Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background

The development of factor VIII (FVIII) inhibitors remains the major hurdle in the clinical management of patients with hemophilia A. FVIII uptake by professional antigen-presenting cells (APC) is the first step involved in initiation of immune responses to FVIII. Studies on FVIII catabolism have highlighted the role played by CD91/LRP as a potential target for increasing FVIII half-life in patients and prolonging treatment efficiency. We investigated the involvement of CD91 in FVIII endocytosis by human dendritic cells (DC), a model of professional APC.

Design and Methods

Immature DC were generated from circulating monocytes from healthy donors. Surface expression of CD91 was assessed by flow cytometry. Uptake of fluoroscein isothiocyanate-conjugated ligands by immature DC was studied in the presence of various blocking agents.

Results

CD91 was expressed on approximately 20% of DC and mediated the internalization of its model ligand, α2-macroglobulin. DC internalized FVIII and activated a human FVIII-specific T-cell clone in a dose-dependent manner. FVIII uptake by DC and subsequent T-cell activation were not inhibited by receptor-associated protein.

Conclusions

Our results indicate that CD91 and other members of the LDL receptor family are not strongly implicated in FVIII internalization by monocyte-derived DC, and suggest the involvement of alternative divalent ion-dependent endocytic receptors.

Key words: hemophilia, factor VIII, inhibitors, dendritic cells, T-cell clone, CD91/LRP.

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Introduction

Factor VIII (FVIII) replacement therapy in patients with hemophilia A results, in up to 25% of the cases, in the development of anti-FVIII alloantibodies that inhibit FVIII procoagulant activity. The development of FVIII inhibitors precludes further therapeutic use of FVIII and remains a major therapeutic challenge. It has been documented that FVIII-specific $CD4^+$ T lymphocytes are involved in the initiation of the humoral immune response to exogenous FVIII.[1,2] In view of their capacity to stimulate naïve T cells, dendritic cells (DC) play a central role in initiation of the primary immune response.[3] Thus, in the context of a primary alloimmunization against FVIII, i.e. when FVIII-specific B lymphocytes are not yet present to internalize FVIII from the circulation and serve as antigen-presenting cells (APC), DC are the major cell type that activate specific $CD4^+$ T lymphocytes.

The low density lipoprotein (LDL) receptor-related protein (LRP), also known as the α2-macroglobulin receptor or CD91, is a member of the LDL receptor family of endocytic receptors that mediates the binding of multiple ligands and their transport from the cell surface to the endosomal compartment.[4] CD91/LRP expressed on transfected fibroblasts mediates at least 50% of FVIII uptake and degradation.[5,6] FVIII catabolism by LRP is facilitated by heparan sulfate proteoglycans (HSPG).[7] Inactivation of the CD91 gene in conditional LRP-deficient mice confirmed the relevance of LRP for clearance of circulating FVIII.[8] The involvement of CD91 in FVIII uptake has been considered with respect to the half-life of therapeutically administered FVIII. CD91-targeted modulation of FVIII clearance has thus been proposed as an approach to prolong the efficiency of replacement treatments. However, its role in the internalization and processing of FVIII for presentation by APC is unknown.

Alloimmunization against exogenous FVIII represents the major hurdle of hemophilia treatment. We hypothesized that, in patients infused with FVIII, the CD91/LRP-dependent endocytic pathway plays an important role in FVIII uptake by professional APC, and is thus critical for the primary events leading to the anti-FVIII immune response. We, therefore, investigated the presence of functional CD91 at the surface of monocyte-derived human DC, and examined its capacity to mediate FVIII endocytosis and FVIII-specific T-cell activation.

Design and Methods

Proteins and antibodies

Methylamine-treated (activated) α-2-macroglobulin-fluoroscein isothiocyanate (α2M-FITC), CD91 and anti-CD91-FITC antibodies were purchased from Biomac (Leipzig, Germany). Receptor-associated protein (RAP) was obtained from Molecular Innovations, Inc. (Kordia Life Sciences, Leiden, The Netherlands).

