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Factor VIII-Pulsed Dendritic Cells Reduce Anti-F.VIII Antibody Formation in the Hemophilia A Mouse Model

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Abstract

Objective—Hemophilia inhibitor formation is a T-cell dependent immune response to infused factor VIII (F.VIII). As immature dendritic cells (DCre) regulate immune response and promote tolerance, we evaluated F.VIII-pulsed DCre, propagated from bone marrow in the presence of GM-CSF and TGFβ, in achieving F.VIII tolerance.

Methods—The effects of intravenous F.VIII-pulsed DCre in C57BL/6 hemophilia A mice were determined by total F.VIII inhibitory antibodies, T cell proliferation, thymidine uptake, cytokine profile, and surface molecule expression.

Results—After tail vein injection of 2.5 U recombinant F.VIII (rF.VIII) on day 0, 2, and 4, anti-F.VIII antibody peaked on day 6, and increased further on day 17 following rF.VIII re-challenge on day 12, 14, and 16, with increased T cell proliferative response to *in vitro* F.VIII. When mice were pretreated with 2×10^6 F.VIII-pulsed immature DCre (deficient NF-κB nuclear protein binding, low CD80, low CD86, high IL-10 phenotype) 7 days before rF.VIII challenge, anti-F.VIII was reduced on day 6 and on day 8, 0.1 ± 0.0 BU/ml (Bethesda units/ml) vs. control PBS-treated hemophilia A mice, 2.0 ± 0.1 BU/ml, $p < 0.01$. Re-challenge with rF.VIII on day 12 produced no increase in anti-F.VIII antibody response. This was associated with high serum IL-10 and low IL-2 levels by ELISA, and splenic T cell hyporesponsiveness to F.VIII, with IL-10 production, high FoxP3 expression by qT-PCR, and T regulatory cell expansion, confirmed in OVA-TCR transgenic mice.

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Conclusions—These findings suggest F.VIII-pulsed *D_{Cre}* reduce anti-F.VIII antibody formation in hemophilia A mice by induction of regulatory T cell-mediated hyporesponsiveness of T helper cells to F.VIII.

Keywords

Hemophilia; anti-F.VIII antibody; dendritic cells; regulatory T cells

Introduction

Hemophilia A is an X-linked bleeding disorder caused by deficiency of coagulation factor VIII (F.VIII), and characterized by spontaneous and traumatic bleeding. A major complication of hemophilia treatment is inhibitor formation, in which alloantibodies directed against infused factor VIII develop in up to 25% of hemophilia A patients after 9 or 10 factor VIII treatment exposures. The anti-F.VIII antibody interferes with clinical response to factor VIII infusion, resulting in significant morbidity and early mortality in affected patients [1,2]. Anti-F.VIII antibody formation is a T cell-dependent immune response [3–6], involving F.VIII peptide binding to HLA class II molecules, antigen presentation to CD4⁺ T cells, and recognition by the T cell receptor (TCR) [7–9]. Thus, there has been a growing interest in suppressing inhibitor formation by blocking F.VIII-specific T cell response.

Among professional antigen presenting cells that play a major role in immune response to foreign antigens are dendritic cells (DCs), which serve as initiators and regulators of immune response to antigen [10–13]. DCs promote immune response to foreign antigens through costimulatory molecule signaling, production of immunostimulatory cytokines, and migration to regional lymphoid tissue to interact with T cells specifically responsive to the foreign antigen, once internalized from the periphery [10,11,13].

In contrast to mature or effector DC (*D_{Ce}*) which promote antigen-specific immunity, regulatory DC, *D_{Cre}*, exhibit low level co-stimulatory CD80, low CD86 expression, and IL-10 positive, IL-12 negative phenotype, and promote self tolerance [10,11]. Recently, FoxP3, a forkhead family transcription factor [12], was shown to be highly expressed by T regulatory cells [14,15] and linked to T cell suppression [15]. Indeed, the exploitation of immature dendritic cells to prevent transplant rejection [16] and diabetes [17] in animal models, has suggested the potential role of these cells in suppression of inhibitor formation in hemophilia. We, therefore, hypothesized that administration of F.VIII-pulsed *D_{Cre}* in the hemophilia A exon 16 knockout C57BL/6 mouse, a model which predictably develops anti-F.VIII antibodies after F.VIII injection [18,19], would reduce or prevent hemophilia anti-F.VIII antibody formation.

