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PHOSPHATIDYLSERINE REDUCES IMMUNE RESPONSE AGAINST HUMAN RECOMBINANT FVIII IN HEMOPHILIA A MICE BY REGULATION OF DENDRITIC CELL FUNCTION

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Abstract

A major clinical complication in the treatment of Hemophilia A using exogenously administered recombinant Factor VIII (FVIII) is the development of neutralizing antibodies. It has been shown previously that FVIII complexed with phosphatidylserine (PS) reduces the development of total and neutralizing antibody titers in hemophilic mice. The effect of complexation of FVIII with PS upon dendritic cell (DC) uptake, maturation and processing, T-cell proliferation and cytokine secretion profiles was investigated. Flow cytometric studies of DC showed that PS inhibited the up-regulation of cell surface co-stimulatory markers (CD86 and CD40). PS reduced T-cell proliferation and increased significantly levels of TGF- β and IL-10 but reduced secretion of IL-6 and IL-17 compared to controls. The data suggest that PS reduces immunogenicity of FVIII by regulating dendritic cell maturation and subsequent T-lymphocyte activity through modulation of cytokine secretion. A possible mechanism for PS-mediated induction of FVIII tolerance is discussed.

Keywords

Immunogenicity; Phosphatidylserine; Tolerance; Factor VIII; Regulatory T cell; Tolerogenic dendritic cell

Introduction

Hemophilia A is a bleeding disorder caused by the deficiency or dysfunction of Factor VIII (FVIII), a multi-domain glycoprotein comprised of six domains [1] that are cleaved during processing to the final form, consisting of a heavy (A1-A2-B) and light chain (A3-C1-C2) [1,2] that are bound together by divalent cations [3]. Replacement therapy employing recombinant FVIII is the first line treatment for Hemophilia A. A major clinical challenge is the development of neutralizing antibody responses that can abrogate the activity of the protein, which occurs in 15–30% of patients [4]. The epitope regions that elicit antibody response are well studied, and it has been determined that the immunodominant epitopes are

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located in the A2, A3, and C2 domains [4–6]. Based upon immunoprecipitation and inhibitor neutralization assays, it was found that anti-light chain antibody titers were the highest [7,8]. This finding is consistent with the presence of several CD4⁺ immunodominant epitopes in the C2 domain of the light chain [8,9]. The C2 domain also contains the lipid binding region, which binds to phosphatidylserine (PS) on the platelet membrane [10].

Because the PS-binding C2 domain also contains a CD4⁺ immunodominant epitope, we hypothesized that lipid binding might alter its conformation and/or shield the epitope region, leading to a reduction in its immunogenicity. Complexation of FVIII with O-phospho-L-serine (OPLS), which comprises the headgroup moiety of PS, or with PS-containing liposomes, improved its stability and reduced its immunogenicity in Hemophilia A mice [11,12]. A role for PS in reducing immunogenicity receives support from the observation that PS exposed on the outer leaflet of apoptotic cells reduces inflammatory and adaptive responses against self-antigens ([13] and references therein). Here, we carried out mechanistic studies in culturing conditions to investigate the immunological significance of reduction in immunogenicity of FVIII by PS. The effect of complexing with PS upon presentation and processing of FVIII by antigen presenting cells (APC), as well as T-cell proliferation following FVIII challenge and cytokine secretion was investigated. The results suggest that PS reduces immunogenicity of FVIII by regulating the maturation of DC and subsequent lymphocytic activity through an effect upon the profile of cytokines elicited.

