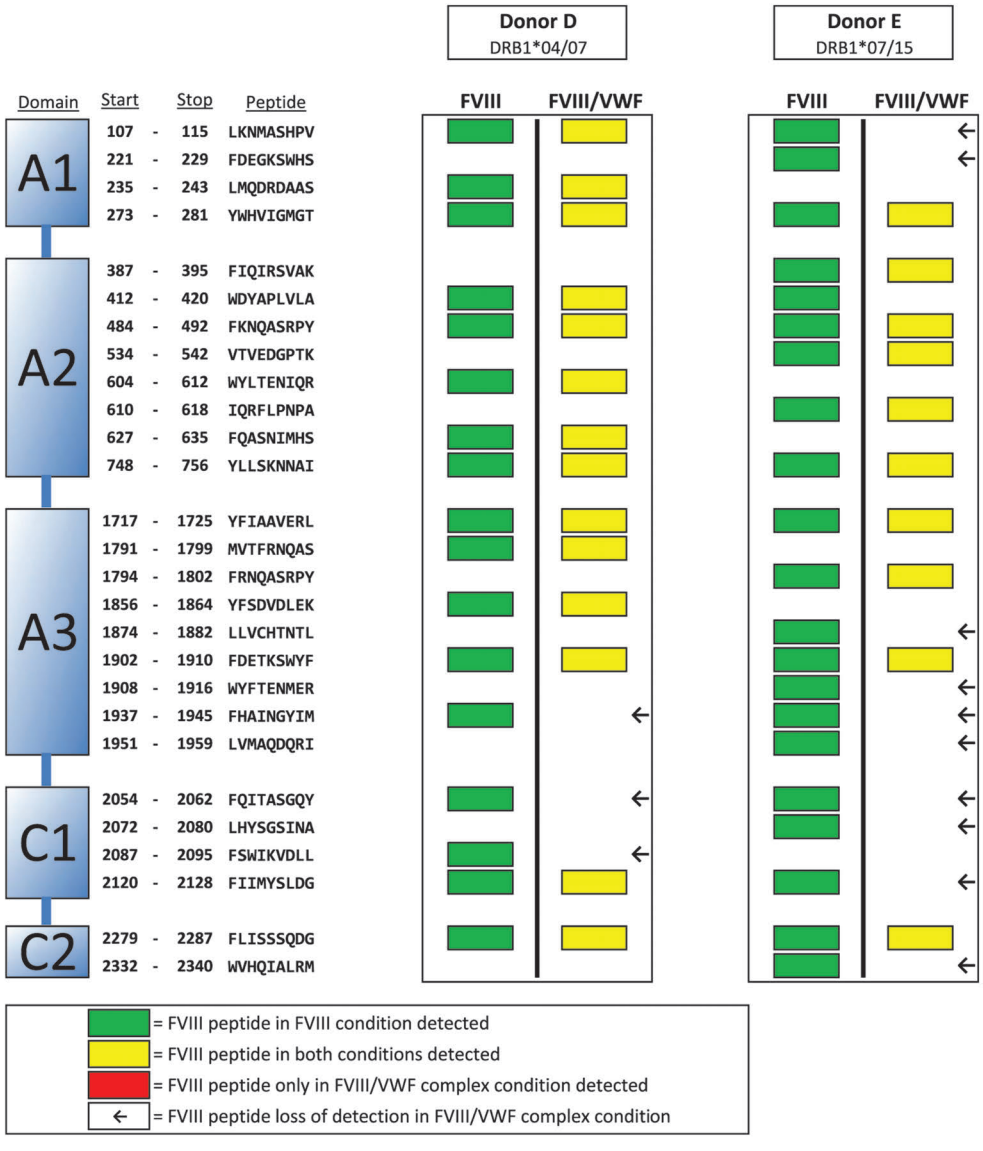

Figure 5. FVIII-derived MHC class II core peptides identified from donors D and E. Cells from donors D and E were pulsed with 25 nM FVIII or a complex of 25 nM FVIII with 250 nM VWF. After maturation of DCs MHC class II ligands were extracted and analyzed using mass spectrometry. FVIII specific peptides were identified using Proteome Discoverer and subsequently HLA-DRB1 genotype specific core peptides were predicted using NetMHCpan 2.8. The first column shows predicted core peptides with corresponding residue numbers and corresponding FVIII domain. The core peptides represent a cluster of identified FVIII derived peptides. The core peptide with the highest predicted affinity was selected when multiple core peptides for the same cluster of peptides were predicted. *Online Supplementary Figure S3* provides an overview of all peptides identified. Subsequent columns depict which peptides are found for each donor for cells pulsed with FVIII or FVIII/VWF complex. Green: FVIII peptide detected following incubation with FVIII; yellow: FVIII peptide detected following incubation with FVIII and FVIII/VWF complex; red: FVIII peptide detection following incubation with FVIII/VWF; arrow: FVIII peptide not detected following incubation with FVIII/VWF.

of 1:5. After uptake, maturation and lysis of the cells, the MHC class II complexes were purified and the eluted peptides were identified, as described above. Figure 5 shows that a wide range of FVIII peptides was detected in samples pulsed with FVIII alone or with FVIII/VWF complex. In addition, under these conditions, VWF modulated the repertoire of FVIII peptides presented on MHC class II. Taken together these data indicate that, although a reduction in FVIII endocytosis by iDCs is observed, when iDCs are pulsed with FVIII/VWF complex there is still an appropriate presentation of FVIII-derived peptides in the presence of VWF.

