Published in final edited form as:

J Pharm Sci. 2012 January ; 101(1): 48–55. doi:10.1002/jps.22746.

Down-regulation of CD40 signal and induction of TGF- β by Phosphatidylinositol mediates reduction in immunogenicity against recombinant human Factor VIII

Puneet Gaitonde¹, Aaron Peng¹, Robert M. Straubinger¹, Richard B. Bankert², and Sathy V. Balu-Iyer^{1,*}

¹Department of Pharmaceutical Sciences, University at Buffalo, State University of New York Amherst, NY 14260

²Department of Microbiology and Immunology, University at Buffalo, State University of New York Amherst, NY 14260

Abstract

Factor VIII (FVIII) is an important coagulation cofactor and its deficiency causes Hemophilia A, a bleeding disorder. Replacement therapy using recombinant FVIII is currently the first line of therapy for Hemophilia A, but the development of neutralizing antibody is a major clinical complication for this therapy. Recently, it has been shown that FVIII associated with Phosphatidylinositol (PI) containing lipidic nanoparticles reduced development of neutralizing antibodies in Hemophilia A mice (1). Here, we investigated the underlying mechanism of this reduction in antibody response in culturing conditions. *In vitro*, PI interfered with the processing of FVIII by cultured dendritic cells, resulting in a reduction in the up-regulation of phenotypic co-stimulatory signal CD40. Furthermore, PI increased secretion of regulatory cytokines Transforming Growth Factor beta1 (TGF- β 1) and Interleukin 10 (IL-10) but reduced the secretion of pro-inflammatory cytokines IL-6 and IL-17. The data suggests that PI reduces the immunogenicity of FVIII by modulating DC maturation and by secretion of regulatory cytokines.

Keywords

Biotechnology; Phospholipids; Nanoparticle; Therapeutic protein; Immunogenicity; Immunology; Dendritic cell; Cell-culture; Co-stimulatory signal; Transforming Growth Factor- β

Introduction

Factor VIII (FVIII) is a large protein containing six domains (NH₂-A₁-A₂-B-A₃-C₁-C₂-COOH), and it is a critical cofactor in the blood coagulation cascade (2, 3). FVIII deficiency or dysfunction causes Hemophilia A, a bleeding disorder. Administration of exogenous FVIII is currently the most common therapy (4). Unfortunately, 15-35% of patients develop FVIII neutralizing antibodies that abrogate the activity of the protein, thus complicating

*Corresponding Author: (Formerly Sathyamangalam V. Balasubramanian Ph.D.) Department of Pharmaceutical Sciences 521 Hochstetter Hall, University at Buffalo State University of New York Amherst, NY 14260 Telephone: (716) 645-4836 Fax: (716) 645-3693 svb@buffalo.edu.

FVIII replacement therapy and clinical management of the disease (4-6). Formation of antibodies following administration of a protein therapeutic can have a profound impact on its pharmacology and efficacy (7). The antibodies can abrogate protein activity and/or alter its pharmacokinetic properties (8-11). Therefore, less immunogenic FVIII would improve the safety and efficacy of replacement therapy. Recently, we showed that FVIII associated with Phosphatidylinositol (PI) reduces antibody development against FVIII in Hemophilia A mice (1). The underlying mechanism by which PI reduces antibody development is not clear but would be helpful to realize broader therapeutic potential of PI.

The processing and presentation of the therapeutic protein by antigen presenting cells (APCs) in the context of the Major Histocompatibility Complex (MHC), interaction of APCs and T-cells mediated by MHC - T-cell receptor (TCR) interaction (in the presence of co-stimulatory signals and cytokine support), followed by T-cell maturation, T-cell - B-cell interaction and B-cell maturation (9, 12, 13) constitute immune response against therapeutic proteins. We investigated the effect of PI on initial stages of immune response, uptake and presentation by APC and cytokine secretion to determine the mechanism of reduction in immune response. The data show that PI interfere with processing and presentation of FVIII by dendritic cells (DC) to naïve T-cells and further modulate the immune response against FVIII by secreting regulatory cytokines.

Materials and Methods

Materials

Albumin-free, recombinant full-length FVIII expressed in CHO cells (Baxter Health Care Duarte, CA) and reconstituted in Tris buffer was used for the studies. Dimyristoyl phosphatidylcholine (DMPC), soybean Phosphatidylinositol (PI), Dimyristoyl phosphatidylglycerol (DMPG) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). The cytokine ELISA kits were obtained from R&D Systems Inc., (Minneapolis, MN). Endosafe-Endochrome-K® endotoxin testing kit was purchased from Charles River Inc. (Cambridge, MA).