Materials

Full-length, purified, excipient-free FVIII (Advate®, Baxter Biosciences, Carlsbad, CA) was obtained as a gift from the Western New York Hemophilia A Foundation. Normal plasma (control), Brain phosphatidylserine (PS), dimyristoylphosphatidylcholine (PC), dimyristoylphosphatidylglycerol (PG) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL) and stored in chloroform at –80°C. They were used without further purification. Sterile, pyrogen-free water and Isoflurane were purchased from Henry Schein Inc. (Melville, NY). O-phospho-L-serine (OPLS), phosphocholine (PChg), O-phospho-D-serine (OPDS), IgG-free bovine serum albumin (BSA), sodium pyruvate, Tween 20, potassium iodide, hydrogen peroxide, phosphorus solution standards and sephadex G75 were obtained from Sigma (Saint Louis, MO). Endosafe-Endochrome-K[®] endotoxin testing kit was purchased from Charles River Inc. (Cambridge, MA). All other buffer salts used in the study were purchased from Fisher Scientific (Fairlawn, NJ). Hydroxypyrene trisulfonate (HPTS) and buffer salts were purchased from Fisher Scientific Inc. (Hanover Park, IL). The Dynal CD4⁺ negative isolation kit, RPMI-1640 culture medium, penicillin, streptomycin, L-glutamine, 2-mercaptoethanol, and Polymyxin B were obtained from Invitrogen Inc., (Carlsbad, CA). ³H-thymidine and Unifilter 96-well plates were obtained from Perkin Elmer Inc. (Boston, MA). Maxisorp 96-well ELISA plates and 6-well flat-bottom sterile plates were purchased from NUNC (Rochester, NY). TGF-β1, IL-10, IL-6, IL-17, TNF-α and IL-12 development kits were obtained from R&D Systems (Minneapolis, MN). Recombinant murine Granulocyte/Macrophage Colony-Stimulating Factor (rmGMCSF) was obtained from Peprotech (Rocky Hill, NJ) and heat-inactivated Fetal Bovine Serum was purchased from Lonza (Basel, Switzerland). BD Fc-block[™], anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, and corresponding isotype control antibodies were purchased from BD Biosciences (San Jose, CA). Anti-MHC-II-FITC, anti-CD11c-FITC, and corresponding isotype control antibodies were purchased from eBioscience (San Diego, CA).

Methods

Preparation of FVIII-PS complexes

Preparation of liposomes and association with FVIII—PS and PC in chloroform were mixed in a 30:70 molar ratio and the chloroform was removed using a rotary evaporator (Buchi Labs, Switzerland), forming a thin lipid film. The molar ratio of PS was kept below 50% to prevent liposome aggregation and to ensure physical stability of liposomes in the calcium containing buffer system [14]. The film was rehydrated using a sterile Ca^{2+} -containing Tris buffer (150 mM NaCl, 25 mM Tris, 5 mM CaCl_2 , pH 7.0) and the solution was extruded multiple times through 200 nm pore-size polycarbonate membranes using a nitrogen gas extruder (Northern Lipids Inc., Burnaby, Canada). The liposomes were sterile-filtered using a 0.22 μm syringe filter. Lipid recovery was determined by the method of Bartlett [15]. FVIII was associated with the liposomes as described previously [16]. The protein:lipid ratio was 1:10,000 (mol:mol) and the mixture was incubated at 37°C for 30 min to achieve association. This procedure yields an association efficiency of 45±16%, and the interaction is mediated by lipid binding region of the protein [16]. Control liposomes of PC (PC:cholesterol, 100:5 mol ratio) and phosphatidylglycerol (PG) (PG:PC:cholesterol, 50:50:5 mol ratio) were prepared using the same procedure as for PS liposomes.

Preparation of FVIII – OPLS/PChg/OPDS complexes—The FVIII-OPLS complex was prepared by diluting FVIII stock solution in a buffer composed of 10 mM OPLS, 300 mM NaCl, 25 mM Tris, 5 mM CaCl_2 and, pH 7.0. The solution was incubated at room temperature for 30 min. The FVIII-PChg and FVIII-OPDS complexes were prepared in the same manner except that 10 mM PChg or 10mM of OPDS was substituted for OPLS. The solutions were prepared using pyrogen-free water and sterile filtered prior to use.

Endotoxin test

All formulations were tested for the presence of endotoxin using the Endosafe Endochrome-K[®] kit (Charles River, MA). Endotoxin levels were below detection limit in all formulations.

Isolation and characterization of dendritic cells

Dendritic cells were isolated from the bone marrow of hemophilic mice by the procedure of Lutz [17]. The prolonged culturing conditions reduce granulocyte contamination and yield about 80% of DC. In order to verify the differentiation of bone marrow cells into DC, the expression of the DC surface markers MHC-II, CD11c, CD40, CD80, and CD86 were monitored using fluorescently-tagged antibodies and flow cytometry. Briefly, on day 10 the cells were harvested, washed twice with ice cold sterile PBS and resuspended at approx. 1×10^6 cells/ml. One mL of cells was dispensed into flow-cytometry tubes and centrifuged at 1000 g for 3–5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in the residual (~100 μL) volume. BD Fc-block antibody was added and incubated for 10 min on ice. An appropriate volume of different fluorescently-tagged antibodies was added to parallel samples and incubated for 15 minutes on ice in dark. The appropriate isotype antibody controls were also included in the study. After incubation, 4 ml of ice cold PBS was added and the cells were centrifuged at 1000 g for 3–5 minutes at 4°C. The supernatant was discarded and 0.5 ml of 2% ultrapure paraformaldehyde was added. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Characterization of DC—To confirm presence of immature DC in the culture, on day 9, the DC were harvested and stimulated for 24h with lipopolysaccharide (LPS) and the cell

surface markers were evaluated by flow cytometry as described above. The fraction of DC in the culture was estimated by measuring the shift in the flow cytometry peak before and after LPS addition.

