Published in final edited form as:

J Pharm Sci. 2016 October ; 105(10): 3039–3045. doi:10.1016/j.xphs.2016.06.008.

Effect of biophysical properties of Phosphatidylserine (PS) particle on immune tolerance induction towards Factor VIII in a Hemophilia A mouse model

Radha Ramakrishnan and Sathy V. Balu-Iyer^{†,*}

Department of Pharmaceutical Sciences

Abstract

A major complication in the replacement therapy of Factor FVIII (FVIII) for Hemophilia A (HA) is the development of unwanted immune responses. Previous studies from our lab have shown that pretreatment of FVIII in the presence of Phosphatidylserine (PS) resulted in hyporesponsiveness to subsequent administration of FVIII alone, due to the ability of PS to convert an immunogen to a tolerogen. We investigated the importance of biophysical properties of PS liposomes on its ability to convert an immunogen to a tolerogen. PS particles were prepared differing in size, protein-lipid topology, lamellarity and % association to FVIII keeping the composition of the particle same. PS particles were prepared in two different sizes with differing biophysical properties, smaller particles in the nanometer range (200 nm) and larger size particles in the micron range (2µm). HA animals treated with both the nanometer and micron size PS particle showed a significant reduction in anti-FVIII antibody titers when compared to animals receiving free FVIII alone. Upon rechallenge with free FVIII animals that received FVIII along with the nanometer size particle continued to show reduced antibody responses. Animals receiving the micron size particle showed a slight increase in titers although they remained significantly lower than the free FVIII treated group. Upon culture with bone marrow dendritic cells (BMDCs), the nanometer size particle showed a reduction in CD40 expression and an increase in TGF- β cytokine production; which was not observed with the micron size particle. These results show that biophysical properties of PS play an important role in tolerance.

Keywords

protein delivery; lipids; phosphatidylserine; immunology; immune tolerance

*Corresponding author: 359 Kapoor Hall, University at Buffalo, The State University of New York, Buffalo, New York 14215, Telephone: (716)-645-4836, Fax: (716)-645-3693, svb@buffalo.edu.

[†]Formerly Sathyamangalam V. Balasubramanian

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Factor VIII (FVIII) is an important clotting factor, which plays a central role in the blood coagulation cascade. Hemophilia A (HA) is a bleeding disorder characterized by a genetic deficiency or dysfunction of FVIII. Replacement therapy with recombinant FVIII is the first line of therapy for this disorder. However, one of the major drawbacks of this therapy is the development of unwanted immune responses that renders the therapy less efficacious. These immune responses include anti-FVIII neutralizing antibodies (Nabs) also known as inhibitors observed in about 30% of patients, as well as binding antibodies. Nabs bind to FVIII and abrogate its activity while binding antibodies increase its clearance affecting its pharmacokinetics¹. Any approach to reduce immunogenicity and induce tolerance to FVIII would improve safety and efficacy of replacement therapy for Hemophilia A.

Our approach to this problem is to administer FVIII in the presence of phosphatidylserine (PS) liposomes. Our previous studies have shown that pre-exposure of FVIII in the presence of PS led to hyporesponsiveness towards FVIII upon subsequent rechallenges with the free FVIII alone in a HA mouse model². This effect was found to be antigen specific. Bone marrow derived dendritic cells (BMDCs) upon exposure of FVIII in the presence of PS showed a downregulation of the costimulatory marker CD40 as well as an increase in the regulatory cytokine TGF- β ³. Further mechanistic studies showed that PS acts by increasing the regulatory T cells (Tregs) production as well as reducing the memory B cell development *in vivo*⁴. These results put together indicate that PS converts an immunogen to a tolerogen.

While we have an insight into the mechanisms by which PS induces tolerance, it is not clear whether the biophysical properties of PS liposomes play a role in this novel regulatory property. Hence, here we investigated the biophysical properties of PS liposomes as a critical requirement for tolerance. PS particles were prepared differing in size, protein-lipid topology, lamellarity and % association to FVIII keeping the composition of the particle same. The studies show that the smaller size particles, in the nanometer range, are more effective in inducing tolerance to FVIII.