Preparation of Protein- Lipid (PI, PC and PG) particles

Lipidic particles were prepared by hydration of a thin lipid film consisting of 50:50:5 (mole percent) DMPC: soy PI: cholesterol (or DMPC: DMPG: cholesterol) and 100:5 (mole percent) DMPC: cholesterol; with Tris buffer (TB) (25mM Tris, and 300mM NaCl, pH=7.0). Multilamellar vesicles (MLV) were extruded through double polycarbonate membranes of 80 nm pore size (GE Osmonics Labstore, Minnetonka, MN) in a high-pressure extruder (Northern Lipids Inc., Burnaby, BC, Canada) and then sterile-filtered through a 0.22 µm Millex™-GP filter unit (Millipore Corporation, Bedford, MA). The concentration of phospholipid and its recovery was determined by inorganic phosphorous assay (14). Particle size was determined using a Nicomp Model CW 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA). The protein was added to lipidic particles at 37°C, and during this process, the Ca²⁺ ion concentration in TB was adjusted to ensure optimal lipid-Ca²⁺ interaction and lipid phase change. The presence of surfactants, carrier protein and other organic solvents can interfere with loading of the protein in the

particle and care was taken to eliminate them from the preparation. The protein: lipid ratio was maintained at 1:10,000, and the total protein and lipid concentrations were same for all experiments, unless stated otherwise. All formulations were tested for endotoxin (Endosafe-Endochrome-K®, Charles River, MA) and the level was below the detection limit of the kit.

Animals

A colony of breeding pairs of Hemophilia A mice (C57BL/6J with a target deletion in exon 16 of the FVIII gene) was established and animals of 8-12 weeks' age were used for the isolation of bone marrow derived DC and FVIII specific T-cells.

Animal Experiments and Ethics Policy

All animal experiments were approved and performed according to the guidelines of Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo.

Isolation and Characterization of Dendritic cells

Dendritic cells were isolated from the bone marrow of naïve hemophilic mice as described previously by Lutz et al (15). Briefly, on day 0, approximately, 2×10^6 bone marrow cells in 10 ml of DC-media (RPMI-1640 media, 10 % heat-inactivated and sterile fetal bovine serum (FBS), 200 U / ml recombinant murine Granulocyte-macrophage colony-stimulating factor (rmGM-CSF, Peprotech Inc., Rocky Hill, NJ), 100 U / ml Penicillin, 100 µg / ml Streptomycin, 50 µM 2-mercaptoethanol, and 2 mM L-glutamine) were plated in sterile petri plates and incubated at 37°C and 5 % CO₂. 10 ml of additional fresh DC-media was added to the plates on Day 3. On day 6 and 8, half of the supernatant was aspirated, centrifuged at 300 x g for 10 min at 4°C and the supernatant was discarded. The cell pellet was resuspended in 10 ml of fresh DC-media and added back to the plate. On day 10, the cells were harvested for future experiments. This prolonged culture procedure reduces granulocyte contamination and yields a purity of about 70-80% mature and immature dendritic cells. A small fraction of other cell types which include granulocytes and B-cells is present and their interaction cannot be ruled out (15). In order to verify sample purity and the differentiation of bone marrow cells into dendritic cells, the expression of DC surface markers MHC-II, CD11c, CD40, CD80, and CD86 were monitored using flow cytometry. The presence of immature DC (iDC) was confirmed by exposure to lipopolysaccharide (LPS); a well-known DC maturation agent.

Dendritic cell uptake studies

HPTS-containing Lipidic particle Preparation—Fluorescent, pH-sensitive dye (hydroxypyrene trisulfonate; HPTS) was used to monitor endocytic uptake of the particles (16). Lipid films (10 µmol) were rehydrated and vortexed in a 35 mM HPTS solution. The resulting vesicles were extruded through double polycarbonate filters with pore diameter of 0.1 µm. The HPTS-containing particles were separated from the free HPTS using a Sephadex G-75 column (1 × 30 cm). The column was equilibrated with the Tris buffer (140 mM NaCl, 25 mM Tris, pH 7.4). Each lipid particle preparation was loaded to the column and 0.5 ml of eluent was collected for each fraction. 200 µl of each fraction was added to the 96-well plate and the fluorescence intensity was determined using a microplate

spectrofluorometer (Spectra Max Gemini, Molecular Devices; Sunnyvale, CA). The sample was excited at 413 nm and the fluorescence emission was detected at 515 nm. Fraction numbers and fluorescence intensity were plotted in linear or log scale and was used to select HPTS-containing particles for the dendritic cell uptake study. Phosphate assay was performed to estimate concentration of phospholipid and its recovery.