DC exposure to free FVIII and FVIII-lipid formulations

In order to assess the direct effect of FVIII and FVIII-lipid formulations on DC, Day 9 naïve bone marrow-derived DC from hemophilic mice were incubated either with free FVIII or FVIII-PS/PC/PG in 24-well plate in a humidified cell-culture incubator at 37°C for 24 hr. Plain DC served as the negative control. The cells were thoroughly washed and 1×10^6 cells were labeled with fluorescently-tagged antibodies and analyzed for cell-surface expression of MHC-II, CD80, CD86 and CD40 markers by flow-cytometry. In a separate experiment involving direct exposure of DC isolated from hemophilic mice either with FVIII or FVIII-lipid formulations, the cell-culture supernatants were collected and analyzed for the presence of TNF- α and IL-12 cytokines using the respective development ELISA kits (R&D systems, Minneapolis, MN).

Liposome-Dendritic Cell Interaction

Preparation of fluorescent liposomes—Thin lipid films of 10 μ mol total lipid were prepared as described above and rehydrated with vigorous vortex mixing in Tris buffer (140 mM NaCl, 25 mM Tris, pH 7.4) containing 35 mM hydroxypyrene trisulfonate (HPTS), which is a fluorescent pH-sensitive dye that has been used to monitor liposome endocytosis [18]. The resulting vesicles were extruded through double-stacked polycarbonate membranes having a pore diameter of 200 nm. Unencapsulated HPTS was removed by size exclusion chromatography using a Sephadex G-75 column (1 X 30 cm) equilibrated with NaCl-Tris buffer.

DC uptake—DC were harvested on day 8 of culture by centrifugation at 300 g for 10 min at 4°C. The cell pellet was resuspended in DC media (RPMI-1640, Penicillin-Streptomycin, 2-mercaptoethanol, 10% heat-inactivated Fetal Bovine Serum; FBS, and 200U/ml of rmGM-CSF, 0.22 μ m filtered) and aliquots of 1×10^6 cells were plated in 35 $\times$ 10mm tissue culture dishes (BD and Co., Franklin Lakes, NJ) that contained a pre-sterilized 22 $\times$ 22 mm coverslip. DC were cultured for 48 hr, washed three times with NaCl-Tris buffer containing calcium/magnesium (0.36 mM CaCl₂, 0.42 mM MgCl₂) and then treated with HPTS-containing liposomes at a concentration of 500 mM phospholipid (0.1 μ mol/200 μ l). DC were incubated for 30 min at 37°C and 5% CO₂, and then washed three times in the calcium/magnesium-containing NaCl-Tris buffer. The coverslip was then mounted on a microscope slide for fluorescence microscopy study.

Fluorescence Microscopy

Cellular uptake of HPTS-containing liposomes was monitored as described previously [18] using a Zeiss Axiovert 200M inverted fluorescence microscope equipped with an AxiocamMR3 camera. Fluorescence in the pH-dependent spectral band was imaged using the Zeiss filter set 10 (excitation band pass 450–490 nm, emission band pass 515 – 565 nm) and fluorescence in the pH-independent spectral band (“total fluorescence”) was monitored using Zeiss filter set 02 (excitation 365 nm and emission long pass 420 nm). Images were acquired using 20X and 63X objective lenses and analyzed using the Axiovision 4.6.3 software provided by the manufacturer.

DC and CD4⁺ T-cell interaction

Hemophilia A mice were immunized with FVIII to provide CD4⁺ T-cells for challenge with DC that had been exposed to either FVIII or FVIII-lipid complexes. Five groups of naïve

hemophilic mice received two weekly sc injections of 2 µg free FVIII in 100µl sterile buffer. Three days after the second injection, the animals were sacrificed and the spleens were collected and homogenized. The cells were centrifuged at 200 g for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 3 ml of sterile, cold Buffer-1 (PBS containing 1% Bovine Serum Albumin) as prepared according to the Dynal CD4⁺ negative isolation kit instruction manual (Invitrogen Inc., Carlsbad, CA). The total T-lymphocyte count was determined using Cell-dyn-1700® instrument (Abbott Labs, Abbott Park, IL).