MATERIALS AND METHODS

Materials

Full length recombinant human FVIII was a generous gift from the Hemophilia Center of Western New York, Buffalo NY. Brain Phosphatidylserine (PS) and Dimyristoyl phosphatidylcholine (DMPC) were purchased from Avanti Lipids (Alabaster, AL). All solvents and buffer salts were obtained from Fisher Scientific (Fairlawn, NJ) or from Sigma (St. Louis, MO). Anti-FVIII monoclonal antibody ESH8 was obtained from American Diagnostica Inc. (Greenwich, CT). Endosafe Endochrome-KR kit was purchased from Charles River Laboratories, (Charleston, SC). NUNC MaxiSorp 96 well plates were from ThermoScientific (Waltham, MA). aPTT reagents were purchased from T-coag Ireland Ltd (Ireland). Normal pooled and deficient human plasma were obtained from PrecisionBiologic (Canada). Antibodies for flow cytometry were purchased from BD Biosciences (San Jose, CA). TGF- β cytokine ELISA kit was purchased from R&D System Inc. (DuoSet) (Minneapolis, MN).

Animals

A colony of hemophilic mice with targeted deletion in the exon 16 of Factor VIII gene (termed HA mice) was maintained on site in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo. The animals were handled and surgical procedures performed as per the protocol approved by the IACUC.

Preparation and characterization of protein lipid complex

Preparation of PS-FVIII (200nm)—PS liposomes were prepared at a 30:70 molar ratio of PS to DMPC as previously described⁵. To get the appropriate size, PS liposomes were extruded multiple times through double stacked polycarbonate membrane of pore size 200nm using high pressure extruder. The size of the liposomes was measured using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems (Port Richley, Florida). This procedure results in small unilamellar vesicles (SUVs). The size of the PS liposomes was found to be 194 ± 61.2 nm (mean $\pm$ SD). The lipid content was assayed by a phosphate assay⁶. The protein to lipid molar ratio used was at 1:10,000. FVIII was associated with liposomes by a trigger-loading mechanism by incubating the preparation at 37°C for 30 minutes. The trigger loading mechanism is a gentle and controlled thermal unfolding procedure employed to promote interaction between FVIII and PS liposomes. The procedure results in approximately 47% of FVIII to be associated with PS liposomes. The remaining FVIII remains unbound to the PS and is left in the preparation prior to injection. The interaction of FVIII with PS liposomes is mainly through the C2 domain of FVIII⁵ and in this configuration the protein is surface bound to the liposome. The FVIII is bound either on the surface of the PS liposomes or is embedded in the inner bilayer of liposome⁷.

Preparation of PS-FVIII (2um)—PS liposomes were prepared at a 30:70 molar ratio of PS to DMPC. The appropriate amounts of lipids were dissolved in chloroform and solvent was evaporated using a rotary evaporator (Buchi- R200, Fisher Scientific) to form a thin lipid film. The FVIII in Tris buffer (300mM sodium chloride, 25mM Tris, pH 7.0) was added slowly to the film (at a 1:10,000 protein to lipid molar ratio) and gently mixed. This mixture was then placed at 37°C for 2 minutes and then gently mixed by rotating in hand. This cycle was repeated for a total of 3 times. This procedure results in the generation of multilamellar vesicles (MLVs). At the end, this PS-FVIII complex was spun at 13000g for 60mins. This is to remove any free FVIII unbound to the PS liposomes. The supernatant is removed and assayed for free FVIII by an activity assay using an activated partial Thromboplastin time (aPTT) assay. This procedure results in about 85% of FVIII to be associated with the PS liposomes. The remaining 15% of the FVIII is free and is removed in the supernatant. This method results in all the FVIII to be associated with PS. All the FVIII is embedded in the inner bilayer of the liposomes. The size of the liposomes was measured using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems (Port Richley, Florida). The size of the PS particle was found to be 2320 ± 271 nm (mean $\pm$ SD). The pellet is resuspended in Tris buffer and the appropriate amount used for studies.

It is important to note that in both preparations, the ratio of protein: lipid and the amount of protein administered is the same. The PS-FVIII complexes in both the cases above were

tested for endotoxin level by using Endosafe Endochrome-K endotoxin assay kit (Charles River Inc., MA) and endotoxin negative samples were used for all studies.

Biophysical properties of the two different size PS particles are summarized in Table 1.

Tolerance induction studies

Animal studies were conducted in HA mice. Animals (n=14) were immunized with four weekly s.c. injections of FVIII (1ug/injection) alone or in the presence of PS (200nm) or PS (2um). This was followed by a two-week washout period. At the end of the sixth week, half the animals in each group were sacrificed and the plasma samples collected as baseline samples for relative immunogenicity analysis of antibody titers. The remaining animals were then aggressively rechallenged with 2 weekly doses of just the free FVIII alone (0.5ug/injection) via the s.c. route to determine tolerance. Two weeks after the last dose, all animals were sacrificed and their plasma samples collected via cardiac puncture into acid citrate dextrose buffer. The plasma samples were stored at -80°C until further analysis.