Dendritic Cell Uptake Study—Dendritic cells were harvested on day 8 of the dendritic cell culture. Briefly, culture supernatant was collected and centrifuged at $300 \times g$ for 10 min at 4°C . The cell pellet was resuspended in RPMI-1640 media and counted under the microscope using a hemocytometer in the presence of Trypan Blue solution (1:1 cell aliquot to Trypan Blue volume). 1×10^6 cells in 4 ml of DC-media were seeded per 35×10 mm tissue culture dish (Becton Dickinson and Company, Franklin Lakes, NJ) that contained a pre-sterilized 22×22 mm micro cover glass (No. 1.5). Cells adhere to the cover glass upon 24 hr of culture and were used for the uptake study. Cells grown on coverslips were washed three times with Tris buffer (140 mM NaCl, 25 mM Tris, 0.36 mM CaCl_2 , 0.42 mM MgCl_2 , pH 7.4) and then were treated with HPTS-containing particles. Briefly, 500 μM of particle preparation (0.1 $\mu\text{mol}/200 \mu\text{l}$) was added to each coverslip of 1×10^6 cells in the dish. Cells were incubated for 30 min at 37°C in the humidified incubator, and were washed three times with Tris buffer. The cell-grown coverslip was carefully mounted (cells facing down) onto a $3'' \times 1'' \times 1.0$ mm microscope slide and fluorescence was observed (Figure 1).

Processing and Presentation of Antigen and Dendritic Cell Maturation—On day 9, immature bone marrow derived dendritic cells (BMDC) from naïve hemophilic mice were divided into groups in 6-well plates. The cells were incubated for 24 hr (Day 10) at 37°C and 5 % CO_2 with free FVIII (2 $\mu\text{g}/\text{ml}$), FVIII-lipid particles or blank particles in DC-media. The cells were then washed by centrifugation at $300 \times g$ for 10 min at 4°C with sterile PBS. Viable cells were counted using a hemocytometer and trypan blue was used to assess viability and 1×10^6 viable DC were added to flow-cytometry tubes. The cells were washed again by centrifugation and incubated with 'Fc-blockTM' antibody on ice. Later, the cells were labeled with fluorescently tagged anti-CD40, anti-CD86 or anti-MHC-II antibodies on ice. Appropriate media controls also were included in the study. Finally, 0.5ml of 2% ultra-pure para-formaldehyde was added to each tube. The expression level of the cell surface markers was determined using a BD[®] FACS Calibur flow-cytometer. Representative plots of three independent studies are shown in Figure 2 and 3.

Cytokine Analysis—Female hemophilia A mice were immunized with free FVIII weekly via the s.c. route for two consecutive weeks. On the third day after immunization, the animals were sacrificed and CD4^+ T-lymphocytes were isolated from spleen using a commercially available CD4^+ negative isolation kit (Invitrogen Inc., CA). Bone marrow was isolated from the femurs and tibiae of naïve hemophilia A mice and differentiated into iDC for 9 days. These Day 9 iDC were challenged with different lipidic FVIII formulations for 24hr (Day 10) and washed by centrifugation. On day 10, 7×10^4 iDC which were previously exposed to the various formulations on day 9 were co-cultured with 2×10^5 splenic CD4^+ T-cells in quadruplicate, in 96-well culture plates at 37°C and 5 % CO_2 . After 72 hr of co-culture, the plates were centrifuged at $300 \times g$ for 10 min at 4°C , and the supernatant was

collected for cytokine analysis using a commercially available ELISA kit (R&D systems, MN). Each point represents data from an individual animal ($n = 3$) (Figure 4).

Statistical Analysis—One-way ANOVA with Dunett's and Bonferroni's post-hoc analysis was performed using SPSS 16 statistical software (IBM Corp. Armonk, NY). $P < 0.05$ was considered as a statistically significant difference.