After harvesting DC cultures on day 9, they were incubated with either FVIII or FVIII-lipid complexes (2 µg protein/ml) in sterile 6-well plates for 24 hr at 37°C and 5% CO₂. The cells later harvested and washed twice by centrifugation with ice cold PBS (pH 7.0), suspended in T-cell media (RPMI-1640, L-glutamine, Penicillin/Streptomycin, Sodium pyruvate, 2-mercaptoethanol, FBS, Polymyxin-B, 0.22 µm) and viable cells were counted using hemocytometer and trypan blue dye. 2×10^5 CD4⁺ T-lymphocytes were plated along with 7×10^4 DC in quadruplicate in 96-well sterile tissue culture plates. T-lymphocytes plated without DC served as the negative control. T-lymphocytes co-cultured with naïve DC (from animals that were not exposed to FVIII) and exposed to Concanavalin A served as the positive control. The cells were incubated for 72 hr at 37°C and 5% CO₂ before analysis of T-cell proliferation and cytokine expression. All studies involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University at Buffalo.

T-cell proliferation study

For analysis of T-cell proliferation, 1µCi/well of ³H-Thymidine was added and cells were incubated for 16 hr at 37°C and 5%CO₂. The cells were then harvested using a plate harvester onto a Uni-filter-96® GF/C plate (Perkin Elmer, Boston, MA), and ³H-Thymidine incorporation was measured using a Top Count scintillation counter (Perkin Elmer, Boston, MA). The Stimulation Index (SI) was calculated as the ratio of the average ³H incorporation of samples to the average ³H incorporation of the negative control.

Cytokine analysis

For analysis of cytokine secretion, the cell-containing plates were centrifuged at 300 g for 10 min at 4°C and the supernatant was collected carefully and stored at -80°C until analysis. The supernatants were analyzed for the concentration of Transforming Growth Factor-beta (TFG-β), Interleukin-10 (IL-10), Interleukin-6 (IL-6) and Interleukin-17 (IL-17) using a developing ELISA kit (R&D systems, Minneapolis, MN).

Statistical Analysis

One-way ANOVA followed by Dunett's and Bonferroni's post-hoc analysis was performed using SPSS-16 (SPSS Inc. IL) statistical software, with a significance level of 0.05.

Results

PS mediated changes in the processing of FVIII by immune system

It was shown previously that association of FVIII with PS liposomes reduces the development of total- and neutralizing FVIII antibody titers in Hemophilia A mice [11,12]. To investigate the mechanisms underlying this reduction in antibody titers, the effect of PS on protein processing by immune system was examined. The immune response against therapeutic proteins is a multi-step process involving processing of the protein by antigen-presenting cells (APCs), presentation by the APCs in the context of the major histocompatibility complex (MHC), interaction of APCs and T-cells (mediated by MHC II

and the T-cell receptor (TCR) in the presence of co-stimulatory signals), T-cell maturation, interaction between T-cell and B-cells, and B-cell maturation [19].

Effect of PS on DC following FVIII exposure—The uptake and processing of proteins by APCs was examined as it constitutes the first step in the immune response towards therapeutic proteins [19]. Because immunogenicity against FVIII is a T-cell dependent process [20] and dendritic cells are the principal initiators of T-cell responses [21], the maturation and activation of DC was investigated in response to FVIII alone or complexed with PS-containing liposomes (Fig. 1).

Phenotypic maturation was followed using MHC II, CD80, CD86 and CD40 as the surface markers. When naïve Hemophilia-A bone marrow derived DC were exposed to free FVIII, an increase in MHC-II, CD86 and CD40 phenotypic markers was observed which was in agreement to observations by Qadura et al [22]. Upon exposure to FVIII-PS complexes, the increase in MHC II (Fig. 1.A) expression was similar to that observed with exposure to free FVIII, suggesting that PS did not interfere with FVIII-mediated maturation of DC. The CD80 expression (Fig. 1.B) also was unaltered when compared between free FVIII and FVIII-PS groups suggesting that CD80 may not be an important co-stimulatory signal for the PS-mediated effects. However, PS reduced the up-regulation of the other co-stimulatory molecules; CD86 and CD40 (Fig. 1.C & 1.D respectively). This lipid-mediated effect was specific for PS liposomes, and was not observed with liposomes of similar electrostatic charge in which PG was substituted for PS.

