5－8）生活上の心配・不安をお聞きします。該当するものを選んで下さい。（複数回答可）

①□こどもへの遺伝が心配　②□身体障害による行動制約　③□就職　④□結婚
⑤□老後の健康不安　⑥□親の介護不安　⑦□特になし　⑧□その他（　　）

5－9）現在、医療面で不安を感じていることはありますか？（複数回答可）

①□現在の病状　②□希望する医療が受けられない　③□病院が遠い　④□医療費
⑤□健康保険　⑥□特になし　⑦□その他（　　）

5－10）現在、経済面で不安を感じていることはありますか？（複数回答可）

①□年金支給　②□生命保険加入　③□給料　④□その他（　　）⑤□特になし

5－11）血友病など凝固異常症を意識して以下のようなことをされているかお聞きします。

1. 診療は健康保険を使わず自費にしていますか？

①□はい　②□いいえ

2. 職場・学校・近所の人に病名を知らせていますか？

①□はい　②□一部の人のみ　③□いいえ

3. 家族の方は病名を知っていますか？

①□はい　②□一部の人のみ　③□いいえ

4. 親戚の方は病名を知っていますか？

①□はい　②□一部の人のみ　③□いいえ

5. 地元の人や知人の会う可能性の少ない病院に受診していますか？

①□はい　②□いいえ

5－12）HIV感染症に関連して、社会生活上不愉快な思いをしたことがありますか？

①□はい　②□いいえ　③□わからない

5－13）社会生活上望むことについてお聞きします。最も望むものを1つだけチェックをして下さい

1. 学校に関して望むことは何ですか？

①□病気への理解　②□差別の解消　③□バリアフリーの向上　④□その他（　　）

2. 職場・仕事に関して望むことは何ですか？

①□病気への理解　②□身体障害者雇用の推進　③□病気への差別解消　④□通院時間の確保
⑤□その他（　　）

3. 保険制度や年金制度に関して望むことは何ですか？

①□健康保険制度の不安除去・プライバシーの確保　②□生命保険の加入のしやすさ
③□年金制度の向上　④□公的サポートシステム　⑤□その他（　　）

4. 医療制度に関して望むことは何ですか？

①□治療の進歩　②□恒久的公的医療費助成　③□ヘモフィリアセンターの充実
④□病院　⑤□診療時間の選択　⑥□差別の解消
⑦□その他（　　）

高校卒相当年齢以下（生年月日が平成元年4月2日以降）の方の設問はこれで終了です。
引き続き12ページの自由記載欄にお進み頂き、ご意見・ご要望等ございましたらご記載下さい。