Materials and methods

Mice and reagents

Hemophilia A C57BL/6 exon 16 knockout mice (referred to as hemophilia A mice) [18], non-hemophilic mice, C57BL/6 (B6 mice), and ovalbumin (OVA)-specific T cell (OT-II) receptor transgenic (OT-II OVA-TCR Tg) mice, all 10–12 weeks old, were obtained from The Jackson Laboratory (Bar Harbor ME) and maintained in a specific pathogen-free facility at the University of Pittsburgh Medical Center. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), University of Pittsburgh. Granulocyte-macrophage colony stimulating factor (GM-CSF), TGF- β , and IL-4 were obtained from Schering Plough Research Institute (Kenilworth NJ). Full-length recombinant F.VIII (rF.VIII, Advate®), kindly provided by Baxter Bioscience, Inc. (Westlake Village CA), was injected into the tail vein of hemophilia A mice at a dose of 2.5 U per injection, a dose previously shown

to induce anti-F.VIII antibody formation in 80% of hemophilia C57Bl/6 mice [4–6]. OVA 323–339 (ISQAVHAAHAEINEAGR) was kindly provided by Dr. Fen Lin, Case Western University (Cleveland OH). Plasma samples were obtained by tail vein. T cells were isolated from mouse spleen and enriched through a nylon wool column. Three to five hemophilia mice and three to five control mice were included in each experiment.

Dendritic cell (DC) propagation and purification

Bone marrow cells (BM) harvested from femurs of B6 mice or hemophilia A mice were cultured in 24-well plates (2×10^6 /well) in RPMI-1640 media supplemented with antibiotics and 10% (vol/vol) fetal calf serum (FCS), referred to subsequently as complete medium, and GM-CSF+IL-4 or GM-CSF+TGF- β . Dendritic cells were selected and purified from the cultures, as previously described [16]. Thus, two dendritic cell (DC) populations were propagated, one of predominantly immature DC with immunoregulatory activities (DCre) and the other of conventional DC with immunoeffector activity (DCE). DCre were propagated from mouse bone marrow and cultured in RPMI-1640 containing GM-CSF (4 ng/ml) and TGF- β (0.2 ng/ml) for 5–7 days [20]. To derive rF.VIII-pulsed DCre, the DCre were then pulsed with rF.VIII 20 U/ml for 24 hr, a dose we determined to be optimal in T proliferation assays. Collection and purification procedures were as previously described [21]. For comparison, conventional DC, professional antigen presenting cells (DCE), were propagated from bone marrow in RPMI-1640 media supplemented with antibiotics and 10% vol/vol FCS containing GM-CSF (4 ng/ml) plus IL-4 (1,000 U/ml). To derive rF.VIII-pulsed DCE, the DCE were pulsed with rF.VIII 20 U/ml for 24 hr, as above [20].

T cell proliferative assays

Purified splenic T cells were assessed for proliferative response to factor VIII by ^3H -thymidine incorporation in day 4 cultures. In this assay, T cells from hemophilia A mice treated with DCre or DCE (as described below) were incubated with rF.VIII and irradiated B6 spleen cells *in vitro*, and T cell proliferative responses were measured by thymidine uptake [20,22].

T cell labeling with CFSE and suppression assay

T cells (10^7 /ml) were labeled with $1 \mu\text{M}$ carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene OR) for 10 minutes at 37°C , and washed before culture. The intensity of CFSE was determined via flow cytometric analysis. To determine the T regulatory cell inhibitory activity, T regulatory cells (Treg) were purified using a $\text{CD4}^+ \text{CD25}^+$ regulatory T cell kit (Miltenyi Biotec, Auburn CA), and were added into CFSE-labeled reporter T cells (10^5 /well in $100 \mu\text{l}$), which were stimulated by anti-CD3 monoclonal antibody (mAb) ($2 \mu\text{g}/\text{ml}$) in the presence of antigen presenting cells (APC). In some experiments, purified spleen T cells from OVA-specific TCR transgenic (OT-II) mice were used as reporter T cells, which were re-stimulated by APC in the presence or absence of OVA peptide 323–339 ($0.3 \mu\text{M} \times 3\text{h}$). The suppressive effects of T regulatory cells were determined by CFSE dilution assay.

Flow cytometry

Expression of DC surface antigens was analyzed by cytofluorography, using an EPICS ELITE flow cytometer (Coulter, Hialeah FL). Cells were stained with primary hamster or rat monoclonal antibodies directed against CD11c, CD40, CD80, CD86 (BD PharMingen, San Diego CA), followed by FITC- or PE-conjugated goat anti-hamster or goat anti-rat IgG2a, as previously described [20,23].