Generation of BMDCs

Bone marrow was isolated from naive HA mice as reported in Lutz et al⁸. Briefly, bone marrow was cultured in RPMI supplemented with fetal bovine serum, 2-mercaptoethanol, Penicillin/Streptomycin and granulocyte macrophage colony stimulating factor (GM-CSF). The media was changed on days 3, 6, and 8 of culture and DCs were used on day 9 as described previously⁹. BMDCs were then harvested and used as needed.

Effect of particle size on Phenotypic Maturation of BMDCs

Next, we investigated the effects of PS particle size on phenotypic maturation of BMDC. BMDCs were exposed to 1 ug/mL FVIII in the presence and absences of PS (200nm) or PS (2um) for 24 h. 24 h later, the cells were harvested, washed twice with ice cold sterile PBS. Cells were blocked using BD Fc-block antibody before labeling with anti-CD40-PE or anti-CD86-PE. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). This study was conducted in triplicates. The flow cytometry data was analyzed using FlowJO software. A histogram analysis was used to determine the Median Fluorescence Intensity (MFI) for the fluorophore used. The histogram plot represents the intensity of the fluorophore on the X-axis and the number of events on the Y-axis. A large number of events detected at a particular intensity will be displayed as a spike in a histogram. A statistical analysis of the histogram plots in FlowJo produces the MFI. The histogram plot shown in Figure 5 represents multiple histogram plots from the control (Isotype control) overlaid along with all treatment groups.

Effect of particle size on TGF- β production by BMDCs

The effects of PS particle size on the regulatory TGF- β cytokine secretion by DCs were investigated. On day 9, BMDCs generated as above, were treated with FVIII (2ug/ml) in the presence or absence of PS (200nm) or PS (2um). Lipopolysaccharide (LPS) was used as an immunogenic control while Vitamin D3+Dexamethasone (VitD3+Dex) was used as a tolerogenic control. Cells were plated in triplicates and cultured for 72 h. At the end of the

incubation period, the cells were spun at 300g for 10 minutes and the supernatant medium was collected for cytokine analysis by ELISA. This study was conducted in triplicates.

Determination of anti-FVIII Nabs and Total Anti-FVIII antibodies

The plasma samples were analyzed for anti-FVIII Nab titers by activated partial Thromboplastin time (aPTT) assay following Nijmegen's modified Bethesda assay and expressed in Bethesda Units (BU/ml)¹⁰. Total anti-FVIII antibody titers were determined by ELISA as described previously¹¹.

Statistical Analysis

One-way ANOVA followed by Tukey's post-hoc analyses was performed using Graphpad Prism (La Jolla, CA) statistical software. $P < 0.05$ was considered as a statistically significant difference.

RESULTS

Both nanometer and micron size PS particle reduces anti-FVIII antibody responses

Animals were immunized with the different treatment groups according to the immunization protocol described in Fig 1. Baseline plasma samples were taken at week 6, before rechallenge to assess relative immunogenicity of FVIII and PS-FVIII formulations. As seen in fig 2a, animals treated with free FVIII alone mounted robust total anti-FVIII antibody responses (2665 ± 482 arbitrary titer units; mean $\pm$ SEM). However, animals treated with both PS-FVIII (200nm) and PS-FVIII (2um) showed significantly lower total anti-FVIII antibody responses (1096 ± 174 and 1491 ± 224 arbitrary titer units respectively; mean $\pm$ SEM).

Similar results were observed with the neutralizing antibody titers (Fig 2b). The nanometer and micron size PS particle showed mean neutralizing antibody titers of 51 ± 27 BU/ml (mean $\pm$ SEM) and 50 ± 18 BU/ml (mean $\pm$ SEM) respectively; which was significantly lower than the free FVIII treated group (197 ± 46 BU/ml; mean $\pm$ SEM).