高校卒相当年齢以降（生年月日が平成元年４月１日以前）の方は、
引き続きこのあとの質問にもお答え下さい。

6）HIV 感染あるいは肝炎について

6－1）HIV ウイルスの感染はありますか？

①□あり　②□なし　③□わからない

＃②③の方は6－2）へ進んで下さい。

＃①「あり」の方に質問します。

a)抗エイズ薬を服用していますか？

①□はい　②□いいえ　③□中断／あるいは休薬中

b)最近の HIV ウイルス量は？

①□検出感度未満　②□（　　）コピー/ml　③□わからない

c)最近の CD4 細胞数は？

①□（　　）個/μL　②□わからない

d)エイズを発症していますか、あるいはしたことがありますか？

①□なし　②□今発症している　③□発症したが今はなおっている　④□わからない

6－2）肝炎ウイルスの感染はありますか？（複数回答可）

①□なし　②□C型　③□B型　④□その他（　　）　⑤□わからない

＃②「C型肝炎」の方に質問します。　それ以外の方は次の項目７）へお進み下さい。

a)HCV 抗体の有無に拘らず血中 HCV ウイルスが無治療で陰性となり自然治癒していますか？

①□はい　②□いいえ　③□わからない

＃②「いいえ」の方に質問します。①③の方は次の項目７）へお進み下さい。

b)現在の病期は？

①□慢性肝炎　②□肝硬変　③□肝癌　④□わからない

c)インターフェロン治療に対する要望がありますか？（複数回答可）

①□在宅治療ができるようにして欲しい
②□もっと治癒率の高い治療法を開発して欲しい
③□副作用がもっと軽くなるようにして欲しい
④□なし　⑤□その他（　　）

d)インターフェロン治療をうけましたか？

①□これまでにうけた　②□現在治療中　③□うけたことはない

＃d)で①「これまでにうけた」および②「現在治療中」の方に質問します。

＃③の「うけたことがない」の方は次頁の g）へお進み下さい。

e)インターフェロン治療の種類は？（複数回答可）

①□インターフェロンのみ
②□ペグインターフェロン
③□インターフェロンとリバビリン併用
④□ペグインターフェロンとリバビリン併用
⑤□わからない

次頁 g)へ

f)効果は？（複数の種類の治療を受けた場合は最近の治療法の効果を記載）

①□ウイルスは消失し、肝機能正常化（完治した）
②□ウイルスは一度消失したが再出現、肝機能正常化
③□ウイルスは一度消失したが再出現、肝機能正常化せず
④□ウイルスは消失したが肝機能正常化せず
⑤□ウイルスは消失せず肝機能正常化せず
⑥□ウイルスは消失しなかったが肝機能正常化
⑦□現在治療中
⑧□副作用などで中止
⑨□わからない

＃d)で③「うけたことはない」を選択した方に質問します。
g)インターフェロン治療をしない理由は？（複数回答可）

①□仕事あるいは学校を休めないので　②□副作用がこわい／つらいので
③□病状が進み治療できないため　④□肝機能が正常であり治療開始時期ではないため
⑤□治療費が高額のため　⑥□その他（　　）

7）就職について

7－1）現在、患者さんは就職していますか？

①□はい　②□いいえ　③□現在就職活動中

＃②方は次頁 c)へお進み下さい。□
＃③方は次頁 d)へお進み下さい。

＃①「はい」を選択した方に質問します。
a)現在、仕事上の不安・心配はありますか？（複数回答可）　裏

①□出血した場合の止血管理　②□職場の理解
③□職場に病気を伝えていないため知られないようにする
④□身体障害による行動制約　⑤□会社や同僚の差別的対応
⑥□通院時間の確保　⑦□希望する仕事に就けない
⑧□給料が少ない　⑨□体調不良や治療の都合で欠勤が多い
⑩□仕事がきつい　⑪□職場の人間関係に恵まれていない
⑫□その他（　　）

b）仕事中に出血した場合、止血管理は主にどうされていますか？

①□速やかに職場の医務室などで自己注射をする
②□速やかに一時帰宅あるいは早退して自己注射をする
③□速やかにかかりつけ医に注射に行く
④□なるべく会社が終わるまで我慢し、帰宅後に自己注射
⑤□なるべく会社が終わるまで我慢し、帰宅後にかかりつけ医で注射
⑥□その他（　　）

＃7－1）で②「いいえ」を選択した方に質問します。

c）仕事をしていない理由は何ですか？（複数回答可）

①□病気を知られたくない	②□出血傾向が強い	
③□全体に体調が悪い	④□身体障害による行動制約が大きい	
⑤□就労での差別不安	⑥□通院時間が確保しにくい	
⑦□入院している	⑧□希望する仕事がない	
⑨□就職する気がない	⑩□給料が少ない	⑪□学生だから

＃7－1）で③「現在就職活動中」を選択した方に質問します。

d）以下の中のどれに該当しますか？（複数回答可）

①□血友病を知らせて就職活動をしている
②□血友病を知らせないで就職活動をしている
③□身体障害者枠での申請をしている
④□ハローワークなどに就労先を探しに行っている