To determine the effect of DCre on T regulatory cell expansion, DCE or DCre propagated from B6 mice bone marrow and co-cultured with splenic CD4^+ Tcells isolated from OVA-specific

TCR transgenic (OT-II) T cells, in the absence or presence of OVA 323–339, were stained with monoclonal antibodies against CD4 (BD, Franklin Lakes NJ) and FoxP3 (eBioscience, San Diego CA), as previously described [24]. For *in vivo* studies, DC subsets were pulsed with OVA 323–339. After washing in PBS, 5×10^5 DC were injected subcutaneously into the footpads of naïve B6 mice, followed immediately thereafter by tail vein injection of 1×10^6 splenic T cells from OVA transgenic (OVA Tg, OT-II) mice, isolated with anti-CD4-conjugated magnetic beads (Miltenyi Biotec, Auburn CA). Three days later, mice were sacrificed and cells from draining popliteal lymph nodes were harvested and stained with monoclonal antibodies to FoxP3 and TCR V α 2 (BD Biosciences, San Jose CA), as previously described [24].

Cytokine profile assays

Serum samples and supernatants from splenic T cells stimulated with rF.VIII were assayed for interleukin-10 (IL-10), interleukin-2 (IL-2), interferon-gamma (IFN- γ), and transforming growth factor beta (TGF- β) by enzyme linked immunosorbent assay (ELISA) kits (BioSource, Camarillo CA) [17].

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed using a commercially available kit (Promega, Madison WI) supplied with an NF- κ B probe oligonucleotide (sense sequence: 5'AGTTGAGGGGACTTTCCCAGGC3') end-labeled with γ - 32 P ATP (NEN, Boston MA). A 25-fold excess of unlabeled oligonucleotide was used as cold probe. Nuclear proteins (1 μ g) were loaded in each lane. NF- κ B translocation in nuclear protein was measured by mobility shift, detected by running the mixture on a 4% acrylamide gel, with visualization of shifted bands by autoradiography [17].

Anti-F.VIII antibody assay

Anti-F.VIII antibody titer was determined by Bethesda assay [25]. This was carried out by incubating mouse plasma samples with 1.25 μ g/ml recombinant human factor VIII in 0.1 mol/L carbonate-bicarbonate (pH 9.6).factor VIII, and measuring residual F.VIII by one-stage F.VIII activity, with one Bethesda unit/ml (BU/ml) defined as the amount of inhibitory activity producing 50% inhibition of F.VIII activity.

FOXP3 quantitative RT-PCR assay

FoxP3 was quantitated by real-time PCR (qT-PCR) in splenic T cells as previously described [14]. Total RNA was purified using TRIzol reagent: 1 μ g aliquot of RNA was treated with deoxyribonuclease I (Invitrogen, Carlsbad, CA). The FoxP3 specific primers (forward: GGC CCT TCT CCA GGA CAG A, reverse: GGC ATG GGC ATC CAC AGT) and 18S rRNA control primers were used in quantitative real-time PCR with an ABI Prism 7500 machine (Applied Biosystems, Foster City CA). The cycle quantity was determined with a standard curve, and expression levels were normalized to 18S rRNA.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Comparison of two means was by Student's t test. Comparison of more than two means was by ANOVA, and Student-Newman-Keuls test. Significance was set at $p < 0.05$.

Results

Hemophilia A mouse anti-F.VIII antibody model

Following injection of 2.5 U (0.2 µg) recombinant factor VIII (rF.VIII) into the tail vein of hemophilia A mice on days 0, 2, and 4, the anti-F.VIII antibodies peaked on day 6, 9.8 ± 2.2 BU/ml vs. 0 ± 0 BU/ml pre-treatment, $p < 0.01$ (Figure 1A). On re-challenge with 2.5 U rF.VIII intravenously on days 12, 14, and 16, there was a further increase in anti-F.VIII on day 16, 15.0 ± 1.3 BU/ml, $p < 0.05$, and on day 17, 24 ± 0.2 BU/ml, $p < 0.01$. Consistent with the previously recognized T cell-dependent antibody response, the development of anti-F.VIII antibody to rF.VIII treatment *in vivo* was associated with high level splenic T cell proliferation in response to rF.VIII stimulation *in vitro*, as measured by ^3H -thymidine incorporation in T cell proliferation assay (Figure 1B). At all time points, *in vitro* proliferative response to rF.VIII by T cells from F.VIII-treated hemophilia A mice was significantly greater, $p < 0.01$, than proliferative response by T cells from control PBS-treated hemophilia A mice (Figure 1B).