Fluorescein conjugation of FVIII

Recombinant human FVIII (1000 IU, Kogenate SF, Bayer) was dialyzed against bicarbonate buffer (pH 9.2) containing 5 mM $CaCl_2$ at 4°C for 2 h, followed by coupling with FITC for 7-8 h at 4°C. Unconjugated FITC was removed by dialyzing against RPMI-1640 media. The specific activity of FVIII-FITC was >4000 IU/mg, as assessed using a chromogenic assay (Dade-Behring), confirming the preservation of the structural and functional integrity of FVIII following FITC conjugation.

Radioactive conjugation of FVIII

Recombinant human FVIII (1000 IU, Kogenate SF, Bayer) was dialyzed against phosphate-buffered saline (PBS) at 4°C for 2 h. It was then labeled with ^{125}I to a specific activity of 915 cpm/fmole, using the iodogen method. Free iodine was removed by desalting on a PD-10 column equilibrated with X-VIVO^{15}-2% bovine serum albumin (BSA).

Generation of dendritic cells

Monocytes from peripheral blood mononuclear cells (PBMC) were isolated by adherence from heparinized buffy coats of healthy donors or from blood samples from two patients with severe hemophilia A (aged 8 years and 14 years, both inhibitor-positive) in RPMI 1640-10% human AB serum, glutamine and antibiotics. Samples were obtained in accordance with the local ethical regulation. Adherent monocytes were cultured in X-VIVO^{15}-1% human AB serum, in the presence of 500 IU/10^6 cells, interleukin-4 (R&D Systems, Lille, France) and 1000 IU/10^6 cells granulocyte-monocyte colony-stimulating factor (ImmunoTools, Friesoythe, Germany). After 5 days of culture, the non-adherent DC-enriched fraction was harvested. The immature phenotype and CD91 expression of 5-day old DC were assessed by FACS using appropriate FITC and phycoerythin (PE)-labeled antibodies (*Supplementary Figure 1*). DC were >90% pure.

Labeling of dendritic cells by conjugated ligands

DC (4×10^5 cells/well) were incubated in 100 μL of X-VIVO^{15} with fluorescent conjugated ligands (FVIII-FITC, α2M-FITC), or with ^{125}I-FVIII, for 15 to 120 min at 4°C and 37°C. To confirm the involvement of endocytic receptors in ligand uptake, cells were incubated for 30 min at 37°C with a 100 molar excess of RAP, anti-LRP antibodies or 5 mM EDTA, prior to the addition of conjugated ligands. Cells were washed and the labeling of the cells by the conjugated ligands was analyzed by FACS or using a γ-counter. In the case of ^{125}I-FVIII, the incubation medium was X-VIVO^{15}-2% BSA. At 20 nM FVIII, the amounts of ^{125}I-FVIII fixed on DC at 37°C and

4°C were 37.0±0.8 fmoles and 6.2±0.5 fmoles per 1×10^6 cells, respectively.

Labeling of mouse embryonic fibroblasts by FVIII-FITC

Mouse embryonic fibroblasts (MEF) were seeded at 2.2×10^4 cells/well onto flat-bottomed, 96-well plates and grown in DMEM containing 10% fetal calf serum (FCS) to 90% confluency. MEF, pre-incubated for 30 min in medium alone or with a 100 molar excess RAP, were incubated in 100 μL medium containing 1% BSA with FVIII-FITC for 5 h at 37°C. The wells were gently washed with PBS and 50 μL of water were added to each well and incubated for 30 min to lyse the cells. Fluorescence was then measured using a spectrofluorometer (TECAN, excitation at 492 nm and emission at 535 nm). Autofluorescence was subtracted.