Results and Discussion

FVIII was associated with PI containing nanoparticles as described in the *Materials and Methods* section. Unincorporated protein was separated from protein associated with lipidic particles using flotation on Dextran gradients, and the concentration of the protein was estimated using both activity and spectroscopic assays. The developed loading procedure yielded an association efficiency of $72 \pm 9\%$ of the added protein. The protein retained biological activity as measured by *in vitro* (activated partial thromboplastin time and chromogenic assays) and *in vivo* assay (1).

The association of FVIII with PI was previously shown to reduce antibody responses in Hemophilia A mice. Neutralizing titers were reduced significantly in animals given FVIII-PI compared to animals receiving free FVIII (1). *In vitro* studies aimed at understanding the mechanisms underlying the observed reduction in antibody responses mediated by PI nanoparticles were carried out. Immune responses against therapeutic proteins involve several steps, including processing and presentation of the protein by antigen presenting cells (APCs), and interaction of APCs and T-cells mediated by MHC II - T-cell receptor (TCR) interaction (in the presence of co-stimulatory signals), followed by T-cell maturation, T-cell - B-cell interaction and B-cell maturation. The key first step in this process is antigen uptake and processing by antigen presenting cells.

Does PI interfere with antigen uptake by antigen presenting cells?

The presence of PI in lipid particles reduces binding of complement proteins, confers “stealth-like” properties, and reduces uptake by Kupffer cells (17). These results suggest PI has an effect upon macrophages, but its effect and uptake by dendritic cells is not clear and PI may reduce the uptake of particles and its cargo. Because DC are important APCs and the primary initiators of immune response toward FVIII (18), DC isolated from naïve hemophilic mice were employed in these studies. In order to investigate whether cellular association and uptake by DC might be less for PI, the FVIII-PI complexes were labeled with the fluorescent, pH-sensitive probe (hydroxypyrene trisulfonate; HPTS) to monitor their endocytic uptake (16). Cationic liposomes containing N-[1-(2,3-dioleoyloxy)propyl] – N,N,N trimethyl ammonium methylsulfate (DOTAP) were used as a positive control, based on previous studies showing high uptake of cationic liposomes by DC (19). After 30 min of incubation of $0.1 \mu\text{mol}$ of PI particles with DC, limited cell-associated fluorescence was observed (Figure 1 Left column), in contrast to the high levels observed following incubation of DC with cationic liposomes (Figure 1 center column) (19). Due to limited cell associated fluorescence of PI particles, a clear image of uptake could not be acquired under identical exposure and other microscope setting conditions. The limited uptake was further confirmed by double labeling of DCs and by flow cytometry, which showed that fluorescent

PI-containing lipid particles were associated with a small fraction of the total DC population (data not shown). The uptake of PI complexes by CD11c-expressing cells was investigated by incubating cells with PI complexes labeled with rhodamine phosphatidylethanolamine (Rho-PE) and staining DC with FITC labeled monoclonal anti CD11c antibody. The fraction of cells positive for both Rho-PE and FITC was monitored by flow cytometry and fluorescence microscopy (Figure 1 right column). As is clear from the figure (Figure 1 right column: top, middle and bottom panels), only a small fraction of DC are double labeled, suggesting a limited uptake of PI complexes by iDC.

The spectral properties of HPTS make it possible to image not only total cell-associated particles (violet illumination, 390-410 nm) but also those in a non-acidified environment (blue illumination, 410-450 nm), thus providing a rapid visual capability to determine the extent of endocytic uptake of the complexes(16). Dual-wavelength imaging provide evidence for the limited endocytosis of some lipid particles within 30 min of initial exposure of DC to PI-containing particles and could likely to be mediated by cell surface receptors. Thus, while the effect of PI may be to alter cellular association of lipidic complexes with cells, it may also exert effects upon the subsequent processing of FVIII by DC.

Does PI influence processing and presentation of FVIII?

Following the uptake of antigens, APCs process and up-regulate MHC-II for efficient presentation of antigenic peptides to naïve T-cells. The antigen processing also typically results in the maturation and migration of DC. Upon maturation, DC display phenotypic changes that involve up-regulation of co-stimulatory signals such as CD40 and CD86. In order to investigate whether PI interferes with phenotypic changes in FVIII-mediated maturation of DC, the DC were exposed to FVIII or FVIIIPI/PC/PG complexes and the up-regulation of CD40, CD86 and MHC-II was monitored by flow cytometry.