Characterization of immature dendritic cells (DCre)

DCre propagated from hemophilia A mouse bone marrow with GM-CSF and TGFβ exhibited low level co-stimulatory molecules CD80 and CD86 (Figure 2A). By contrast, mature DCe, propagated from hemophilia A mouse bone marrow with GM-CSF+IL-4 exhibited high level co-stimulatory molecules, CD80 and CD86. Further, DCre also demonstrated deficient NF-κB binding activity in nuclear protein in gel shifting assay (Figure 2B), as compared with DCe, which demonstrated enhanced NF-κB binding (Figure 2B). These data indicate that TGFβ contributes to suppression of co-stimulatory molecule expression on DCre, which is associated with inhibition of NF-κB translocation in nuclear protein.

rF.VIII-pulsed DCre reduce anti-F.VIII antibody in hemophilia A mice

To determine whether DCre regulate immune responses to F.VIII, 2×10^6 rF.VIII-pulsed DCre ($20 \text{ IU/ml} \times 24 \text{ h}$) were injected by tail vein into hemophilia A mice 7 days before rF.VIII was given on day 0, 2, and 4. By day 6 (day 13 after DCre), there was a reduction in anti-F.VIII antibody, mean 2.0 ± 0.1 BU/ml, (with 75% of the mice developing anti-F.VIII antibody) compared with control (PBS-treated) hemophilia A mice, 3.0 ± 0.3 BU/ml (with 90% of the mice anti-F.VIII positive) and further reduction on day 8, mean 0.1 ± 0.0 BU/ml (with 50% of the mice anti-F.VIII antibody free) vs. 2.0 ± 0.1 BU/ml, in the control group (75% remaining anti-F.VIII antibody positive) both $p < 0.01$ (Figure 3A). By contrast, high levels of anti-F.VIII antibody were detected in mice pretreated with rF.VIII-pulsed DCe, on day 6, mean 16.4 ± 0.2 BU/ml (100% anti-F.VIII antibody positive), and on day 8, mean 11 ± 0.5 BU/ml (75% anti-F.VIII antibody positive), as compared with control, each $p < 0.01$. These data indicate that rF.VIII-pulsed DCre induce reduced anti-F.VIII immune responses in hemophilia mice. By contrast, administration of F.VIII-pulsed DCe markedly augmented anti-F.VIII immune responses.

Reduced T cell proliferation is associated with reduced anti-F.VIII antibody after DCre treatment

Re-challenge of DCre-treated mice with rF.VIII *in vivo* on day 12 resulted in no increase in the anti-F.VIII antibody by day 17 (data not shown), suggesting that rF.VIII-pulsed DCre induce hyporesponsiveness to *in vivo* F.VIII challenge (injection) in hemophilia A mice. To determine the mechanism of the reduced anti-F.VIII antibody response, we evaluated immunoregulatory activity of DCre by measuring T cell proliferative responses in T proliferation assay. In this assay, splenic T cells obtained from hemophilia mice 7 days after *in vivo* treatment with 2×10^6 rF.VIII-pulsed DCre (or DCe) were restimulated *in vitro* with and without rF.VIII (Figure 3B). Splenic T cells from rF.VIII-pulsed DCre-treated mice

showed significantly lower proliferative responses to *in vitro* rF.VIII restimulation than did splenic T cells from rF.VIII-pulsed DCe-treated mice, $p < 0.01$ (Figure 3B, right). Splenic T cells from control PBS-treated mice demonstrated low proliferative responses to *in vitro* r.FVIII restimulation (Figure 3B, right). In the absence of rF.VIII restimulation *in vitro*, the proliferative responses of T cells from all groups, including from rF.VIII-pulsed DCe-treated and r.FVIII-pulsed DCe-treated hemophilia mice, remained low (Figure 3B, left). These findings indicate that T cell proliferation in culture is antigen-dependent, and that administration of rF.VIII-pulsed DCe induces T cell hyporesponsiveness that is antigen (rF.VIII)-dependent.

Cytokine profile of T cells from mice treated in vivo with rF.VIII-pulsed DCe

The reduction in anti-F.VIII antibody formation in mice following treatment with rF.VIII-pulsed DCe was also associated with changes in the serum cytokine profile measured by ELISA. There were significantly higher serum IL-10 levels in rF.VIII-pulsed DCe-treated mice as compared with rF.VIII-pulsed DCe-treated mice or DCe-treated mice, both $p < 0.01$ (Figure 3C). By comparison, no significant differences in IFN- γ , IL-2 or TGF- β levels between these groups were observed.