Activation of a FVIII-specific T-cell clone

DC were generated from monocytes from a healthy donor (MHC haplotype: DRB1*1501/DRB5*01) in RPMI-1640 supplemented with 10% FCS. Five-day old DC were resuspended in DMEM:F12 (1:1) media containing 10% FCS and were distributed (10,000 cells/well) in 96-well round-bottomed, cell culture plates. DC were cultured with 5000 T cells (a human FVIII-specific $CD4^+$ T-cell clone, D9E9) in DMEM:F12 (1:1) medium containing 10% FCS and 20 U/mL recombinant human interleukin-2 (Sigma), with FVIII (36 nM or 10 μg/mL, 20 nM, 10 nM or 5 nM) or FVIII-FITC (2, 10 or 40 μg/mL) for 20 h at 37°C. Production of interferon-γ was measured in the supernatants using the human interferon-γ Duo Set (DY285, R&D Systems). When indicated, DC were pre-incubated with a 100 molar excess of RAP for 30 min at 37°C. Controls included: T cells incubated alone, or T cells incubated with DC alone or in the presence of human recombinant factor IX (36 nM, Benefix, Baxter).

Results

Immature monocyte-derived DC express functional CD91

Five day-old immature DC revealed heterogeneous expression of CD91 (Figure 1A), which was high on 20.4±9.9% of $CD11c^+$ DC (ranging from 5.9 to 42.3; mean fluorescence intensity [MFI]: 251.1±109.1 in the case of 13 healthy donors). We explored the endocytic potential of CD91 on DC, using activated α2M as a model ligand for CD91. Labeling of DC with α2M-FITC was dose-dependent, with an optimal concentration of 100 μg/mL of α2M following 2 h of incubation (Figure 1B). Labeling by α2M-FITC was dependent upon time and temperature (Figure 1C). Large ΔMFI values between 37°C and 4°C (160.1 and <20 MFI for 10 μg/mL α2M-FITC, respectively) demonstrate active endocytosis of α2M by DC. RAP is an inhibitor of receptors belonging to the family of LDL receptors, and is commonly used to substantiate CD91 as an endocytic receptor.[5,6,9] A 100 molar excess of RAP abrogated α2M endocytosis by DC (Figure 1D), thus confirming the involvement of CD91 in α2M internalization.

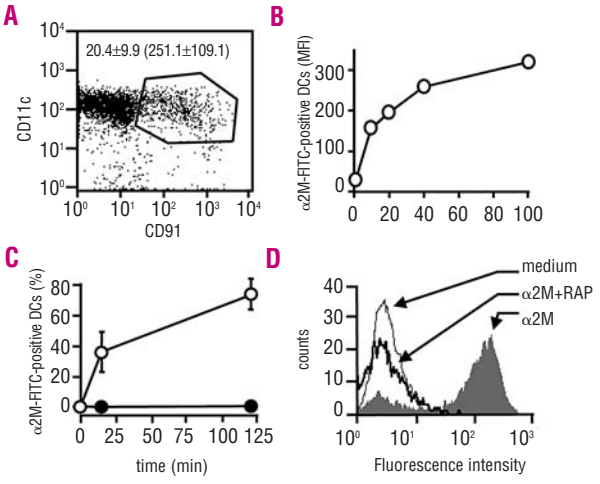

Figure 1. Functional expression of CD91 by immature monocyte-derived DC. A. DC were stained for surface expression of CD91 on CD11c-positive cells (gating indicated). Mean values of percentage (and mean fluorescence intensity) of $CD11c^+CD91^+$ cells ± SD, were calculated for DC from 13 healthy donors. B. α2M-FITC was incubated at various doses with DC in X-VIVO[15] for 2 hr at 37°C. Values depict the MFI measured among α2M-FITC-positive cells by FACS. C. DC were incubated with 10 μg/mL α2M-FITC at 4°C (closed circles) and 37°C (open circles) for 15 and 120 min (mean ± SD from experiments with three healthy donors). D. RAP (1.39 μM) was incubated with DC for 30 min at 37°C prior to the addition of α2M-FITC (10 μg/mL, 0.0139 μM) for an additional 120 min. Data are from one representative experiment of three independent experiments.