8）自由記載欄

血液凝固異常症に関する医療制度、治療あるいは社会生活に関して日ごろお考えになっていること、ご意見・ご希望などございましたら、ご記載下さい（足りない場合は別紙を添付して下さい）。

これで終了です。有難うございました。

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ORIGINAL ARTICLE

Induction of factor VIII-specific unresponsiveness by intrathymic factor VIII injection in murine hemophilia A

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Summary *Background:* Hemophilia A is a congenital bleeding disorder caused by a deficiency of coagulation factor VIII. Approximately 30% of hemophilia A patients develop inhibitors against FVIII following replacement therapy. We have reported that neonatal exposure of FVIII antigen can induce antigen-specific immune tolerance by interferon-γ (IFN-γ)-dependent T-cell anergy in hemophilia A mice. *Objective:* The thymus plays crucial roles in self-tolerance, with negative selection of self-reactive effector T cells and positive selection of self-reactive regulatory T cells. We investigated the possibility of the induction of antigen-specific immune tolerance by intrathymic injection of FVIII in hemophilia A mice. *Methods:* Hemophilia A mice were injected with recombinant FVIII into the thymus under real-time high-resolution image guidance. *Results:* Anti-FVIII inhibitory antibody titers in mice challenged with intravenous administration of FVIII were significantly lower in mice (n = 22) that had received thymic FVIII injection than in mice (n = 18) without thymic injection (9.4 ± 2.3 vs. 122.5 ± 27.6 BU mL^{-1}, respectively, P = 0.00078). The $CD4^+$ T cells from thymic-injected mice could not proliferate or produce interleukin (IL)-2, IL-12 and IFN-γ in response to FVIII. The $CD4^+CD25^+$ T cells generated from thymic-treated mice but not from naïve mice efficiently suppressed the *in vitro* proliferative response of $CD4^+$ T cells and blocked the *in vivo* development of anti-FVIII antibodies in the adoptive transfer. *Conclusion:* These data suggest that intrathymic administration of FVIII could result in immune tolerance by induction of FVIII-specific regulatory T cells.

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Introduction

Hemophilia A is an X-linked hereditary bleeding disorder caused by deficiency in coagulation factor VIII [1]. Plasma-derived or recombinant FVIII is sufficiently available to permit its use for primary prophylaxis to avoid bleeding in patients with severe hemophilia A. A major complication of hemophilia A treatment is the development of neutralizing antibodies against the infused FVIII [2]. We have previously demonstrated that exposure to FVIII antigen within 24 h of birth induces antigen-specific immune tolerance by interferon (IFN)-γ-dependent T-cell anergy in hemophilia A mice [3].

The thymus plays a major role not only in the development of self-tolerance but also in acquired tolerance in autoimmunity and organ transplantation [4,5]. There are two mechanisms in the thymus to establish a self-tolerance system, consisting of negative selection of self-reactive effector T cells, and positive selection of self-regulatory $CD4^+CD25^+$ T cells [6,7,4,8]. The $CD4^+CD25^+$ T cells are known to be weakly reactive to antigenic stimulation and able to mediate suppression of $CD25^-$ naïve T cells [9,10]. In the thymus, $CD4^+CD25^+$ T cells are detected during the fetal period in humans, and during the perinatal period in mice [11]. The $CD4^+CD25^+$ T cells may be responsible for the translation of tolerance from an antigen-inoculated thymus to a mature but naïve peripheral immune system [12] [13]. Injection into the thymus of organs or cells has been successful in the induction of T-cell-mediated immunologic tolerance [14,15,16]. Allogenic grafts of pancreatic islets had better survival when grafted in the thymus, and provided long-term protection against spontaneous autoimmune diabetes if grafted during the early period after birth [17]. The expression or presentation of nominal antigen in the thymus might lead to enhanced deletion of autoreactive T cells or to induction of a number of antigen-specific regulatory T cells. In this study, we investigated the possibility of the induction of antigen-specific immune tolerance by intrathymic

injection of FVIII in hemophilia A mice. Our study may open new perspectives for the manipulation of FVIII-specific tolerance in the thymus of hemophilia A patients.