To further characterize the T cell differentiation induced by DC, the cytokine profile of T cells in response to F.VIII stimulation were analyzed. Splenic T cells from hemophilia A mice, 7 days after rF.VIII-pulsed-DCe or rF.VIII-pulsed-DCe or PBS treatment, were re-stimulated *in vitro* with rF.VIII and irradiated spleen cells as antigen presenting cells (APC). Cytokine profiles were analyzed on the T cell supernatants by ELISA. T cells from the rF.VIII-pulsed DCe-treated mice produced significantly higher IL-10 and lower IL-2 in T cell supernatants from the rF.VIII-pulsed DCe-treated mice, as compared with the rF.VIII-pulsed DCe treated group, $p < 0.01$ (Figure 3D). These findings suggest that rF.VIII-pulsed-DCe and -DCe treatments promote differences in T cell differentiation in hemophilia A mice, and, possibly, that rF.VIII-pulsed DCe induce T regulatory cells.

FoxP3 expression increases in response to rF.VIII-pulsed DCe

Consistent with these findings, Foxp3 levels measured by real time PCR (qT-PCR) on RNA isolated from splenic T cells from rF.VIII-pulsed DCe-treated hemophilia A mice were significantly higher than from rF.VIII-pulsed DCe-treated mice, $p < 0.01$, DCe-treated mice, $p < 0.001$, or control PBS-treated mice, $p < 0.001$ (Figure 3E).

Antigen-pulsed DCe preferentially promote T regulatory cell differentiation

To determine whether antigen-pulsed DCe preferentially promote antigen-specific T regulatory cell differentiation, *in vitro* and *in vivo* experiments were conducted on antigen-specific DCe and DCe, specifically with OVA peptide as antigen and splenic CD4⁺ T cells isolated from OVA-specific TCR transgenic mice (OT-II mice). In the *in vitro* experiments, DCe and DCe propagated from B6 mice bone marrow were cultured with splenic CD4⁺ T cells isolated from OT-II mice at 1:10 ratio DC:T cells, in the presence or absence of 0.3 μ M OVA 323–339. After 4 days, DCe presenting OVA peptide to OT-II T cells induced greater T regulatory cell expansion, by FoxP3⁺ and CD4⁺ double staining, than DCe, 12.4% vs. 7.2% (Figure 3F). In the *in vivo* experiments, DCe and DCe from B6 mice were incubated with OVA 323–339, washed, and injected subcutaneously, at 5×10^5 , into the footpads of naïve B6 mice, immediately followed by tail vein injection of 1×10^6 splenic CD4⁺ T cells from OT-II mice, identified with V α 2 mAb. Cells harvested 3–4 days later from draining popliteal lymph nodes were analyzed by flow cytometry. As can be seen in Figure 3G, more V α 2⁺ T cells from OVA-pulsed DCe-treated mice were FoxP3⁺, than from OVA-pulsed DCe-treated mice, 9.0% vs. 2.7%, or from control PBS-treated mice, 0.8%, indicating OVA-pulsed DCe administration favors expansion of T regulatory cells in antigen-specific CD4⁺ T cells.

The immunoregulatory activity of T regulatory cells elicited by DCre was further determined in a suppressive assay *in vitro*. T regulatory cells isolated from 4 day culture of OT-II T cells and OVA 323–339-pulsed DCre were added into CFSE-labeled OT-II T cells in the presence or absence of OVA 323–339. As shown in Figure 3H, the isolated CD4⁺CD25⁺ T regulatory cells effectively suppressed proliferative responses of OT-II T cells to OVA peptide stimulation, with decreasing effect associated with decreasing T regulatory cell dose. These findings suggest that T regulatory T cells elicited by DCre are capable of inhibiting T cell response, and in a dose-dependent manner.

To verify whether the inhibitory activity of DCre-expanded T regulatory cells was antigen-specific, the OT-II T regulatory T cells were added into CFSE-labeled B6 splenic T cells in the presence of anti-CD3 mAb and irradiated B6 spleen cells (providing co-stimulation). As noted in Figure 3I, the OVA-pulsed DCre expanded T regulatory cells demonstrated comparable inhibitory effect on T cell proliferation induced by anti-CD3 mAb, indicating the inhibitory effect is not antigen specific.