Internalization of FVIII by DC leads to the activation of FVIII-specific $CD4^+$ T cells

The kinetics and molecular mechanisms underlying the endocytosis of FVIII by APC have not been described as yet. Internalization of FVIII by DC was studied using FITC-conjugated FVIII. We first confirmed that conjugation of FVIII with FITC does not alter the structure of FVIII; FITC-conjugation of FVIII did not modify the clotting factor's interaction with a series of monoclonal anti-FVIII antibodies (Figure 2A) or with von Willebrand factor;[10] CD91 bound to FVIII-FITC to a similar extent as to native FVIII, as assessed using an enzyme-linked immunosorbent assay (Figure 2B); furthermore, the MEF cell line, which expresses surface CD91, was labeled by FVIII-FITC in a dose-dependent manner (Figure 2C).

Incubation of FVIII-FITC with DC resulted in a time- and dose-dependent increase of both labeling intensity and percentage of labeled cells at 37°C (Figures 3A, 3B and *data not shown*). Optimal FVIII endocytosis occurred following 2 h of incubation. Confocal microscopy confirmed that FVIII was internalized by DC (*data not shown*). At 4°C, 40 μg/mL FVIII resulted in baseline labeling of 25 to 35 MFI, which was stable between 15

and 120 min of incubation (Figure 3B), thus suggesting saturation of the membrane-exposed receptors for FVIII after 15 min of incubation.

We validated that FVIII endocytosis by DC resulted in presentation of FVIII-derived peptide to T lymphocytes. For this purpose, DC from MHC-matched donors were incubated with the human FVIII-specific $CD4^+$ T-cell clone D9E9[11] in the presence of increasing amounts of FVIII. D9E9 was activated in a dose-dependent manner by FVIII-FITC, as assessed by the production of interferon-γ in the culture supernatant (Figure 3C). Interferon-γ was not detected when DC were incubated alone or when DC and D9E9 were incubated in the presence of an irrelevant antigen (Figure 3D). Importantly, DC loaded with FVIII and with FVIII-FITC activated D9E9 to a similar extent (Figure 3D), further supporting the fact that FITC-conjugation does not alter endocytosis and endosomal processing of FVIII.