Materials and methods

Hemophilia A mice

FVIII-deficient mice (B6; $_{129}S_4$-$F8^{tm1Kaz}$/J) with targeted destruction of exon 16 of the FVIII gene were previously described and kindly provided by H. H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA) [18]. The experimental protocol was approved by the institutional Animal Care and Concern Committee of Jichi Medical University.

Intrathymic injection under the real-time high-resolution imaging system

Hemophilia A mice, 1–3 days old, were anesthetized by inhalation with 2.5% isoflurane in the anesthesia unit (Univentor, ZTN 08, Malta), and were imaged with a 30–50-MHz mechanical sector transducer with 50-μm axial and 115-μm lateral resolution (Vevo 770; Visualsonic Inc., Toronto, Canada). Two-dimensional real-time imaging of the thymus was accomplished with a 12 × 12-mm field of view and an optimal depth of 12.5 mm. When a cross-section with the thymus was located, a glass microcapillary needle (Becton Dickinson, San Jose, CA, USA) was placed at the parasternal area on the chest in the ultrasound imaging plane. Under real-time image guidance, the thymus was punctured with the needle, and 0.05 u g^{-1} body weight (BW) of highly purified, albumin-free preparations of recombinant FVIII (Kogenate FS; Bayer Healthcare, Leverkusen, Germany) or 0.005 μg g^{-1} BW of human albumin (Sigma-Aldrich, St Louis, MO, USA) was injected precisely using a microinjector remote control system (Fig. 1A,B). Mice were then stimulated with intravenous FVIII (0.05 u g^{-1} BW) every 2 weeks, from 10 to 18 weeks of age. Blood samples were obtained 2 weeks after each of the injections from the jugular vein, and were added at a 9 : 1 (v/v) ratio to 0.38% sodium citrate; plasma was then separated by centrifugation. The plasma samples were subsequently stored at − 80 °C until further analysis.

Assay for FVIII inhibitors

FVIII inhibitor levels were measured according to the Bethesda methods. In brief, mouse plasma (50 μL) was incubated with 50 μL of normal pooled human plasma at 37 °C for 2 h. Residual human FVIII activity was measured in a one-stage assay using 50 μL of FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and a 50-μL sample from the previous incubation. Samples were mixed with 100 μL of phospholipid activator, incubated at 37 °C for 3 min, and then mixed with 100 μL of 20 mmol L^{-1} $CaCl_2$. Clotting times were measured with a coagulometer (CA-500; Sysmex, Kobe, Japan). Coagutrol N (Kokusai-Shiyaku) was diluted with Owren's Veronal Buffer to