The exposure of murine bone marrow derived dendritic cells from Hemophilia A mice to human FVIII showed an unexpected increase in expression level of co-stimulatory marker CD40, but the extent of expression was less than what was observed following 2 µg of lipopolysaccharide (LPS) stimulation. Waters et al (20) observed that the up-regulation of maturation markers in human DC upon interaction with FVIII occurs only in *in vivo* conditions, but not in *in vitro* culture conditions. A possible cause for this discrepancy was not clear, but could be in part due to the *in vivo* microenvironment within which FVIII is presented to the cells of the immune system (20). However, the mannose receptor mediated endocytosis of FVIII by human DCs effectively presents FVIII to T-cells. This efficient presentation and processing of FVIII by dendritic cells, support the fact that FVIII causes activation of dendritic cells (20). A control experiment was carried out in which DCs isolated using an identical procedure from wild type, Hemophilia A mice, C3H/HeJ LPS-resistant mice and Von Willebrand Factor knockout mice ($vWF^{-/-}$ mice) were exposed to the same batch of FVIII (Figure 2). If the up-regulation of co-stimulatory marker was due to the presence of immunologically active molecules/contamination (such as LPS) in the FVIII formulation or due to experimental conditions such as DC isolation and culturing, an increase in the level of the co-stimulatory marker in all mice samples would be expected. The flow cytometric analysis showed surprising, but reproducible results of up-regulation

only in DC from Hemophilia A mice, and no up-regulation of CD40 was observed following FVIII exposure on DC from wild type, C3H/HeJ and vWf knockout mice (mice strains that have endogenous FVIII). On the basis of these studies, we speculate that FVIII is an effective antigen and/or presence of an immunologic stimulator/factor present either in FVIII (thrombin (21), glyco groups, aggregates, degradation or presence of sub measurable TLR activators such as less than 0.05 EU/ml of LPS) or in its formulation and/or complex interaction between dendritic cells and other cell types, impart the ability to DCs from Hemophilia A mice to distinguish between self and non-self proteins. This discrimination between self and non-self proteins could be mediated by C-type lectin receptors and its cross talk with cell surface receptors such as toll like receptors (22). However, more studies are necessary to further investigate the underlying cause of the up-regulation of co-stimulatory signals in dendritic cells isolated from Hemophilia A mice upon human FVIII exposure §.

The uptake and processing of FVIII in free form or associated with lipid particles of differing composition was investigated to determine the effect of the lipid structures on the maturation of iDC. Controls included FVIII mixed with lipid particles (1:10,000 mol ratio) containing the neutral phospholipid PC or by charge-matching the PI-containing particles by substituting the anionic phospholipid PG. Protein-free lipid particles were tested as well. Observations by Rossi et al (23) and Ewenstein et al (24) showed that blockade of CD40-CD40L interaction leads to reduced immune response against FVIII in mice and in humans. The effect of lipidic particle on the FVIII mediated up-regulation of CD40 in particular was investigated. An up-regulation of CD40 was observed following the exposure of FVIII. This is consistent with previous observation of a significant up-regulation of CD40 by Qadura et al (22) following 12 hr exposure of bone marrow derived DC with canine FVIII. Minimal, but not significant, up-regulation of MHC-II and CD86 was also observed (data not shown). It is appropriate to mention here that, we observed the up-regulation of CD86 and MHC-II possibly due to difference in the culturing condition in the present study wherein, IL-4 was not added to the cell culture and also included prolonged culturing and prolonged exposure of human full-length FVIII to DC as per the method of Lutz (15). The prolonged culturing has been shown to enhance purity and yield of immature and mature dendritic cells. The culturing from day 8-10 is optimal for T-cell presentation and is associated with progressively increasing over-expression of MHC-II (15). Qadura et al also have shown that there is genetic strain dependent difference in FVIII immunogenicity in mice model (25). The anti-FVIII antibody response and the extent of increase in the up-regulation of CD40 and MHC-II level in antigen presenting cells observed in C57BL/6 mice was higher than Balb/c mice (25).

Up-regulation of the co-stimulatory marker CD40 was clearly reduced following exposure of DC to FVIII complexed with PI (Figure 3), whereas particles containing PC and PG did not reduce CD40 expression and the expression level was similar to that of free FVIII ($p < 0.05$). PI alone, in the absence of FVIII, had minimal observable effect on the maturation of

§It is appropriate to mention here that freshly reconstituted bulk protein formulated for clinical use (Advate®) showed minimal or no up-regulation of co-stimulatory signals upon exposure to dendritic cells, but showed up-regulation in the presence of sub-measurable concentration of LPS (less than 0.05 EU/mL). However, the administration of formulated proteins for clinical use such as Advate® and Refacto® elicited intense anti-FVIII antibody response in this mice model and complexation of these proteins with phospholipids reduced the development of the antibody response.