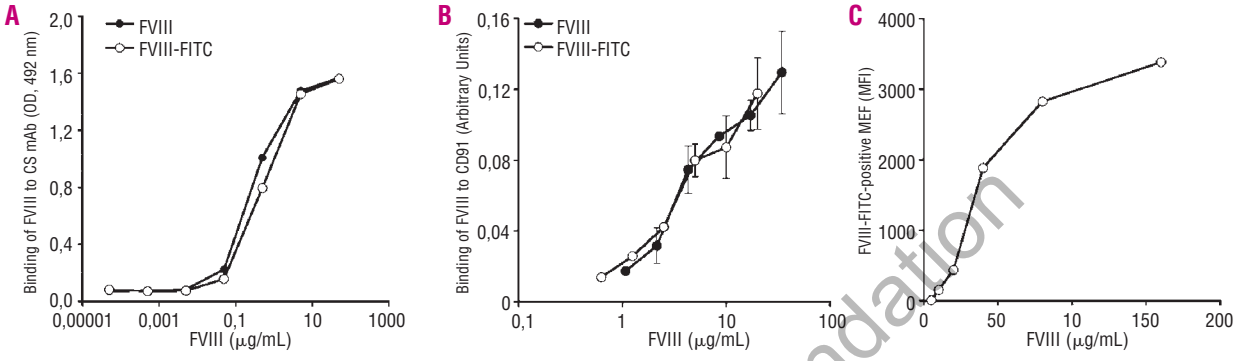

Figure 2. FITC conjugation of FVIII does not alter the binding properties of the clotting factor. A. FVIII and FVIII-FITC were coated onto enzyme-linked immunosorbent assay (ELISA) plates in serial dilutions (0 to 50 μg/mL). Following blockade of the plates with PBS-1% BSA, the anti-A1 IgG C5 (4 μg/mL) was incubated. Bound C5 was revealed using a polyclonal anti-murine IgG coupled to peroxidase and its substrate. Data representative of a series of monoclonal anti-FVIII antibodies. B. FVIII (full circles) and FITC-labeled FVIII (empty circles) were coated in serial dilutions (0.5 to 40 μg/mL) onto ELISA plates. Following blockade with TBS-3% BSA, CD91 (10 μg/mL, Biomac, Leipzig, Germany) was incubated in TBS-Tween 20 containing 5 mM $CaCl_2$ and 3% BSA. Fixed CD91 was then revealed using a monoclonal anti-CD91 IgG (10 μg/mL, Biomac) and polyclonal rabbit anti-murine IgG coupled to horse-radish peroxidase (Jackson Research Laboratories). The non-specific binding of CD91 to uncoated wells was subtracted from all values. Results are shown as arbitrary units. Error-bars depict the variation between duplicates in a single experiment. The data are representative of two independent experiments. C. Mouse embryonic fibroblasts (MEF) positive for the expression of CD91/LRP were seeded at 2.2×10^4 cells/well onto flat-bottomed 96-well plate and were grown in DMEM media containing 10% FCS to 90% confluency. FVIII-FITC at different doses (0, 5, 10, 20, 40, 80 and 160 μg/mL) was incubated in medium containing 1% BSA for 5 h. After gentle washing, cells were lyzed by addition of 50 μL of water. Fluorescence was measured using a spectrofluorometer (TECAN, excitation at 492 nm and emission at 535 nm). Autofluorescence was subtracted. The data shown are from one representative experiment.

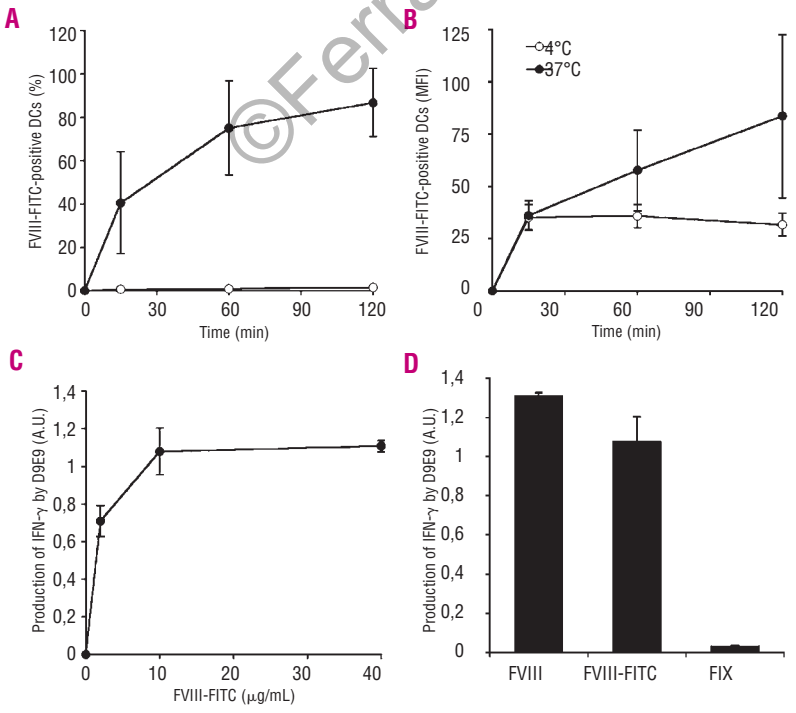

Figure 3. Endocytosis of FVIII by monocyte-derived DC and subsequent activation of FVIII-specific T cells. A and B. Five-day-old immature human monocyte-derived DC were incubated in X-VIVO[15] with FVIII-FITC (0.143 μM, 40 μg/mL) for 15, 60 and 120 min at 4°C (open circles) or at 37°C (closed circles). Values in panel A depict the percentage of FVIII-FITC-positive cells. Panel B depicts MFI measured among FVIII-FITC-positive cells. C and D. DC (10,000 cells/well) were generated from DRB1*1501/DRB5*01 healthy blood donors and incubated with FVIII-specific D9E9 T cells (5,000 cells/well) in the presence of unlabeled FVIII (0.036 μM or 10 μg/mL), FVIII-FITC (2, 10 or 40 μg/mL) or human recombinant FIX (0.036 μM), and 20 UI/mL recombinant human interleukin-2 (rhIL-2) for 20 hr at 37°C. The production of interferon-γ was assessed by ELISA.