Presentation of VWF-derived peptides on MHC class II molecules

To investigate whether iDCs are able to present VWF-derived peptides, iDCs were pulsed with either 250 nM of VWF alone or in complex with FVIII at a FVIII:VWF ratio of 1:5 (donors A, B and C) or 1:10 (donors D and E). After uptake and maturation of iDCs, cells were lysed and the MHC class II complexes were purified. The eluted peptides were analyzed by mass spectrometry. No VWF-derived peptides were detected upon incubation with VWF alone (Figure 6), although a similar amount of endogenous peptides was eluted from the MHC class II

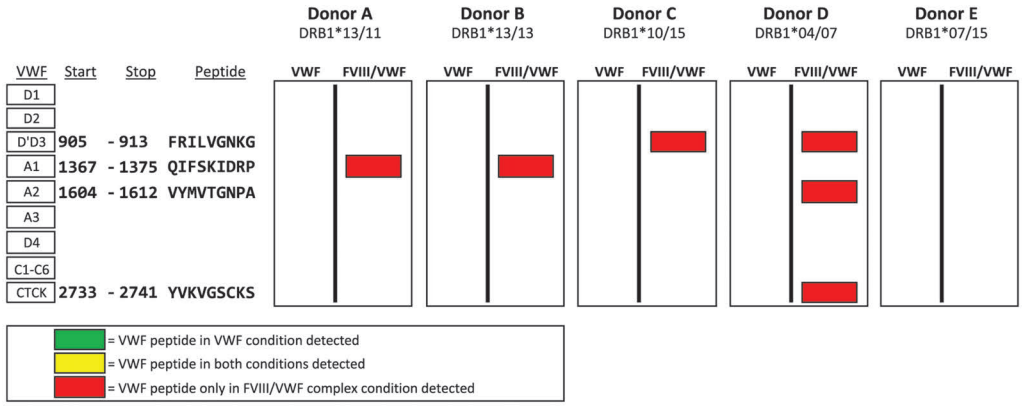

Figure 6. VWF-derived MHC class II core peptides identified from donors A-E. Cells from donors A to E were pulsed with 250 nM VWF or complex of 25 nM FVIII with 250 nM VWF. After maturation MHC class II ligands were extracted and analyzed using mass spectrometry. VWF specific peptides were identified using Proteome Discoverer and subsequently HLA-DRB1 genotype specific core peptides were predicted using NetMHCpan 2.8. The first column shows predicted core peptides with corresponding residue numbers and corresponding VWF domain. Predicted core peptides are used to indicate the location of VWF-derived peptides identified. Subsequent columns depict which peptides are found for each donor for cells pulsed with VWF only or cells pulsed with FVIII/VWF complex. Green: VWF peptide detected following incubation with VWF; yellow: VWF peptide detected following incubation with VWF and FVIII/VWF complex; red: VWF peptide detection following incubation with FVIII/VWF.

molecules (*Online Supplementary Figure S2*). These results are consistent with the observation that VWF is not internalized by iDCs. Strikingly, when iDCs were pulsed with FVIII/VWF complex at a ratio of 1:5 (donors A, B and C) or 1:10 (donors D and E), VWF peptides were identified in 4 out of 5 donors (Figure 6). Donors A and B present peptides derived from the VWFA1 domain of VWF while donors C and D presented a single peptide derived from the VWFD3 domain. Donor D also presents two other peptides from the VWFA2 and CTCK domain, respectively, while for donor E, no VWF-derived peptides were identified. Taken together these data suggest that VWF, when in complex with FVIII, is internalized and can subsequently be presented on MHC class II molecules. The number of VWF peptides identified suggests that only a limited amount of VWF is being internalized under these conditions.