dendritic cells[¥]. It is appropriate to mention here that it has been shown that prolonged culturing conditions progressively increase expression of MHC II (15). The altered uptake of FVIII mediated by PI leads to inhibition of the expression of co-stimulatory signals such as CD40 on DC and to their inefficient activation and maturation which can lead to reduction in antibody response. Such effects upon DC by protein-carrying PI particles could result in tolerizing rather than activating T-cells. This is consistent with our observation that the mean stimulation index (SI), which is the ratio of ³H-thymidine incorporation in the presence of antigen vs. the absence of antigen, was lower for PI treatment group compared to CD4⁺ T-lymphocytes harvested from animals treated with free FVIII (1). In addition to co-stimulatory signals, T-cell activation also requires secretion of cytokines.

Does PI alter cytokine profile following DC and T-cell interaction?

TGF- β plays a critical role in regulating T-cell dependent immune responses (26), and therefore the possible contribution of TGF- β and other immuno-inhibitory cytokines to the decrease in the observed *in vivo* antibody response to FVIII-PI was examined. CD4⁺ T-cells isolated from animals administered free FVIII were co-cultured with dendritic cells that were challenged with either free FVIII or with lipidic nanoparticle formulations of FVIII, and cytokine secretion was quantified by ELISA. The results (Figure 4) show a significant ($p < 0.05$) increase in the level of regulatory cytokines such as TGF- β 1 (Figure 4.A) and IL-10 (Figure 4.B) upon challenge with FVIII-PI particles compared to the response following challenge with free FVIII, lipidic FVIII formulations from which PI was omitted (*i.e.*, pure PC), or charge-matched formulations in which PG was substituted for PI (Figure 4). Also, FVIII-PG had significantly reduced levels ($p < 0.05$) whereas, FVIII-PC exhibited similar levels ($p > 0.05$) of these regulatory cytokines when compared to free FVIII. The secretion of pro-inflammatory cytokines such as IL-6 (Figure 4.C) and IL-17 (Figure 4.D) levels was significantly lower upon challenge with FVIII-PI, compared to challenge with free FVIII and the other lipidic FVIII formulations ($p < 0.05$). Further, FVIII-PC and FVIII-PG exhibited similar levels ($p > 0.05$) of these pro-inflammatory cytokine when compared to free FVIII. Another anionic lipid Phosphatidylserine (PS) exposed to human peripheral blood DC also showed reduction in up-regulation of co-stimulatory signals but had no effect on regulatory cytokines (27).

The underlying mechanism of PI mediated effect could be due to multiple mechanisms. As we recently showed that PI particle shields substantial surface area of protein involving several epitopes within the protein, this shielding may lead to an immunological ignorance (1). In addition, immunomodulatory properties of PI could play a significant role in the observed inhibition of the response to FVIII-PI. It has been shown that PI directly inhibits T-lymphocytes (28) and could contribute to observed reduction in the antibody response to FVIII. However, the study design presented here involves exposure of PI to DC and not to T-lymphocytes. Thus the observed changes are due to the direct effect of PI on DC maturation and presentation, but not on T-lymphocytes. Our data establish that the exposure of PI to DC results in the low expression of co-stimulatory signal (CD40) and high production of TGF- β and IL-10. These functional characteristics correspond to those that

[¥]PI particles associated with FVIII also reduced the up-regulation of MHC-II and CD86 on DC.

have been observed previously with tolerogenic DC (29). One of the mechanisms by which tolerogenic DC can modulate immunogenicity is by inducing tolerance via regulatory T-cells (Tregs). Previous studies (26, 30) have established that TGF- β 1 induces the generation of Tregs, while TGF- β 1 with IL-6 skews T-cells into IL-17 producing (T_H17) cells. On the basis of these findings, our data is consistent with the possibility that FVIII-PI may polarize the generation of naïve T cells into Tregs by up-regulating TGF- β 1 and suppressing of IL-6. An expansion of Tregs could explain the decreased T cell dependent anti-FVIII antibody response observed with FVIII-PI.