However, DC exposed to PC liposomes associated with FVIII showed lower MHC-II and CD86 expression compared to cells exposed to free FVIII, but the up-regulation of CD40 expression was not inhibited. Thus the data suggest consistent PS-mediated reduction in the up-regulation of both co-stimulatory signals upon FVIII exposure. Similar PS-mediated inhibition of co-stimulatory markers was observed for human dendritic cells [23] and for bone marrow-derived mouse dendritic cells in response to LPS stimulation [13]¹. TNF- α and IL-12 are cytokines which are known to be secreted by DC in response to an antigenic stimulation. Hence, the direct effect of PS on DC secretion of these two immunomodulatory cytokines in the presence of FVIII was evaluated. We determined that there was a decrease in the level of both the cytokines (~ 10 % reduction) when FVIII was complexed with PS, as compared to free FVIII, suggesting that PS might mask the DC cytokine activation property of FVIII.

In order to investigate the role of cellular association and endocytic uptake of PS liposomes by DC in the reduced expression of co-stimulatory cytokines, liposomes were labeled with the fluorescent, pH-sensitive probe HPTS [18]) (Fig. 2). Cationic liposomes containing N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethyl ammonium methylsulfate (DOTAP) were used as a positive control, owing to their avid binding to DC [24]. High levels of cell-associated fluorescence were observed for cationic liposomes (data not shown), and comparatively, lower level of fluorescence was observed when cells were exposed to PS liposomes for 30 min of incubation (Fig. 2C).

The spectral properties of HPTS permit visualization of both total cell-associated liposomes (violet illumination, 390–410 nm) and those exposed to a non-acidified environment (blue illumination, 410–450 nm), thus providing a facile method to monitor uptake of liposomes

¹Significant difference could not be established in the case of bone marrow derived dendritic cells in studies describe in 13. Hoffmann, P.R., et al., *Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo*. J Immunol, 2005. 174(3): p. 1393-404., possibly due to experimental conditions in which the cells were primed with PS first and were followed by prolonged exposure to LPS.

into an acidic endocytic environment [18]. Dual-wavelength imaging of cells exposed for 30 min to PS-containing liposomes showed punctate fluorescence in the pH-independent violet excitation spectral band both at the margin of cells and within the cell outline (Fig. 2B). In contrast, imaging of cells with the pH-dependent (blue) excitation band showed only annular punctate fluorescent uptake (Fig. 2C). The abundant fluorescent punctates within the cell outline that are visible with violet but not blue excitation represent liposome contents exposed to the acidic environment of the endocytic pathway [18]. This experiment confirms that PS liposomes can be bound and endocytosed by DC. This finding is consistent with the observation that bone marrow derived DC can accomplish endocytic uptake of exosomes which is mediated by PS on the exosome membrane surface [25].

Effect of PS on FVIII specific CD4⁺ T-cell activation

CD4⁺ T-cell proliferation is an additional key step in the immune response against FVIII, and the effect of PS-mediated reduction of co-stimulatory cytokine secretion by DC was investigated in terms of downstream stimulation of T-cell proliferation. Immature DC that were exposed to free FVIII or FVIII-lipid complexes were co-cultured with CD4⁺ T-cells obtained from animals immunized with free FVIII (Fig. 3). The T-cell stimulation index (SI) for the PS treatment group (32.9 ± 4 SEM) was significantly lower than that observed for co-cultures containing DC challenged with free FVIII (51.4 ± 5 SEM) or FVIII-PC liposomes (50.1 ± 11 SEM). The reduced capacity of DC exposed to FVIII-PS complex to stimulate T-cell proliferation correlates well with the observed reduction in the expression of co-stimulatory signals by the DC. The nature of the FVIII formulation administered to the immunized T-cell donor animals had no effect on the results; the suppression of T-cell proliferation upon challenge of DC with FVIII-PS was also observed for CD4⁺ T-cells isolated from animals that were immunized with FVIII-PS.