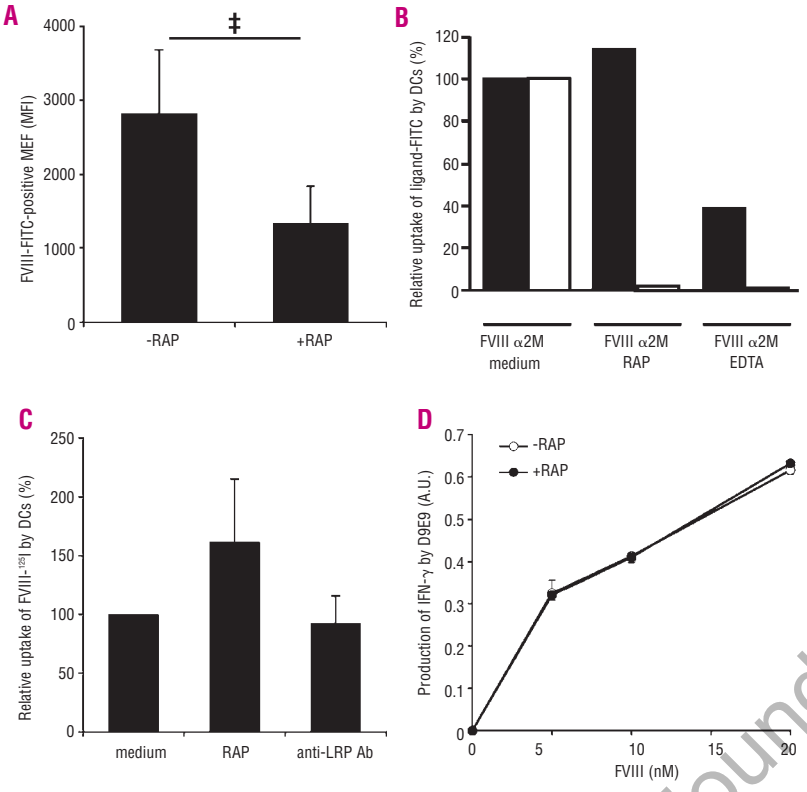

Figure 4. CD91 does not mediate FVIII endocytosis by monocyte-derived DC. **A.** MEF were seeded at 2.2×10^4 cells/well onto 96-well plates and grown in DMEM containing 10% FCS to 90% confluency. Following a 30 min pre-incubation of MEF with RAP (28.6 μM) at 37°C, FVIII-FITC (286 nM) was added to the cells and incubated for 5 h. Differences were assessed for significance using the Mann-Whitney test. The depicted data are from one representative experiment. **B.** DC were seeded at 4×10^5 cells/well. Uptake of FVIII-FITC (0.143 μM) and α2M-FITC (0.0139 μM) was studied for 120 min, following a 30 min pre-incubation period of DC in medium alone or in the presence of a 100 molar excess of RAP or 5 mM EDTA. The reported values depict the relative antigen uptake defined as $[(^{37^\circ C}MFI_{inh}\text{-}^{4^\circ C}MFI_{medium})/(^{37^\circ C}MFI_{medium}\text{-}^{4^\circ C}MFI_{medium})]\times100$, where *inh* stands for the MFI detected in the presence of the inhibitor. Data are from one representative of at least three independent experiments. **C.** DC were seeded at 4×10^5 cells/well. Following a 30 min pre-incubation of DC at 37°C alone, with RAP (2 μM) or with anti-LRP antibodies (2 μM), FVIII-^{125}I (20 nM) was added to the cells and incubated for 2 h. The reported values depict the relative FVIII uptake defined as $[(37^\circ C\,CPM_{inh}\text{-}4^\circ C\,CPM_{medium})/(37^\circ C\,cCPM_{medium}\text{-}4^\circ C\,cCPM_{medium})]\times100$, where *cpm* stands for counts per minute. **D.** 10,000 DC/well were pulsed with 0, 5, 10 or 20 nM FVIII following pre-incubation with or without a 100 molar excess of RAP. 5000 D9E9 cells were added to each well and co-cultured with the DC for 20 h. The production of interferon-γ was assessed by ELISA.