The nanometer size particle is more effective than the micron size particle in inducing immunological tolerance to FVIII

Plasma samples were taken at week 11 to assess tolerance. Upon rechallenge with free FVIII alone, animals pretreated with free FVIII continued to mount increased total anti-FVIII antibody responses (12789 ± 1680 arbitrary titer units; mean $\pm$ SEM) (Fig 3a). However, it was interesting to note that while both the nanometer and micron size particle showed significantly lower total anti-FVIII antibody titers at baseline, only the animals pretreated with PS-FVIII (200nm) was able to induce effective tolerance. This is because the PS-FVIII (200nm) continued to maintain lower total anti-FVIII antibody titers (1806 ± 220 arbitrary titer units; mean $\pm$ SEM) upon multiple rechallenges with free FVIII alone even after PS therapy was stopped. This was significantly lower than the free FVIII treatment group. The micron size particle, on the other hand, showed an increase in total anti-FVIII antibody titers (4624 ± 485 arbitrary titer units; mean $\pm$ SEM) upon rechallenge with free FVIII although

they still remained significantly lower than the free FVIII treatment group (12789 ± 1680 arbitrary titer units; mean $\pm$ SEM).

These observations were also reflected in the anti-FVIII neutralizing antibody titers (Fig 3b). Animals receiving the free FVIII alone showed significantly higher anti-FVIII neutralizing antibody titers (751 ± 179 BU/ml; mean $\pm$ SEM) when compared to the animals that were pretreated with the PS-FVIII (200 nm) (76 ± 25 BU/ml; mean $\pm$ SEM) and the PS-FVIII (2 μ m) (317 ± 61 BU/ml; mean $\pm$ SEM).

Figure 4 shows the comparison of anti-FVIII antibody titers for the different treatment groups at baseline and following rechallenge with free FVIII alone. This shows that animals that continued to receive free FVIII increased antibody production between baseline and following rechallenge. While the PS-FVIII (2 μ m) showed an increase in antibody titers between baseline and following rechallenge, the graph clearly shows that there is not much change in the anti-FVIII antibody responses when animals were pretreated with PS-FVIII (200nm) indicating tolerance. It is interesting to note here that although the PS-FVIII (200nm) has only 47% FVIII associated, it was more effective in inducing tolerance. However, we believe that we are unable to see a complete absence of antibody titers due to the presence of free fraction of FVIII in the injection preparation.

The nanometer size particle is effective in converting dendritic cells to a tolerogenic phenotype

CD40 expression in BMDCs is reduced in the presence of PS-FVIII (200nm)—

In vitro studies were conducted with bone marrow derived dendritic cells (BMDCs) to determine if the particle size affects the processing by the BMDCs. The BMDCs were exposed to FVIII in the presence or absence of PS (200nm) and PS (2 μ m) particle and the surface expression markers analyzed by flow cytometry. BMDCs exposed to the free FVIII alone resulted in an increase in the MFI of co-stimulatory marker CD40 (174 ± 34.2 Arbitrary Units; mean $\pm$ SD), which was similar to the PS-FVIII (2 μ m) (162 ± 24.7 Arbitrary Units; mean $\pm$ SD) (Figure 5a and 5c). However, the PS-FVIII (200nm) resulted in a decrease in the MFI of CD40 expression (97.3 ± 20.4 Arbitrary Units; mean $\pm$ SD). Similarly, the PS-FVIII (200nm) resulted in a slight decrease in the expression of the late maturation marker CD86 (404 ± 6.36 Arbitrary Units; mean $\pm$ SD) when compared to the free FVIII (417 ± 15.6 Arbitrary Units; mean $\pm$ SD) and the PS-FVIII (2 μ m) (445 ± 15.2 Arbitrary Units; mean $\pm$ SD) treatment groups (Figure 5b and 5c). Although the differences seen are small and not statistically significant, these differences were reproducible upon multiple repeats of the experiment. This suggests that the phenotypic changes in DCs observed could potentially explain the differences in the antibody titers detected.

PS-FVIII (200nm) increases the TGF- β production in BMDCs—Cytokine production by DCs is an important factor converting it from an immunogenic to a tolerogenic phenotype. Upon exposure of Free FVIII to the BMDCs, there is TGF- β production of 222 ± 60.0 pg/ml; mean $\pm$ SEM (Figure 5d). These were similar to the immunogenic control LPS (126 ± 7.21 pg/ml; mean $\pm$ SEM). However, when the DCs were exposed to the PS-FVIII (200nm), there is an increase in the TGF- β production (692 ± 88.7

pg/ml; mean $\pm$ SEM) and this was similar to the tolerogenic control group, VitaminD3+Dexamethasone (817 $\pm$ 37.8 pg/ml; mean $\pm$ SEM). It is interesting to note that, the PS-FVIII (2 μ m) did not increase the TGF- β production (197 $\pm$ 62.2 pg/ml; mean $\pm$ SEM) and showed levels similar to the free FVIII treatment. These results indicate that the PS-FVIII (200nm) is able to convert the DCs to a tolerogenic phenotype; while the PS-FVIII (2 μ m) is far less effective in producing tolerogenic DCs.