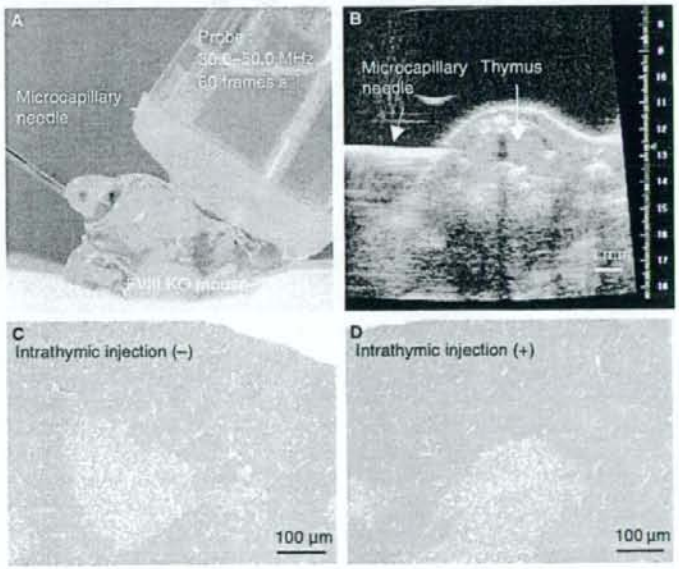


Fig. 1. Intrathymic injection of FVIII antigen using a high-resolution ultrasound system. Under real-time imaging of the thymus (A), a glass microcapillary needle was placed at the parasternal area and used to puncture the thymus (B). (C) The thymic sections were analyzed with hematoxylin and eosin staining 5 days after without (left panel) or with (right panel) of in day 1 thymic injection of FVIII in hemophilia A mice.

produce a standard curve of FVIII activity. The measurements were made in the linear portion of the response range.

Anti-FVIII IgG measurements

Anti-FVIII IgG concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) in microtiter wells (Nunc, Roskilde, Denmark) coated with 1 μg mL^{-1} recombinant human full-length FVIII (Kogenate FS). After blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), serial dilutions of murine plasma were added at 4 °C for 16 h. Each well was washed with 0.5% BSA in PBS containing 0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) was added at 37 °C for 1 h. ABTS Microwell substrate (KPL, Gaithersburg, MD, USA) was added, and the absorbance at 405 nm was read. Anti-FVIII antibody concentrations were estimated from the linear portion of a standard curve obtained using anti-human FVIII monoclonal antibodies (kindly provided by The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), which bind to FVIII.

Determination of IgG subclass of anti-FVIII antibodies

Microtiter wells were coated with 1 μg mL^{-1} recombinant human FVIII in PBS for 16 h at 4 °C. After blocking with 5% BSA in PBS, serial dilutions of murine plasma were added for 2 h at 37 °C. The wells were washed with 0.5% BSA in PBS containing 0.05% Tween-20. The IgG subtypes of anti-FVIII antibodies bound to immobilized human FVIII were determined by incubation with isotype-specific rabbit anti-mouse IgGs (Mouse Typer; BioRad, Hercules, CA, USA) for 1 h at 37 °C. After being washed with 0.5% BSA in PBS containing 0.05% Tween-20, the wells were incubated with goat anti-rabbit HRP conjugate for 1 h at 37 °C. Substrate development was performed for 15 min at 25 °C, using ABTS Microwell substrate as described above.

Tetanus immunization of FVIII-deficient mice

Mice were injected intraperitoneally with 1 Limit of flocculation of tetanus toxoid (TT) vaccine (Takeda Chemical Industries, Tokyo, Japan). Plasma samples were obtained after 3 weeks, and anti-TT antibody titers were determined by ELISA as previously described. In brief, microtiter plates were coated with 5 μg mL^{-1} formaldehyde-inactivated tetanus toxin, *Clostridium tetanii* (Calbiochem, Darmstadt, Germany), for 16 h at 4 °C. After washing and blocking with Tris-buffered saline containing 5% BSA, mouse plasma samples were added to the wells and incubated for 2 h at 37 °C. After washing with 0.5% BSA in PBS containing 0.05% Tween-20, 100 μL of HRP-conjugated goat anti-mouse IgG was added for 1 h at 37 °C. Then, the peroxidase substrate was added and the absorbance at 405 nm was measured.

Cell preparation

Mice $CD4^+$ T cells were prepared by depletion of non-$CD4^+$ T cells with the autoMACS cell sorting system (Miltenyi Biotech GmbH, Bergish Gladbach, Germany), according to the manufacturer's instructions. $CD4^+CD25^+$ T cells were isolated by $CD25^+$ positive selection from $CD4^+$ T cells with magnetic cell sorting, using a CD4CD25 Isolation Kit (Miltenyi Biotech). The purity of sorted $CD4^+CD25^+$ T cells was confirmed to be more than 85% by flow cytometric analysis. Antigen-presenting cells were prepared from mice splenocytes by depletion of T cells using the magnetic sorting system with anti-CD90 (Thy1.2)-conjugated microbeads (Miltenyi Biotech), followed by irradiation with a single dose of 20 Gy (Gamma Cell; Norton International, ON, Canada), to prevent nonspecific proliferative responses during the *in vitro* FVIII stimulation assay.