Discussion

Formation of inhibitory antibodies in hemophilia A patients can lead to serious and even life-threatening complications.[1] Endocytosis of FVIII by antigen-presenting cells is the initial step in this process, which can eventually lead to the development of long-living plasma cells which produce antibodies against FVIII.[25] While VWF has been shown to modulate FVIII endocytosis by DCs, to our knowledge, no data regarding the interaction of VWF with iDCs have been described. Dendritic cells have robust antigen-presenting capacity due to the wide variety of endocytic mechanisms that are operational in these cells.[26,27] Both receptor-mediated endocytosis, driven by ligand-binding to specific receptors, as well as non-specific internalization pathways such as macropinocytosis, have been shown to contribute to the efficient sampling of antigens from their environment.[28] Unexpectedly, despite its binding to the cell surface, VWF is not internalized by iDCs. The lack of internalization of VWF is consistent with the observation that VWF-derived peptides are not presented on MHC class II (Figure 5). To our knowledge, this provides a unique property of VWF since the majority of antigens are rapidly and efficiently endocytosed by iDCs. Based on the clusters of VWF that were observed by confocal microscopy, we speculate that VWF binds to discrete domains on the plasma membrane which apparently prohibit its endocytosis. Several cell surface receptors have previously been implicated in clearance of VWF (see review by Lenting *et al.*[29]). Recently, the C-type lectin receptor CLEC4M, the carbohydrate receptor Siglec-5 and scavenger receptor class A member 5 (SCARA5) have been shown to interact with VWF.[30-32] Interestingly, transfected HEK293 cells over-expressing CLEC4M or Siglec-5 were shown to efficiently internalize VWF.[30-32] The lack of VWF internalization observed in iDCs suggests that CLEC4M, Siglec-5 and SCARA5 are not involved in binding of VWF to dendritic cells.

It has been established that VWF is predominantly cleared from the circulation by macrophages in spleen and liver.[33,34] These observations have been confirmed using *in vitro* assays, which have demonstrated that shear stress is critical for endocytosis of VWF by human monocyte-derived macrophages.[35] It is still unclear how shear stress affects VWF uptake. Shear stress may induce changes in cell-surface receptors present on macrophages; alternatively, it may induce conformational changes in VWF, thereby allowing for the exposure of structural determinants necessary for its uptake.

As mentioned previously, VWF-derived peptides are not presented upon incubation of iDCs with increasing concentrations of VWF. Unexpectedly, upon incubation with FVIII/VWF complex a limited set of VWF-derived peptides was presented on MHC class II in 4 out of 5 donors analyzed (Figure 6). These data indicate that, in the presence of FVIII, small amounts of VWF are being internalized and processed for presentation on MHC class II. This suggests that FVIII facilitates VWF endocytosis by DCs. We speculate that VWF is co-internalized through its ability to inter-

act with FVIII. Development of allo-antibodies to VWF in response to replacement therapy occurs in approximately 5%-10% of the patients with severe von Willebrand disease.[30] Our knowledge on the etiology and characteristics of this unwanted immune response remains limited.[36] Genetic studies have shown that allo-antibody development to VWF occurs in patients with deletions, frameshift and nonsense mutations.[37] It is likely that internalization of VWF by antigen-presenting cells is needed to allow CD4+ T cells to recognize VWF-derived peptides presented on MHC class II. Our study is the first to define which VWF-derived peptides are being presented on MHC class II. Peptides derived from the D'D3 domain assembly, the A1 and A3 domain and the carboxy-terminal CTCK domain were identified in this study. In view of its large size, the number of VWF-derived core peptides presented on MHC class II appears to be limited. This may be related to the lack of internalization of VWF by iDCs. We speculate that this may contribute to the low frequency of allo-antibody development in patients with type 3 von Willebrand disease.

In agreement with results from a previous study, we show that internalization of FVIII by dendritic cells is reduced in the presence of VWF.[13,18] However, our data also show that VWF modulates the repertoire of FVIII-derived peptides presented on MHC class II. Overall, the number of FVIII peptides presented on MHC class II is reduced in the presence of VWF; depending on the donor analyzed, 1-10 peptides are not identified in the presence of VWF (Figures 4 and 5). This subset includes peptides containing the core sequence FIIMYSLDG, which has been identified as a promiscuous CD4+ T-cell epitope in the C1 domain of FVIII.[38] In addition, peptides with core sequence FRNQASPRY (A3 domain; residues 1766-1786) are presented less efficiently in the presence of VWF. Interestingly, this peptide is presented by multiple MHC class II alleles[9,10] and has also been described as a functional T-cell epitope in humanized E17 HLA-DRB1*1501 mice.[39] These observations raise the possibility that VWF modulates CD4+ T-cell responses by affecting FVIII peptide presentation by antigen-presenting cells. The potential modulating effect of VWF on FVIII immunogenicity has been the subject of intense discussion.[40] While hemophilia A patients have normal levels of VWF and are treated with VWF containing FVIII products, they can still develop inhibitors. This is in agreement with our data, as we show that even though FVIII endocytosis by iDCs is significantly reduced in the presence of VWF, it is still sufficient to allow for appropriate presentation of FVIII-derived peptides on MHC class II. We obtained evidence that the repertoire of FVIII peptides presented on MHC class II is modulated by VWF. Whether this effect translates into a potential benefit for FVIII/VWF concentrates when compared to highly purified plasma-derived or recombinant FVIII concentrates remains to be established.

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