The endocytosis of FVIII by DC is not mediated by CD91

We then investigated whether CD91 is implicated in FVIII endocytosis. Internalization of FVIII-FITC by MEF, which naturally express surface CD91, was inhibited by pre-incubation of the cells in the presence of a 100 molar excess of RAP ($p<0.05$), as previously reported (Figure 4A).[6] However, a 100 molar excess of RAP did not prevent the endocytosis of FVIII-FITC by DC, while it completely abrogated that of α2M (Figure 4B). Under similar conditions, RAP did not inhibit the endocytosis of FVIII-FITC by DC from two patients with severe hemophilia A (*data not shown*). A combination of RAP and heparinase III had no effect of FVIII-FITC uptake (*data not shown*). Because the lack of inhibitory effect of RAP on the uptake of FVIII-FITC could be related to the elevated FVIII concentrations used in our assays, we repeated the experiments with 20 nM iodine-conjugated FVIII, as described previously.[5] Labeling of DC from healthy donors with ^{125}I-FVIII was dose-dependent (*data not shown*). Pre-incubation of DC with a 50 or 100 molar excess of RAP or with 100 molar excess of anti-LRP antibodies did not prevent the uptake of 20 nM ^{125}I-FVIII (Figure 4C). Furthermore, activation of D9E9 by DC loaded with native unconjugated FVIII was not reduced when an excess of RAP was added to the co-culture for 20 h (Figure 4D), confirming that the anti-endocytic role of RAP on FVIII, while significant to FVIII catabolism by non-immune cells, is negligible in the context of FVIII endocytosis by professional APC leading to activation of immune effectors.

Receptor-mediated endocytosis of antigens by DC involves both divalent ion-dependent and independent receptors. Co-incubation of DC with EDTA, which functionally blocks bivalent ion-dependent receptors such as CD91, abrogated α2M uptake, while it inhibited FVIII uptake by 70% (Figures 4B and 5). It is known that EDTA mediates the dissociation of FVIII subunits.[12] The inhibitory effect of EDTA was, however, not due to EDTA-induced FVIII dissociation, since dissociation of FVIII subunits requires a higher concentration of EDTA than that used in our assays (i.e., 5 mM), and a longer period of incubation.[12]

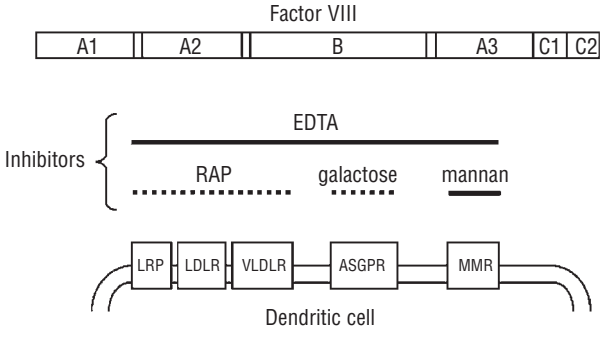

Figure 5. Internalization of FVIII by monocyte-derived DC involves divalent ion-dependent endocytic receptors. Endocytosis of FVIII by monocyte-derived DC is blocked (full lines) by EDTA and to a significant extent by mannan.[22] It is not blocked (dotted lines) by the presence of the competitive inhibitors RAP and galactose (*data not shown*).