The precise mechanism by which FVIII-PI reduces FVIII antibody responses *in vivo* is complex and remains to be further defined. It is now important to determine whether the decrease is a reflection of Treg-mediated suppression of specific immune responses and/or immune tolerance that would result in a durable, specific, non-responsiveness/tolerance to subsequent exposures to the antigen (31). In conclusion, the approach of complexing a therapeutic protein with lipidic PI nanoparticles results in an immune ignorance and possibility of a tolerance induction which is not yet completely understood, but potentially highly beneficial to patients.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01 HL-70227) to SVB. We are grateful to Drs. Kazazian and Sarkar of the University of Pennsylvania for providing the initial FVIII knockout mice model, and to Dr. Bernstein of Western New York Hemophilia Foundation, for providing albumin-free recombinant Factor VIII. We thank Dr. Raymond Kelleher for his guidance with flow-cytometry.

REFERENCES

1. Peng A, Straubinger RM, Balu-Iyer SV. Phosphatidylinositol containing lipidic particles reduces immunogenicity and catabolism of factor VIII in hemophilia a mice. *AAPS J*. 2010; 12(3):473–81. Epub 2010/06/03. [PubMed: 20517659]
2. Foster PA, Zimmerman TS. Factor VIII structure and function. *Blood Rev*. 1989; 3(3):180–91. Epub 1989/09/01. [PubMed: 2506958]
3. Fay PJ. Factor VIII structure and function. *Thromb Haemost*. 1993; 70(1):63–7. Epub 1993/07/01. [PubMed: 8236116]
4. Klinge J, Ananyeva NM, Hauser CA, Saenko EL. Hemophilia A--from basic science to clinical practice. *Semin Thromb Hemost*. 2002; 28(3):309–22. Epub 2002/07/05. [PubMed: 12098093]
5. Fijnvandraat K, Bril WS, Voorberg J. Immunobiology of inhibitor development in hemophilia A. *Semin Thromb Hemost*. 2003; 29(1):61–8. Epub 2003/03/18. [PubMed: 12640567]
6. Lollar P. Molecular characterization of the immune response to factor VIII. *Vox Sang*. 2002; 83(Suppl 1):403–8. Epub 2003/03/06. [PubMed: 12617176]
7. Ferraiolo, BL.; Mohler, MA.; Gloff, CA. Protein pharmacokinetics and metabolism. Plenum; New York: 1992.
8. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther*. 2002; 24(11):1720–40. discussion 19. Epub 2002/12/28. [PubMed: 12501870]
9. Braeckman, R. Pharmacokinetics and pharmacodynamics of protein therapeutics.. In: Reid, RE., editor. Peptide and protein drug analysis. Marcel Dekker, Inc; New York: 2000. p. 633-69.
10. Braun A, Kwee L, Labow MA, Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res*. 1997; 14(10):1472–8. Epub 1997/11/14. [PubMed: 9358564]
11. Scherthaner G. Immunogenicity and allergenic potential of animal and human insulins. *Diabetes Care*. 1993; 16(Suppl 3):155–65. Epub 1993/12/01. [PubMed: 8299472]