DISCUSSION

Plasma samples taken at baseline to assess the relative immunogenicity showed that both nanometer and micron size particles were able to significantly reduce the immune responses to FVIII when compared to the free FVIII treatment group. However, when treatment was stopped and all animals were rechallenged with free FVIII alone, animals that were pretreated with PS-FVIII (200nm) continued to show reduced immune responses to FVIII. The PS-FVIII (2 μ m) that was able to reduce the immunogenicity to FVIII, upon rechallenge showed an increase in antibody responses to FVIII.

There could be several reasons for why the different size PS particles behave differently. The micron size particle could just be shielding the FVIII from the immune system resulting in immune ignorance rather than immune tolerance; as is clear from the observed reduction in antibody titers at baseline but not after rechallenge. Since uptake is a critical step in the immune response, we speculate that the dendritic cells could be processing the particles differently, leading to differences in immune responses. The nanometer size particle could be taken up by the DCs by a more specific process like a receptor-mediated process. Several PS receptors have been identified that mediate the uptake of PS¹²⁻¹⁴. Among these, TIM-4 (T cell/transmembrane immunoglobulin and mucin) is an important receptor as it is exclusively expressed on antigen presenting cells and has been linked to PS mediated immune modulation^{15,16}. The nanometer size particle could be interacting with this receptor on DCs to induce tolerance. The micron size particle, on the other hand, could be taken up by the DCs by a more non-specific process such as macropinocytosis or could altogether be processed by macrophages rather than DCs. Several studies investigating particle sizes for vaccine development have shown that nanometer size particles are efficiently taken up by DCs compared to the micron size particles¹⁷⁻²¹. Support also comes from the inherent processing of viruses and bacteria by the immune system. In general, viruses (20–200nm) are more efficiently taken up by dendritic cells while bacteria (>500nm) are taken up by macropinocytosis or phagocytosis by macrophages²²⁻²⁴.

This PS mediated effect is believed to act on multiple immune cells. When the dendritic cells take up the FVIII, it is processed and the dendritic cells undergo full maturation by upregulating the maturation markers like CD80 and CD86. It also upregulates the costimulatory marker CD40 which interacts with its ligand on the T cells to activate it. The activated T cells now proliferate and interact with the B cells to produce the antibodies against FVIII. On the other hand, when the FVIII is presented along with the PS, the dendritic cells uptake the particle. However, the PS particle inhibits the maturation of the dendritic cells. There is a reduction in CD86 expression and a reduction in CD40 expression, resulting in reduced T cell activation. These tolerogenic DCs secrete TGF- β . PS also acts by

increasing FoxP3 expressing Tregs, which are immunosuppressive in nature. PS either directly or indirectly through Tregs are capable of inhibiting memory B cell development. These properties of PS together are capable of converting an immunogen to a tolerogen²⁻⁴.

To investigate if the dendritic cells are indeed processing the particles differently, *in vitro* studies were conducted using BMDCs. Upon antigen uptake, dendritic cells undergo phenotypic maturation by up-regulating maturation marker like CD86 and co-stimulatory marker like CD40. The mature DC produces pro-inflammatory cytokines and is capable of mounting an immune response by interacting with the T cells. The maturation status of the DCs and the cytokine produced by it determines if there will be an immunogenic or a tolerogenic response. A reduction in CD40 expression and an increase in the regulatory cytokine, TGF- β indicates production of DCs with a tolerogenic phenotype. Although the phenotypic differences in the DCs are small, they were highly reproducible. These results indicate that the PS-FVIII (200nm) could be producing tolerogenic DCs, which could potentially explain the tolerance that is observed *in vivo*. The PS-FVIII (2 μ m) on the other hand did not produce tolerogenic DCs indicating that it could be processed differently by the immune system, which results in a reduction of immune response. Taken together, these results indicate that the nanometer size particle induces tolerance to FVIII by producing tolerogenic DCs.