Discussion

The role of CD4⁺ T cell activation in immune response to F.VIII, which was initially recognized in AIDS patients with CD4⁺ T cell counts below 200/μl whose anti-F.VIII antibody disappeared and response to exogenous F.VIII was restored [3], has been confirmed in the hemophilia A mouse model [4,5]. Despite this, suppression of anti-F.VIII antibody formation remains an unsolved clinical challenge, with significant morbidity and limited treatment options in those affected. The findings of this study demonstrate that manipulation of the immune response may suppress anti-F.VIII inhibitor antibody formation. Specifically, we demonstrate that immature dendritic cells (DCre), pulsed with rF.VIII, are effective in suppressing anti-F.VIII antibody formation in the hemophilia A mouse model when given prior to factor VIII challenge. The approach used in this study was adapted from the successful prevention of transplant rejection [16] and diabetes [17] in animal models, specifically by induction of T cell hyporesponsiveness to antigen by immature dendritic cells. In this study, dendritic cells were modified to have an immature phenotype (low expression of co-stimulatory molecules and high production of IL-10), and then pulsed with rF.VIII. Injection of these rF.VIII-pulsed DCre into hemophilia A mice resulted in reduced anti-F.VIII antibody levels following *in vivo* rF.VIII challenge (injection). Concomitant with the reduction in anti-F.VIII antibodies, splenic T cells from these mice demonstrated reduced proliferative responses to rF.VIII *in vitro*, and the reduced anti-F.VIII antibody response persisted after *in vivo* re-challenge with rF.VIII. The increase in IL-10 production observed in mouse T cell supernatants following rF.VIII-pulsed DCre is consistent with the immunoregulatory role of the IL-10 gene in immune response and antibody production in autoimmune disorders [26], as well as the recent identification of polymorphisms in and near the IL-10 gene associated with hemophilia inhibitor development in mice [27] and humans [28]. Whether IL-10 production is a marker for inhibitor antibody formation in humans, however, remains unknown.

The mechanism by which DCre prevent the antibody response to rF.VIII, although not proven, appears to involve induction of hyporesponsiveness to rF.VIII arising from increased expression of FoxP3, a mediator of T cell suppression. Specifically, the observed serum cytokine profile pattern in DCre-treated mice is consistent with suppression of T cell-dependent immune response and induction of rF.VIII unresponsiveness. The concomitant high level FoxP3 expression by splenic T cells suggests that the reduction in anti-F.VIII antibody levels and T cell hyporesponsiveness to factor VIII arises as a result of expansion of T regulatory cells by DCre, consistent with the recently recognized role of FoxP3 in T cell immunosuppression [11,14,15]. Further, this response appears to be antigen-specific, as FoxP3 staining identified that while both DCre and DCE are capable of presenting antigen that leads

to OT-II proliferation, D C_{re} are more potent antigen-specific T regulatory inducers. However, the inhibitory activity of T regulatory cells expanded by antigen-pulsed D C_{re} is antigen non-specific, since the T regulatory cells expanded by OVA-pulsed D C_{re} are capable of suppressing the proliferation of both OVA-activated OT-II CD4 $^{+}$ and anti-CD3 mAb-activated CD4 $^{+}$ T cells.

There are some limitations to this study. First, even though a smaller proportion of rF.VIII-pulsed D C_{re} -treated mice developed anti-F.VIII antibody and anti-F.VIII antibody titers were reduced, it is important to note that not all hemophilia mice receiving rF.VIII-pulsed D C_{re} showed complete suppression of anti-F.VIII antibody formation. This could suggest suboptimal inhibition of immune reactivity due to an inadequate number of injected tolerogenic dendritic cells (D C_{re}), or limited persistence of these immature cells in tissues to maintain the immune response. While immature DC propagated from mouse BM with GM-CSF and TFG- β are not stable, they may mature under the condition of inflammation. Similar numbers of DCs with immature phenotype, propagated from BM with oligodeoxynucleotides (ODNs) which contain binding sites for NF- κ B, have been successfully used in other immunologic diseases [20]. These NF- κ B decoy-propagated DC (NF- κ B DC) are stable immature DC, more resistant to inflammation-induced maturation due to blockage of the NF- κ B signaling pathway. It has been shown that administration of NF- κ B DC more effectively prolongs heart allograft survival [20]. Whether decoys may improve the response rate in hemophilia mice is currently under study. Alternatively, an inadequate dose of rF.VIII for pulsing dendritic cells might also play a role in failure to suppress anti-F.VIII antibodies in all mice. It should be noted, though, that the rF.VIII concentration used to incubate DCs was physiologic. Secondly, although F.VIII specific CD4 $^{+}$ T cells were not available to determine if the increase in T regulatory cell expansion was antigen specific, we were able to study this question using OVA 323–339 peptide as specific antigen and OVA-specific CD4 $^{+}$ T cells (OT-II), which could be identified by V α 2 $^{+}$ monoclonal antibody. Specifically, the greater FoxP3 $^{+}$ V α 2 $^{+}$ levels in the antigen (OVA)-pulsed D C_{re} group, as compared with the OVA-pulsed D C_{e} group, both *in vitro* and *in vivo*, suggests but does not prove that increased FoxP3 mRNA levels in the hemophilia A mouse model treated with rF.VIII-pulsed D C_{re} are due to T regulatory cell expansion, which suppressed anti-F.VIII inhibitor antibody response.