Effect of PS on Cytokine profile

The immunogen-elicited DC cytokine profile is crucial for T-cell proliferation, survival, and subsequent help provided to B cells for the ultimate plasma cell production of antibodies. The spectrum and concentrations of secreted cytokines by the DC result in either the activation or suppression of T-cell functions. Therefore, the secretion of key cytokines was monitored in order to investigate the role of PS in suppressing DC responses to complexed FVIII. TGF- β has been shown to have an essential role in regulating T-cell dependent immune responses, and also for the development of tolerogenic DC [26]. A significant increase in TGF- β levels was observed for the FVIII-PS treatment group compared to the free FVIII and control treatment groups (FVIII-PC and charge-matched liposomes in which PG was substituted for PS; Fig. 4.A). A similar increase was observed for IL-10, which is also a critical cytokine in regulation of T-cells, in the FVIII-PS treated group (Fig. 4.B). The secretion of other cytokines, such as IL-6 and IL-17 were reduced for the FVIII-PS treated group (Fig. 4.C & 4.D respectively), in contrast to the significant increase in secretion of these cytokines observed for the control FVIII-PC/PG treatment groups.

Effect of PS headgroup, O-Phospho-L-Serine (OPLS) on immune response to FVIII

In the blood coagulation cascade, the O-phospho-L-serine (OPLS) moiety of the PS headgroup mediates the binding of FVIII to the platelet membrane [27]. This interaction involves the lipid binding region of FVIII, which also contains CD4⁺ T-cell epitopes. We previously observed that complexing FVIII with OPLS also reduced FVIII antibody development in Hemophilia A mice [11], and therefore the effect of OPLS on T-cell proliferation and cytokine profiles (Fig. 5) was investigated. T-cell proliferation was reduced for the FVIII-OPLS group (41.89 ± 12 ; $n = 3$) compared to free FVIII (51.4 ± 5 ; $n = 3$), or for FVIII mixed with PChg (48.1 ± 12 ; $n = 3$). An increase was observed in TGF- β levels in the FVIII-OPLS group compared to free FVIII and FVIII-PChg (Fig. 5). Furthermore, IL-10

levels were elevated for the FVIII-OPLS treatment compared to treatment with free FVIII and the other control lipid formulations. The differences in levels of IL-6 did not reach significance, but the secretion of IL-17 was reduced significantly for the FVIII-OPLS group. The OPLS mediated effect was stereo-selective; because the effect of FVIII mixed with O-phospho-D-serine (OPDS) was comparable to that of free FVIII (data not shown). These data are consistent with the notion that the effect of PS to suppress immune responses to FVIII is specific to the OPLS moiety of the PS headgroup.

Discussion

The complexing of FVIII with PS-containing liposomes reduced the development of antibody responses to FVIII in Hemophilia A mice [11,12,28], which have a complete absence of active FVIII, and therefore provide a model for treatment of the disease in patients that possess no intrinsic tolerance to FVIII. This patient population is most susceptible to the formation of neutralizing antibodies. Here we investigated the possible mechanisms by which PS significantly decreases the immunogenicity of FVIII. One plausible mechanism is that the binding to the PS liposome membrane could shield the lipid-binding domain of FVIII, rendering the CD4⁺ T-cell epitopes of that domain cryptic to the immune system. Although there is some support for this mechanism, some data suggest additional mechanisms are operant. Steric shielding of the lipid binding domain of FVIII would only be possible if FVIII remained associated with the PS liposome following administration. The binding affinity of FVIII for PS-containing liposomes is on the same order of magnitude (nM) as its affinity for von Willebrand factor (vWf), a carrier protein that extends the circulating half-life of Factor VIII [28,29]. Therefore, FVIII could remain associated with PS liposomes following administration. Pharmacokinetic studies support this hypothesis, in that the systemic exposure of FVIII is reduced by complexing with PS liposomes because of rapid clearance of PS particles by the Reticuloendothelial system (RES) following intravenous administration [30]. Thus, persistent association of FVIII with PS-containing liposomes and consequently epitope shielding in the systemic circulation is plausible.

However, findings with FVIII-OPLS complex *in vivo* appear inconsistent with epitope shielding as the sole mechanism for reduction in immunogenicity is dependent upon the PS headgroup. The binding affinity of FVIII with OPLS is weak, and considerably lower than that of FVIII-vWf interaction. Based upon this weak binding, the FVIII-OPLS complex may dissociate upon administration. In terms of pharmacokinetics, we observed no difference in the systemic exposure and circulating half-life of FVIII in the presence of OPLS (data not shown). Nonetheless, OPLS significantly reduced development of antibody responses in Hemophilia A mice [11]. Based on this finding, other mechanisms in addition to epitope shielding may also play a significant role in the reduction of FVIII immunogenicity.