These studies may provide insight into key biological functions of PS. PS is a naturally occurring phospholipid present in all eukaryotic cells. Under normal conditions, PS is present on the inner leaflet. However, during apoptosis when the cell is dying it flips the PS from its inner leaflet to the outer leaflet. This exposure of PS is known to suppress immune responses to the self-antigens maintaining tolerance²⁵⁻³⁰. Our studies show that apoptotic event is not an immunologically silent event but acquires tolerogenic potential via the ability of PS to convert an immunogen to a tolerogen. PS is not only present on apoptotic cells but is also expressed on secretory vesicles like exosomes, microvesicles and apoptotic bodies³¹. These naturally present extracellular vesicles play an important role in maintaining peripheral tolerance. While the key similarity between these secreted vesicles is the presence of PS in its outer membrane, one of the major differences is in its size. While exosomes and microvesicles are smaller in size ranging from 40–1000nm; apoptotic bodies are larger about 1–5 μ m³²⁻³⁴. We hypothesize that our nanometer size PS particle behaves in a manner similar to the exosomes and microvesicles while the micron size PS particle mimics apoptotic bodies. We speculate that self-proteins packaged in exosomes and microvesicles may lead to tolerance whereas apoptotic bodies serve as clean up action.

Our approach is to exploit this novel property of PS particle clinically to reduce immunogenicity of therapeutic proteins. We expect that administering FVIII along with PS prior to therapy will desensitize the patients so that they will be unable to immunologically respond to subsequent administrations of FVIII. Additionally, PS is on the FDA's list of Generally Regarded as Safe or GRAS ingredients for its oral use as a dietary supplement and a nutrient. We have also conducted a complete toxicological evaluation of PS liposomes in mice and non-human primates (unpublished results). Our studies indicate that PS liposomes have a wide margin of safety at its anticipated clinical dose, allowing for ease of translation to clinic.

CONCLUSION

In conclusion, the biophysical properties of PS are important for its ability to convert an immunogen to a tolerogen. Both the nanometer size and the micron size particle were effective in reducing immunogenicity to FVIII; however the nanometer size particle was more effective and durable in inducing tolerance by producing tolerogenic DCs.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01 HL-70227) to Dr. Balu-Iyer. The authors thank Pharmaceutical Sciences Instrumentation facility, University at Buffalo, State University of New York, for the use of the shared microplate reader. We are very grateful to the Hemophilia Center of Western New York, Buffalo, NY for providing recombinant Factor VIII. We acknowledge the assistance of the Confocal Microscope and Flow Cytometry Facility in the School of Medicine and Biomedical Sciences, University at Buffalo.

ABBREVIATIONS USED

FVIII	Factor VIII
HA	Hemophilia A
PS	phosphatidylserine
Nabs	anti-FVIII Neutralizing antibodies
BMDCs	Bone Marrow Derived Dendritic Cells

REFERENCES

1. Lollar P, Healey JF, Barrow RT, Parker ET. Factor VIII inhibitors. *Advances in experimental medicine and biology*. 2001; 489:65–73. [PubMed: 11554592]
2. Gaitonde P, Ramakrishnan R, Chin J, Kelleher RJ Jr, Bankert RB, Balu-Iyer SV. Exposure to factor VIII protein in the presence of phosphatidylserine induces hypo-responsiveness toward factor VIII challenge in hemophilia A mice. *The Journal of biological chemistry*. 2013; 288(24):17051–17056. [PubMed: 23649621]
3. Gaitonde P, Peng A, Straubinger RM, Bankert RB, Balu-Iyer SV. Phosphatidylserine reduces immune response against human recombinant Factor VIII in Hemophilia A mice by regulation of dendritic cell function. *Clin Immunol*. 2011; 138(2):135–145. [PubMed: 21094627]
4. Ramakrishnan R, Davidowitz A, Balu-Iyer SV. Exposure of FVIII in the Presence of Phosphatidyl Serine Reduces Generation of Memory B-Cells and Induces Regulatory T-Cell-Mediated Hyporesponsiveness in Hemophilia A Mice. *Journal of pharmaceutical sciences*. 2015; 104(8): 2451–2456. [PubMed: 26038127]
5. Ramani K, Miclea RD, Purohit VS, Mager DE, Straubinger RM, Balu-Iyer SV. Phosphatidylserine containing liposomes reduce immunogenicity of recombinant human factor VIII (rFVIII) in a murine model of haemophilia A. *Journal of pharmaceutical sciences*. 2008; 97(4):1386–1398. [PubMed: 17705286]
6. Bartlett GR. Phosphorus assay in column chromatography. *The Journal of biological chemistry*. 1959; 234(3):466–468. [PubMed: 13641241]
7. Purohit VS, Ramani K, Kashi RS, Durrani MJ, Kreiger TJ, Balasubramanian SV. Topology of factor VIII bound to phosphatidylserine-containing model membranes. *Biochimica et biophysica acta*. 2003; 1617(1–2):31–38. [PubMed: 14637017]

8. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *Journal of immunological methods*. 1999; 223(1):77–92. [PubMed: 10037236]
9. Gaitonde P, Balu-Iyer SV. In vitro immunogenicity risk assessment of therapeutic proteins in preclinical setting. *Methods Mol Biol*. 2011; 716:267–280. [PubMed: 21318912]
10. Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thrombosis and haemostasis*. 1995; 73(2):247–251. [PubMed: 7792738]
11. Purohit VS, Ramani K, Sarkar R, Kazazian HH Jr, Balasubramanian SV. Lower inhibitor development in hemophilia A mice following administration of recombinant factor VIII-O-phospho-L-serine complex. *The Journal of biological chemistry*. 2005; 280(18):17593–17600. [PubMed: 15728582]
12. Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, Kwon TH, Park RW, Kim IS. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell death and differentiation*. 2008; 15(1):192–201. [PubMed: 17962816]
13. Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. *Immunological reviews*. 2010; 235(1):172–189. [PubMed: 20536563]
14. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. *Nature*. 2007; 450(7168):435–439. [PubMed: 17960135]
15. Albacker LA, Karisola P, Chang YJ, Umetsu SE, Zhou M, Akbari O, Kobayashi N, Baumgarth N, Freeman GJ, Umetsu DT, DeKruyff RH. TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. *J Immunol*. 2010; 185(11):6839–6849. [PubMed: 21037090]
16. Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, Butte MJ, Nagumo H, Chernova I, Zhu B, Sharpe AH, Ito S, Dranoff G, Kaplan GG, Casasnovas JM, Umetsu DT, DeKruyff RH, Freeman GJ. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity*. 2007; 27(6):927–940. [PubMed: 18082433]
17. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. *International journal of pharmaceutics*. 2005; 298(2):315–322. [PubMed: 15961266]
18. Mathaes R, Winter G, Siahaan TJ, Besheer A, Engert J. Influence of particle size, an elongated particle geometry, and adjuvants on dendritic cell activation. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV*. 2015; 94:542–549.
19. Kumar S, Anselmo AC, Banerjee A, Zakrewsky M, Mitragotri S. Shape and size-dependent immune response to antigen-carrying nanoparticles. *Journal of controlled release : official journal of the Controlled Release Society*. 2015; 220(Pt A):141–148. [PubMed: 26437263]
20. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nature reviews Immunology*. 2010; 10(11):787–796.
21. Pacheco P, White D, Sulchek T. Effects of microparticle size and Fc density on macrophage phagocytosis. *PloS one*. 2013; 8(4):e60989. [PubMed: 23630577]
22. Gamvrellis A, Leong D, Hanley JC, Xiang SD, Mottram P, Plebanski M. Vaccines that facilitate antigen entry into dendritic cells. *Immunol Cell Biol*. 2004; 82(5):506–516. [PubMed: 15479436]
23. Xiang SD, Scholzen A, Minigo G, David C, Apostolopoulos V, Mottram PL, Plebanski M. Pathogen recognition and development of particulate vaccines: does size matter? *Methods*. 2006; 40(1):1–9. [PubMed: 16997708]
24. Reis e Sousa C, Stahl PD, Austyn JM. Phagocytosis of antigens by Langerhans cells in vitro. *The Journal of experimental medicine*. 1993; 178(2):509–519. [PubMed: 8393477]
25. Tanaka M, Asano K, Qiu CH. Immune regulation by apoptotic cell clearance. *Annals of the New York Academy of Sciences*. 2010; 1209:37–42. [PubMed: 20958314]
26. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol*. 1992; 148(7):2207–2216. [PubMed: 1545126]