Whether the use of modified dendritic cells to reduce anti-F.VIII antibody response in the hemophilia A mouse model equates to suppression of anti-F.VIII antibody in humans with hemophilia remains unknown. Specifically, we evaluated D C_{re} in the prevention of anti-F.VIII response, but not in the suppression of an already existing anti-F.VIII response, e.g. immune tolerance, nor the duration of such a response. Future studies will be needed to address these issues as well as to determine if findings in mice apply to humans, given the immunologic differences anticipated in scale up from mice to men, as recognized in hemophilia gene transfer [29]. The recent successful induction of transplant tolerance by immature DC in mouse transplant models, is currently being studied in larger animal models [30], including rhesus monkeys; and a clinical trial of immature DC, shown to be successful in the treatment of diabetes in mouse models has been initiated in patients with type 1 diabetes [31]. Hopefully, these preliminary studies of DC immunotherapy will provide preliminary insights into the application of this strategy in the prevention and suppression of anti-F.VIII antibodies in individuals with hemophilia.

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Figure 1A. Recombinant F.VIII induces F.VIII antibody inhibitor formation in the hemophilia A mouse

Recombinant factor VIII (rF.VIII) by tail vein injection leads to formation of anti-F.VIII antibodies in hemophilia A mice (dark squares). Hemophilia A mice were infused intravenously with rF.VIII, 2.5 U (0.2 μ g) on days 0, 2 and 4, and again in re-challenge on days 12, 14, and 16. Anti-F.VIII antibody measured by Bethesda assay peaked in serum on day 6, and after re-challenge, continued to increase on day 17. Three to five mice were used in each experiment.

Figure 1B. Splenic T cells from hemophilia A mice show strong response to rF.VIII

Splenic T cells isolated from hemophilia A mice (exon 16 knockout KO mice) treated with rF.VIII intravenously (iv) (dark squares), but not from those treated with PBS (open squares), were strongly responsive to *in vitro* stimulation with rF.VIII. Splenic T cells isolated from hemophilia A mice treated with rF.VIII by tail vein injection, were assessed for proliferative response to *in vitro* rF.VIII in T cell proliferation assay by ³H-thymidine incorporation in day 4 cultures. Cells from three to five mice were used in each experiment.

Figure 2A. Dendritic cells express low level of co-stimulatory molecules

Dendritic cells propagated from bone marrow of hemophilia A mice with GM-CSF + TGF- β exhibit immature phenotype (DCre), with expression of low levels of co-stimulatory molecules on their surface. In contrast, DC propagated with GM-CSF + IL4 exhibit mature DC phenotype (DCe). DC surface antigens were analyzed by flow cytometry, as shown in representative histograms. The scales are logarithmic. Three to five mice were used per experiment.

Figure 2B. NF- κ B binding activity in nuclear protein of DCre

NF- κ B binding activity in nuclear protein of DCre, propagated from bone marrow of hemophilia A mice with GM-CSF + TGF- β , is inhibited, while NF- κ B binding activity of DCe, propagated with GM-CSF+IL-4 is enhanced, as evidenced by NF- κ B translocation in nuclear protein detected in an electrophoretic mobility shift assay. NS represents nonspecific binding. Three to five mice were used for each experiment.

Figure 3.

Figure 3A. Pretreatment of hemophilia A mice with rF.VIII-pulsed DCre inhibits anti-F.VIII antibody formation. Hemophilia A mice received 2×10^6 rF.VIII-pulsed DCre intravenously at day -7, followed by rF.VIII 2.5 U on day 0, 2, and 4. Blood was drawn on day 6 and day 8 for anti-F.VIII antibody detection. By day 6 there was a reduction in anti-F.VIII antibody, with a further reduction on day 8, both $p < 0.01$, as compared with control PBS-treated mice. Three to five mice were used at each time point.

Figure 3B. T cell hyporesponsiveness of hemophilia A mice pre-treated with rF.VIII-pulsed DCre. Splenic T cells obtained from rF.VIII-pulsed DCre-treated mice showed lower proliferative responses to *in vitro* rF.VIII restimulation than splenic T cells from rF.VIII-pulsed

DCE-treated mice (on the right). By contrast, splenic T cells from control PBS-treated mice demonstrated low proliferative responses (on the right). In the absence of *in vitro* rF.VIII restimulation *in vitro* (on the left), the proliferative responses of T cells from all groups remained, low by ³H-thymidine incorporation in T cell proliferation assay. Splenic T cells obtained from three to five mice were used for each experiment.