Flow cytometric analysis

Cells from teased organs were labeled in PBS containing 1% BSA and 2 mmol L^{-1} EDTA at 4 °C for 30 min in the dark under continuous agitation. The following antibodies were used for phenotypic analysis: allophycocyanin-labeled anti-CD25 IgG (PC61.5; eBioscience, San Diego, CA, USA), fluorescein isothiocyanate-conjugated anti-CD4 IgG, phycoerythrin (PE)-labeled anti-CD45 IgG (30-F11; BD Pharmigen), and forkhead family transcription factor (Foxp3)–PE IgG (eBio7979; eBioscience), used according to the manufacturer's instructions. Isotype-matched irrelevant antibodies (BD Pharmigen) were used as controls. At least three events were analyzed on a FACS Aria (Becton Dickinson).

Proliferation assay with [^{3}H]thymidine incorporation

To measure T-cell proliferation, 1×10^5 cells per well were cultured with 0–3 nmol L^{-1} human FVIII at 37 °C for 72 h in complete RPMI-1640 (Gibco BRL, Rockville, MD, USA). [^{3}H]Thymidine (Amersham Bioscience, Uppsala, Sweden) was added (0.037 MBq per well) at 37 °C for 18 h. The cells were harvested, and [^{3}H]thymidine incorporation was determined by scintillation counting (Top count; Packard, Meriden, CT, USA).

Cytokine assays

Splenocytes were incubated in 24-well plates at 1.0×10^6 cells per well in the absence or presence of 3 nmol L^{-1} human recombinant full-length FVIII (Kogenate FS) at 37 °C in 5% CO_2. Production of the cytokines interleukin (IL-2), IL-4, IL-12 and IFN-γ by $CD4^+$ T cells derived from each mouse was analyzed at 72 h with the ELISA kits (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions. In addition, levels of IL-10 were measured at 96 h by the ELISA system (Biotrak ELISA System).

Adoptive transfer of CD4⁺CD25⁺ regulatory T cells into syngeneic hemophilia A mice

Single-cell suspensions were prepared and pooled from the spleens of hemophilia A mice without intrathymic administration of FVIII (non-IT mice) or those with intrathymic administration of FVIII (FVIII-IT mice). The pooled cells were purified to obtain $CD4^+CD25^+$ T-cell populations as described above. A total of 0.5×10^6 $CD4^+CD25^+$ T cells per body in 100 μL of PBS was injected into syngeneic naïve hemophilia A mice via the jugular vein. Mice were challenged with repeated intravenous stimulation by 0.05 u g^{-1} BW FVIII every 2 weeks, and inhibitory antibody titers were followed over time.