12. Tang L, Persky AM, Hochhaus G, Meibohm B. Pharmacokinetic aspects of biotechnology products. *J Pharm Sci.* 2004; 93(9):2184–204. Epub 2004/08/06. [PubMed: 15295780]
13. Chirino AJ, Ary ML, Marshall SA. Minimizing the immunogenicity of protein therapeutics. *Drug Discov Today.* 2004; 9(2):82–90. Epub 2004/03/12. [PubMed: 15012932]
14. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem.* 1959; 234(3):466–8. Epub 1959/03/01. [PubMed: 13641241]
15. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999; 223(1):77–92. Epub 1999/02/26. [PubMed: 10037236]
16. Straubinger RM, Papahadjopoulos D, Hong KL. Endocytosis and intracellular fate of liposomes using pyranine as a probe. *Biochemistry.* 1990; 29(20):4929–39. Epub 1990/05/22. [PubMed: 2163672]
17. Gabizon A, Shiota R, Papahadjopoulos D. Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long circulation times. *J Natl Cancer Inst.* 1989; 81(19):1484–8. Epub 1989/10/04. [PubMed: 2778836]
18. Dasgupta S, Navarrete AM, Andre S, Wootla B, Delignat S, Repesse Y, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica.* 2008; 93(1):83–9. Epub 2008/01/02. [PubMed: 18166789]
19. Foged C, Arigita C, Sundblad A, Jiskoot W, Storm G, Frokjaer S. Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. *Vaccine.* 2004; 22(15- 16):1903–13. Epub 2004/05/04. [PubMed: 15121302]
20. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *Journal of thrombosis and haemostasis.* 2009; 7(9):1446–56. [PubMed: 19583822]
21. Skupsky, Zhang, Su, Scott. A role for thrombin in the initiation of the immune response to therapeutic Factor VIII. *Blood.* 2009
22. Qadura M, Othman M, Waters B, Chegeni R, Walker K, Labelle A, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *Journal of thrombosis and haemostasis.* 2008; 6(12):2095–104. [PubMed: 18826393]
23. Rossi G, Sarkar J, Scandella D. Long-term induction of immune tolerance after blockade of CD40-CD40L interaction in a mouse model of hemophilia A. *Blood.* 2001; 97(9):2750–7. [PubMed: 11313267]
24. Ewenstein BM, Hoots WK, Lusher JM, DiMichele D, White GC 2nd, Adelman B, et al. Inhibition of CD40 ligand (CD154) in the treatment of factor VIII inhibitors. *Haematologica.* 2000; 85(10 Suppl):35–9. Epub 2001/02/24. [PubMed: 11187868]
25. Qadura M, Waters B, Burnett E, Chegeni R, Hough C, Othman M, et al. Immunoglobulin isotypes and functional anti-FVIII antibodies in response to FVIII treatment in Balb/c and C57BL/6 haemophilia A mice. *Haemophilia : the official journal of the World Federation of Hemophilia.* 2010 Epub 2010/11/26.
26. Li MO, Flavell RA. TGF-beta: a master of all T cell trades. *Cell.* 2008; 134(3):392–404. Epub 2008/08/12. [PubMed: 18692464]
27. Chen X, Doffek K, Sugg SL, Shilyansky J. Phosphatidylserine regulates the maturation of human dendritic cells. *J Immunol.* 2004; 173(5):2985–94. Epub 2004/08/24. [PubMed: 15322157]
28. Caselli E, Baricordi OR, Melchiorri L, Bellini F, Ponzin D, Bruni A. Inhibition of DNA synthesis in peripheral blood mononuclear cells treated with phosphatidylserines containing unsaturated acyl chains. *Immunopharmacology.* 1992; 23(3):205–13. Epub 1992/05/01. [PubMed: 1500286]
29. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol.* 2007; 7(8):610–21. Epub 2007/07/14. [PubMed: 17627284]
30. Wan YY, Flavell RA. TGF-beta and regulatory T cell in immunity and autoimmunity. *J Clin Immunol.* 2008; 28(6):647–59. Epub 2008/09/17. [PubMed: 18792765]
31. Horwitz DA, Zheng SG, Gray JD. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol.* 2008; 29(9):429–35. Epub 2008/08/05. [PubMed: 18676178]

Figure 1.

DC uptake of PI particles (Left column) and DOTAP containing liposomes (Middle column) (63X magnification; Top panels: *bright field image*; Middle panels: *FITC staining*; Bottom panels: *HPTS-dye*). Dendritic cell uptake of Rho-PE containing PI particles (Right column; 20X magnification; Top panel: *bright field image*; Middle panel: *FITC anti-CD11c staining*; Bottom panel: *Rho-PE*).

Figure 2. Up-regulation of Dendritic cell surface marker CD40, upon exposure to antigen

Naïve Dendritic cells from Wild-type (C57BL/6J, Violet), LPS-resistant (C3H/HeJ, Orange), VonWillebrand Factor knock-out (vWF^{-/-}, Green) and Factor VIII knock-out (HA, Blue) mice were exposed to free Factor VIII for 24 hr in culturing conditions.

A positive control group of naïve DC from HA mice were exposed to LPS (Red). Appropriate isotype control signal also is shown (Black). The vertical line marker was chosen based on the untreated DC grey peak. The figure shown is representative of three independent studies.

Figure 3. Bar plot of expression level of cell surface co-stimulatory marker CD40 after DC exposure to free FVIII or FVIII-lipid particles (PI, PC, PG) for 24h

CD40 expression level was analyzed by flow-cytometry using fluorescently-tagged anti-CD40 antibody. The data is from three independent studies (Mean Fluorescence Intensity $\pm$ SEM).

Figure 4. Cytokine analysis of supernatants by ELISA from FVIII or FVIII-lipid (PI, PC and PG) exposed dendritic cells and FVIII immunized splenic CD4⁺ T-cells co-culture (A) TGF- β 1, (B) IL-10, (C) IL-6 and (D) IL-17

Level of cytokines in media (negative) control was below the detection limit of the respective ELISA kits.