Figure 3C. Increased serum levels of IL-10 after administration of rF.VIII-pulsed DCre. Higher serum IL-10 levels were detected by ELISA in rF.VIII-pulsed DCre-treated mice, as compared with F.VIII-pulsed DCE-treated mice, $p < 0.01$ (bottom left). By comparison, no significant difference was noted in serum IFN- γ , IL-2, or TGF- β levels between these groups. There were three to five mice per group for each experiment.

Figure 3D. Enhanced IL-10, reduced IL-2 production by T cells after rF.VIII-pulsed DCre. Cytokine profiles by ELISA on the supernatants of splenic T cells from hemophilia A mice 7 days after DCre or DCE or PBS treatment are shown, following *in vitro* re-stimulation with rF.VIII and spleen cells as antigen presenting cells. Higher IL-10 and lower IL-2 levels were detected in T cell supernatants from the rF.VIII-pulsed DCre-treated mice, as compared with T cell supernatants from rF.VIII-pulsed DCE-treated mice, $p < 0.01$. T cell supernatants from three to five mice were used for each experiment.

Figure 3E. FoxP3 levels in splenic T cells from rF.VIII-pulsed DCre-treated hemophilia A mice. FoxP3 levels, measured by real time PCR (qt-PCR) on RNA isolated from splenic T cells from rF.VIII-pulsed DCre-treated hemophilia A mice were significantly higher than from RNA isolated from splenic T cells from rF.VIII-pulsed DCE treated mice, $p < 0.01$, or control PBS-treated mice, $p < 0.001$. Values are given as fold-increase over an 18S rRNA (housekeeper gene) control. Splenic T cells were obtained from three to five mice per group for these studies.

Figure 3F. CD4⁺FoxP3⁺ T cell regulatory expansion by OVA-pulsed DCre *in vitro*. Flow cytometry of T cells, stained for CD4 and FoxP3, demonstrated that DCre presenting OVA peptide to OT-II T cells induced more T regulatory cells, as measured by FoxP3⁺ staining in CD4⁺ T cells, than DCE presenting OVA peptide, 12.4% vs. 7.2%. Results of three representative experiments, with three mice per experiment, are shown.

Figure 3G. Administration of OVA-pulsed DCre-expanded T regulatory cells from OT-II T cells *in vivo*. OT-II T cells were adoptively transferred into B6 mice following administration of OVA 323–339-pulsed B6 DCre or DCE. Flow cytometry of T cells harvested from lymph nodes of the treated mice, stained for V α 2 and FoxP3, demonstrated significantly greater FoxP3⁺ T regulatory cell expansion in OVA-specific TCR CD4⁺ T cells, as shown by a greater number of FoxP3⁺ V α 2⁺ T cells, 9.0%, than T cells from lymph nodes of OVA-pulsed DCE-treated mice, 2.7%, or from control PBS-treated mice, 0.8%. Results of two independent experiments, pooling lymph nodes from three mice per group, are shown.

Figure 3H. Suppressive effect of T regulatory T cell expansion by OVA-pulsed DCre on OVA-specific OT-II CD4⁺ T cell response. Splenic T cells isolated from OT-II mice were labeled with CFSE and cultured with APC (spleen cells) in the presence or absence of OVA peptide 323–339 (see Methods). The proportion of OVA-specific CD4⁺ T cells in the whole cell population was determined with PE-anti V α 2 mAb. The CFSE dilution was determined in the V α 2 positive cells. Division of the reporter cells (splenic T cells) was markedly inhibited by T regulatory cells which were isolated as CD4⁺CD25⁺ from OT-II T cells cultured with DCre-OVA323–339 (see Methods), indicating the inhibitory effect is dose-dependent.

Figure 3I. Suppressive effect of T regulatory cell expansion by OVA-pulsed DCre on T cell activation is not antigen-specific. CFSE-labeled splenic T cells from B6 mice were cultured in anti-CD3 mAb-coated plates, in the presence or absence of isolated T regulatory cells, previously expanded with DCre-OVA323–339 and OT-II cell co-cultures (see Methods). The proportion of CD4⁺ T cells in the whole cell population was determined by staining with PE-anti CD4 mAb. The CFSE dilution of the gated CD4⁺ T cells in response to anti-CD3 mAb stimulation, was significantly suppressed by OVA-pulsed DCre-expanded T regulatory cells, indicating that the inhibitory effect of T regulatory cells is not antigen-specific.