Statistical analysis

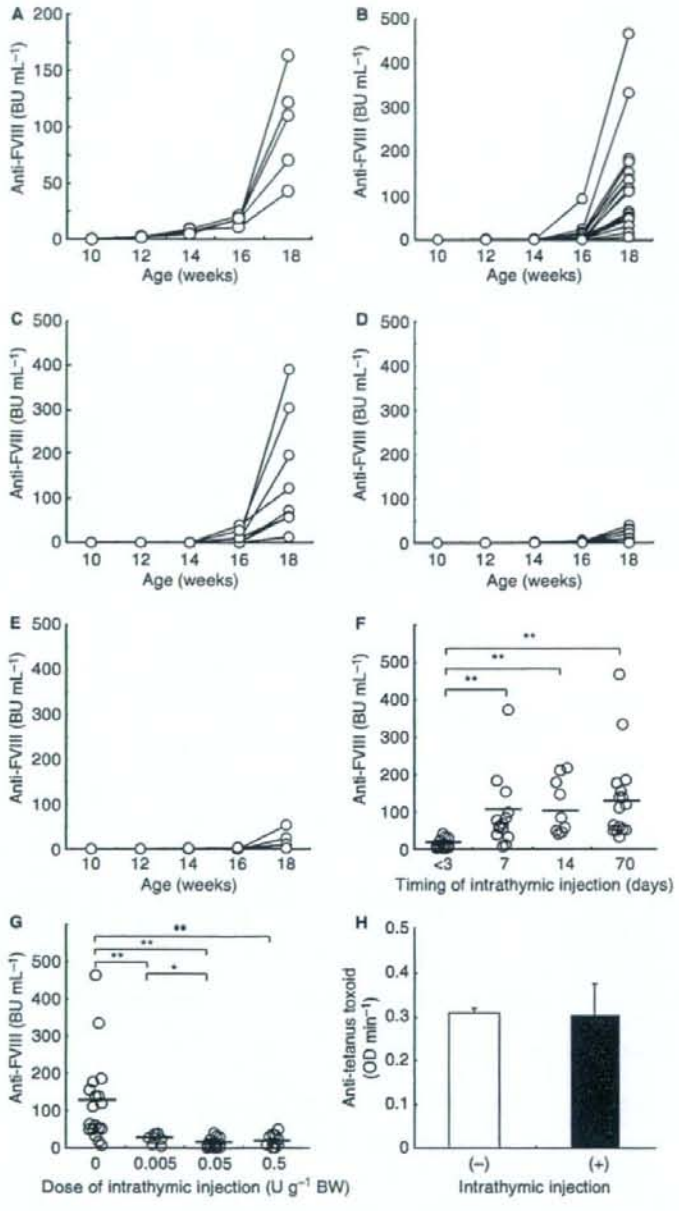
Two-tailed unpaired *t*-tests with 95% confidence intervals were performed using SPSS software (SPSS, Chicago, IL, USA). Mean values were considered to be statistically significant if the *P*-values were below 0.05.

Results

Effect of intrathymic administration of FVIII on anti-FVIII inhibitory antibody formation in hemophilia A mice