Discussion

CD91/LRP is a catabolic receptor for FVIII. It is thus a plausible candidate as a specific receptor that mediates endocytosis of FVIII by APC, and may be involved in the elevated immunogenicity of therapeutically administered FVIII. In order to validate this hypothesis we used monocyte-derived DC as model APC, since DC represent the terminal stage of monocyte differentiation under physiological conditions both *in vitro* and *in vivo*.[13-15]

Here, we describe the kinetics of FVIII internalization by professional APC. APC internalize soluble antigens by different mechanisms, including receptor-mediated endocytosis, which requires only a few hours and, in most cases, is dependent on divalent ions. Incubation of FVIII with immature DC for 2h enabled a large majority of the cells to endocytose FVIII. In contrast, parallel control experiments performed with lucifer yellow, an established ligand for macropinocytosis, resulted in ligand endocytosis by only a marginal proportion of cells (*data not shown*). Furthermore, pre-incubation of the cells with 5 mM EDTA reduced FVIII uptake significantly, implying the involvement of a divalent ion-dependent endocytic pathway. Together with the dose-, time- and temperature-dependency of the endocytic process, our data are in favor of an active receptor-mediated internalization of FVIII. Importantly, we validated the functional presentation of FVIII-derived peptides on MHC II molecules to T lymphocytes.

CD91/LRP and other members of the LDL receptor family, such as VLDL,[16] have demonstrated affinity for FVIII in a RAP-sensitive manner. Expression of CD91 has been reported on various cell types including monocytes, macrophages and B lymphocytes.[17,18] In our experiments, the surface expression of CD91/LRP on human DC was found to be 20% of the $CD11c^+$ DC population at a given time point, while 2h of incubation were sufficient for α2M to be internalized by more than 70% of the cells, resembling results of previous studies[19] and illustrating the elevated turn-over of CD91.[4] CD91/LRP, like various other endocytic receptors, is essentially located intracellularly and is in constant flux between intracellular storage and surface exposure. Interestingly, the expression of CD91 at the surface of immature DC was enhanced up to 2-fold following incubation of the cells in protein-free medium alone for 30 min prior to analysis by flow cytometry (*data not shown)*, thus highlighting the probable contribution of serum proteins to LRP/CD91 internalization. Taken together, our data demonstrate the expression of CD91 on human immature DC and validate the CD91-dependent endocytic pathway in antigen uptake, as shown with α2M. Importantly, antigens endocytosed through CD91 are known to be presented to T lymphocytes in both an MHC I- and MHC II-restricted manner.[20,21]

The importance of CD91/LRP to FVIII entry into professional APC and to the associated FVIII immunogenicity remains elusive. Our data demonstrate that endocytosis of FVIII by DC is not inhibited by RAP or by polyclonal anti-LRP IgG, irrespective of the detection method employed (i.e., FITC- or ^{125}I-conjugated FVIII) or the concentration of FVIII used. Cell surface HSPG have been shown to assist CD91/LRP in FVIII catabolism.[7] A combination of RAP and heparinase III, a specific inhibitor of HSPG, also failed to block FVIII endocytosis by DC at all time points tested (*data not shown*). Furthermore, RAP did not prevent DC from internalizing, processing and presenting FVIII to T lymphocytes. The absence of RAP-sensitivity on FVIII uptake by DC was unchanged in the absence or presence of molar excess of von Willebrand factor (*data not shown*).

The failure of CD91 and related receptors of the LDL-receptor family to play a dominant role in FVIII endocytosis by DC may be explained by several non-exclusive factors: CD91 is targeted by multiple ligands that compete with each other for binding to the receptor;[4] in the case of a large multivalent antigen such as FVIII, internalization may involve a series of alternative endocytic receptors specific for various glycoprotein moieties of the molecule (Figure 5). Indeed, our recent results confirm the involvement of divalent ion-dependent mannose-sensitive receptors in the internalization of FVIII by monocyte-derived DC and subsequent presentation to $CD4^+$ T lymphocytes.[22] Taken together, our results indicate that CD91 and other members of the LDL receptor family cannot be major therapeutic targets for reducing the immunogenicity of exogenous FVIII.

Authorship and Disclosures

All authors at UMR S 872 participated in the design of the study, generation and analysis of the data, discussion and writing of the manuscript. ES, JMS-R and MJ contributed essential material. The authors reported no potential conflicts of interest.

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