27. Hoffmann PR, Kench JA, Vondracek A, Kruk E, Daleke DL, Jordan M, Marrack P, Henson PM, Fadok VA. Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J Immunol.* 2005; 174(3):1393–1404. [PubMed: 15661897]
28. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature.* 2000; 407(6805): 784–788. [PubMed: 11048729]
29. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. *The Journal of experimental medicine.* 2002; 196(8):1091–1097. [PubMed: 12391020]
30. Chaurio RA, Janko C, Munoz LE, Frey B, Herrmann M, Gaipf US. Phospholipids: key players in apoptosis and immune regulation. *Molecules.* 2009; 14(12):4892–4914. [PubMed: 20032867]
31. Li XB, Zhang ZR, Schluesener HJ, Xu SQ. Role of exosomes in immune regulation. *Journal of cellular and molecular medicine.* 2006; 10(2):364–375. [PubMed: 16796805]
32. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, Laszlo V, Pallinger E, Pap E, Kittel A, Nagy G, Falus A, Buzas EI. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and molecular life sciences : CMLS.* 2011; 68(16):2667–2688. [PubMed: 21560073]
33. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nature reviews Immunology.* 2009; 9(8):581–593.
34. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nature reviews Immunology.* 2002; 2(12):965–975.
35. Ramani K, Balasubramanian SV. Fluorescence properties of Laurdan in cochleate phases. *Biochimica et biophysica acta.* 2003; 1618(1):67–78. [PubMed: 14643935]

Figure 1.

Immunization protocol: Study design used for *in vivo* tolerance induction study. Animal studies were conducted in HA mice. Animals (n=14) were immunized with four weekly s.c. injections of FVIII (1ug/injection) alone or in the presence of PS (200nm) or PS (2um). This was followed by a two-week washout period. At the end of the sixth week, half the animals in each group were sacrificed and the plasma samples collected as baseline samples for relative immunogenicity analysis of antibody titers. The remaining animals were then aggressively rechallenged with 2 weekly doses of just the free FVIII alone (0.5ug/injection) via the s.c. route to determine tolerance. Two weeks after the last dose, all animals were sacrificed and their plasma samples collected for analysis of antibody titers.

Figure 2.

Animals were immunized with the different treatment groups according to the immunization protocol described in Fig 1. Baseline plasma samples were taken at week 6, before rechallenge to assess relative immunogenicity of FVIII and PS-FVIII formulations. Both PS-FVIII (200nm) and PS-FVIII (2um) reduces anti-FVIII antibody responses at baseline. Total anti-FVIII antibody titers (a) and anti-FVIII Nabs (b) at baseline.

Figure 3.

Animals were immunized with the different treatment groups according to the immunization protocol described in Fig 1. Plasma samples were taken at week 11 to assess tolerance. The PS-FVIII (200nm) is more effective and durable in inducing tolerance to FVIII. Total anti-FVIII antibody titers (a) and anti-FVIII Nabs (b) after rechallenge.

Figure 4. Comparison of titer levels before and after rechallenge with free FVIII; total anti-FVIII antibody titers (a) and anti-FVIII Nabs (b).

(MFI $\pm$ SD)	LPS	Free FVIII	PS-FVIII (200 nm)	PS-FVIII (2um)
CD40	417 $\pm$ 9.24	174 $\pm$ 34.2	97.3 $\pm$ 20.4	162.3 $\pm$ 24.7
CD 86	1161 $\pm$ 9.49	417 $\pm$ 15.6	404 $\pm$ 6.36	445 $\pm$ 15.2

Figure 5.

The PS-FVIII (200nm) is effective in converting dendritic cells to a tolerogenic phenotype. *In vitro* studies were conducted with bone marrow derived dendritic cells (BMDCs) to determine if the particle size affects the processing by the BMDCs. Phenotypic characterization of BMDCs exposed to FVIII alone or in the presence of PS (200nm) or PS (2um) was done using flow cytometry. A representative histogram plot showing CD40 expression (a) and CD86 expression (b). (c) Table showing the Median Fluorescence Intensity (MFI) of CD40 and CD86 expression. The PS-FVIII (200nm) showed a decrease in CD40 expression and a slight decrease in CD86 expression. (d) TGF- β production by BMDCs following exposure to FVIII alone or in the presence of PS (200nm) or PS (2um). The PS-FVIII (200nm) showed a significant increase in TGF- β production similar to the tolerogenic control (Vit D3 + Dex).

TABLE 1

Biophysical characterization of PS preparations: Table summarizes the biophysical properties of PS liposomes including protein:lipid ratio, size, % association, lamellarity and protein:lipid topology.

Biophysical Property	PS-FVIII (200nm)	PS-FVIII (2um)
Protein: Lipid Ratio	1:10000	1:10000
Size (Mean $\pm$ SD)	194 $\pm$ 61.2 nm	2320 $\pm$ 271 nm
% Association ⁵	47%	100%
Lamellarity	Small Unilammelar Vesicles (SUV)	Multilamellar Vesicles (MLV)
Protein-lipid Topology ^{7,35}	Surface association via C2 domain	Surface association and incorporation into bilayer as a result of multilamellarity