One possibility is that the OPLS and PS-containing liposomes could reduce aggregation and inflammation at injection site. FVIII is susceptible to aggregation mediated by conformational changes in the lipid-binding domain, and it is well recognized that aggregates can increase immunogenicity of proteins [31]. We demonstrated that PS reduces aggregate formation [32], but our studies also showed that the FVIII aggregates generated by thermal stress did not increase immunogenicity of FVIII; rather, the aggregated protein acted as a distinct antigen [33]. It is possible that aggregates formed during administration or at the injection site could differ from those generated by thermal stress. PS could also reduce inflammation at the injection site, which could, in turn, reduce the immunogenic response, as suggested by Hoffman et al [13]. That work suggested that PS suppressed the immune response against protein antigens by reducing injection site inflammation, and implicated PS interaction with the PS receptor on cells at the injection site such as macrophages,

fibroblasts, epithelial cells and endothelial cells. Here naïve DC were obtained without prior exposure to PS, and T-cells were isolated from animals injected with free FVIII, not FVIII-PS complexes. Our results indicate that other mechanisms in addition to injection site responses must be contributing to the decrease in the immunogenicity of the FVIII-PS. In the present studies, we focused upon the immunomodulatory effect of FVIII upon complexing with PS, at the level of dendritic cell presentation and maturation, T-cell proliferation, and the cytokine profiles resulting from antigen interactions with DC. The immunomodulatory effect of PS-containing liposomes has been the focus of numerous studies following the observation that PS on the outer leaflet of apoptotic cells can suppress the immune response against self antigens [34]. Chen et al have shown that such immunomodulation occurs through the inhibition of DC maturation [23].

The *in vitro* studies reported here established that PS does not interfere with antigen uptake and presentation in the context of MHC II complex (Fig. 1.A & Table 1.1) as is clear from the flow-cytometry data, and liposomes were indeed endocytosed and progressed to an acidic intracellular compartment (Fig. 2). Neither free FVIII nor FVIII complexed with lipid formulations up-regulated the CD80 co-stimulatory signal (Fig. 1.B), but FVIII-PS decreased the up-regulation of CD86 (Fig. 1.C & Table 1.2) and CD40 (Fig. 1.D & Table 3) co-stimulatory signals. The down-regulation of the co-stimulatory signals could lead to tolerization rather than activation of T-cell responses. Such a possibility is consistent with the observed reduction in the stimulation index of T-cell proliferation of the PS treatment groups, compared to that observed for free FVIII and control liposomes (Fig. 3). It has been shown that PS liposomes can be internalized by lymphocytes and exert a direct inhibition of lymphocyte activity [35]. Such an effect upon lymphocyte activity is plausible *in vivo*, but under the experimental conditions employed here, there was no direct exposure of lymphocytes to PS. FVIII-PS complexes were exposed only to naïve DC, which were washed multiple times before co-culturing with CD4⁺ T-cells.

We hypothesized that the PS-mediated effect involves DC interaction and immunomodulation by alteration of the cytokine secretion profile. Immune responses against most T-dependent antigens will likely depend upon the type and intensity of DC secreted cytokines. Here we observed that the FVIII-PS treatment group showed increased secretion of TGF- β (Fig. 4.A), an important cytokine in the T-cell mediated response, and this could regulate both DC function and lymphocyte activity. The secretion of TGF- β could transform immature DC into immuno-regulatory DC, which have lower co-stimulatory activities and which can promote tolerance via a regulatory T-cell (T_{reg}) mechanism [20,36]. This hypothesis is consistent with the flow-cytometry studies, which showed lower co-stimulatory functions when DC were exposed to FVIII-PS (Fig. 1). The generation of immuno-regulatory DC could contribute to the reduction in T-cell proliferation and possibly tolerization. The observed reduced secretion of TNF- α and IL-12 cytokines during FVIII-PS exposure to DC suggest a lower activation state of DC. Finally, TGF- β could affect T-cell activity and tolerance directly [26,36]. It has been shown that TGF- β 1 induces the generation of T_{regs}, while TGF- β 1 with concomitant IL-6 skews T-cells into IL-17-producing T_{H17} cells [26]. Therefore, the presence of TGF- β could prompt naïve T-cells to acquire Foxp3 expression and differentiate into induced T_{regs} [26,36–38]. The observed up-regulation of IL-10 upon exposure to FVIII-PS could also contribute to TGF- β mediated effects, promoting DC transition into tolerogenic DC having impaired co-stimulatory functions [39] and inducing peripheral T-cell -mediated tolerance [40,41]. Based on these observations, we conclude that exposure of DC to FVIII in the presence of PS results in the regulation of functional properties of DC, which consequently down-regulate lymphocyte activity via TGF- β and IL-10 secretion. This observation raises the interesting question of whether PS reduces immunogenicity of FVIII by inducing FVIII tolerance involving T_{regs} and immuno-regulatory DC, which will require additional investigation.

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Abbreviations

PS	Phosphatidylserine
OPLS	O-Phospho-L-Serine
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
FVIII	Recombinant Factor VIII
IL-6	Interleukin 6
IL-17	Interleukin 17
TGF-β	Transforming growth Factor beta
Treg	Regulatory T-cells
DC	dendritic cells

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Fig. 1.

Flow-cytometry analysis of the phenotypic maturation of bone marrow-derived DC following stimulation with PS, PC and PG liposomes associated with or without FVIII. The cell-surface markers were identified by FITC-MHC II antibody (1.A), PE-CD80 antibody (1.B), PE-CD86 antibody (1.C) or PE-CD40 antibody (1.D). Grey peak presents unstimulated DC (negative control).

Fig. 2.

DC uptake and endocytosis of HPTS-labeled PS liposomes. DC were exposed to liposomes containing the pH-indicating fluorophore for 30 min at 37°C, washed free of unbound liposomes, and examined by fluorescence microscopy. (2.A): differential interference contrast image. (2.B): violet excitation band (total cell-associated fluorescence). (2.C): blue excitation band (non-acidified compartment) of the same field as in A and B.

Fig. 3.

PS mediated inhibition of T-cell proliferation as measured by ^3H -thymidine incorporation. T-cell proliferation was measured for CD4^+ T-cells isolated from animals immunized by subcutaneous (sc) administration of FVIII and re-stimulated *in vitro* with DC that were exposed to FVIII in the absence or presence of liposomes (PS or PC).

Fig. 4. PS mediated modulation of cytokine secretion as measured by ELISA. Cytokine secretion of TGF- β (4.A), IL-10 (4.B), IL-6 (4.C) and IL-17 (4.D) was measured following co-culturing of CD4⁺ T-cells isolated from FVIII-immunized animals with naïve DC exposed to FVIII in the absence or presence of liposomes (PS, PC and PG).

Fig. 5.

OPLS mediated modulation of cytokine secretion as measured by ELISA. Cytokine secretion of TGF- β (5.A), IL-10 (5.B), IL-6 (5.C) and IL-17 (5.D) was measured following co-culturing of CD4⁺ T-cells isolated from FVIII immunized animals with naïve DC exposed to FVIII in the absence or presence of liposomes (PS, PC and PG).

Table 1

Table of Mean fluorescence intensity (MFI) and percent positive cells as determined from the flow-cytometric analysis of MHC-II (1.1), CD86 (1.2) and CD40 (1.3) cell-surface markers upon DC exposure either to free FVIII or FVIII-PS/PC/PG formulations.

1.1 Mean fluorescence intensity for MHC-II

Group	Average Mean Fluorescence Intensity (MFI) $\pm$ SEM (n = 3)	% positive cells $\pm$ SEM
Free rFVIII	1297 $\pm$ 108	83.5 $\pm$ 7
rFVIII-PS	1240 $\pm$ 131	72.8 $\pm$ 5
rFVIII-PC	1081 $\pm$ 201	76.2 $\pm$ 3
rFVIII-PG	1373 $\pm$ 120	82.3 $\pm$ 5

1.2

Mean Fluorescence Intensity for CD 86

Group	Average Mean Fluorescence Intensity (MFI) $\pm$ SEM (n = 3)	% positive cells $\pm$ SEM
Free rFVIII	1952 $\pm$ 93	89.0 $\pm$ 5
rFVIII-PS	879 $\pm$ 137 *	68.0 $\pm$ 9
rFVIII-PC	1125 $\pm$ 145 *	70.5 $\pm$ 4
rFVIII-PG	1797 $\pm$ 97	89.5 $\pm$ 6

* denotes a significance of $P < 0.05$.

1.3

Mean fluorescence Intensity for CD 40

Group	Average Mean Fluorescence Intensity (MFI) $\pm$ SEM (n = 3)	% positive cells $\pm$ SEM
Free rFVIII	142 $\pm$ 32	78.0 $\pm$ 4
rFVIII-PS	22.1 $\pm$ 19 *	40.0 $\pm$ 6 *
rFVIII-PC	159 $\pm$ 23	75.0 $\pm$ 6
rFVIII-PG	172 $\pm$ 33	76.6 $\pm$ 3

* denotes a significance of $P < 0.05$.