We performed histologic analysis of hemophilia A mice 1 day after direct thymic injection of human recombinant FVIII. We analyzed thymus sections 5 days after thymic injection with hematoxylin and eosin staining, and confirmed that there was no bleeding or histologic change in thymic structure, similar to what was seen with untreated mice (Fig. 1C). Then, we studied the impact of intrathymic injection of FVIII on the immune response in hemophilia A mice. For this, we administrated FVIII or albumin as control antigen into the thymus of naïve hemophilia A mice, and analyzed anti-FVIII inhibitory antibody formation after repeated intravenous stimulation with FVIII (0.05 u g^{-1} body weight). All non-IT mice developed high titers of anti-FVIII antibodies (n = 22, 122.5 ± 27.6 BU mL^{-1}; Fig. 2B), confirming that human FVIII is highly immunogenic in hemophilic mice [3,19]. In addition, mice that had been given intrathymic injections of human albumin (Alb-IT mice) showed high titers of antibody against FVIII (n = 10, 129.0 ± 40.6 BU mL^{-1}, Fig. 2C). By contrast, FVIII-IT mice had undetectable or low titers of anti-FVIII antibodies (n = 18, 9.4 ± 2.3 BU mL^{-1}; Fig. 2D). Moreover, FVIII-IT mice did not develop of high titers of anti-FVIII antibodies even after the boosted immune challenges with a combination of FVIII and Freund's adjuvant (20.1 ± 8.1 BU mL^{-1}, n = 5; Fig. 2E). As shown in Fig. 2F, mice treated on day 7, day 14 and day 70 developed high titers of anti-FVIII antibodies after the fifth intravenous stimulation with FVIII: 118.3 ± 34.9 BU mL^{-1} (n = 15), 113.9 ± 31.5 BU mL^{-1} (n = 10), and 120.5 ± 37.6 BU mL^{-1} (n = 16), respectively. Several researchers have demonstrated that induction of antigen-specific tolerance by intrathymic inoculation of soluble antigens is dose-dependent, and that an optimal dose of soluble antigen is required to induce antigen-specific unresponsiveness [20,21]. We injected FVIII into the thymus of neonatal hemophilia A mice at variable doses (0.005–0.5 u g^{-1} BW), and followed this with repeated intravenous stimulation with FVIII at 10, 12, 14, 16 and 18 weeks. As shown in Fig. 2G, intrathymic administration of 0.005, 0.05 and 0.5 u g^{-1} BW resulted in lower titers of anti-FVIII inhibitory antibodies (24.5 ± 4.4, 9.4 ± 2.3, and 18.9 ± 4.8 BU mL^{-1}, respectively) than those seen without thymic treatment. Interestingly, mice injected intrathymically with 0.005 u g^{-1} BW of FVIII developed significantly higher titers of anti-FVIII antibodies than those injected with 0.05 u g^{-1} BW of FVIII (P = 0.036), suggesting that there is some dose-dependency in the ability to induce immune tolerance. Taken together, these findings show that the intrathymic injection of FVIII antigen within 3 days after birth minimizes neutralizing antibody formation in FVIII-deficient mice. To determine whether the suppression of antibody against FVIII was specific, FVIII-IT mice and non-IT mice were immunized intraperitoneally with TT vaccine 2 weeks after the final challenge with FVIII. As shown in Fig. 2H, FVIII-IT mice were able to mount a T-cell-dependent immune response to a different antigen, and the antibody response was similar to that in non-IT mice. These results indicated that the immune suppression observed in our mouse model was FVIII-specific.

Fig. 2. Effect of intrathymic administration of FVIII on anti-FVIII inhibitory antibody formation in hemophilia A mice. Normal B6 control mice [(A), n = 5], and hemophilia A mice without thymic treatment [(B), n = 22], or with thymic injection of human albumin [(C), n = 10] or human FVIII [(D), n = 18] within 3 days after birth, were injected intravenously with human FVIII (0.05 u g^{-1} body weight) at 10, 12, 14, 16 and 18 weeks. The anti-FVIII inhibitor titer was determined by Bethesda assay. (E) FVIII-deficient mice with prior intrathymic injection of FVIII were repeatedly stimulated with FVIII and Freund's adjuvant every 2 weeks, and anti-FVIII inhibitor titers were measured. (F) The initial intrathymic injection of FVIII was given within 3 days (n = 22), 7 days (n = 15), 14 days (n = 10) or 70 days (n = 18) after birth. Each mouse was then given repeated intravenous injections at 10, 12, 14, 16 and 18 weeks. The mice were bled on day 4 after the fifth treatment, and anti-FVIII inhibitor titers were measured. Bars show means. $^{**}P < 0.03$ (G) Zero unit per gram body weight (saline control, n = 18), 0.005 u g^{-1} body weight (saline control, n = 10), 0.05 u g^{-1} body weight (saline control, n = 22) or 0.5 u g^{-1} body weight (saline control, n = 11) of FVIII were injected into the thymus within 3 days after birth, and the mice were then treated with 0.05 u g^{-1} body weight of FVIII at 10, 12, 14, 16 and 18 weeks. The mice were bled on day 4 after the fifth treatment, and anti-FVIII inhibitor titers were measured by Bethesda assay. Bars show means. $^{*}P < 0.05$, $^{**}P < 0.03$ (H) Hemophilia A mice without (n = 14, open bar) or with (n = 15, closed bar) thymic administration of FVIII were given five doses of intravenous FVIII (0.05 u g^{-1} body weight) every 2 weeks. Each mouse was injected intraperitoneally with one Limit of flocculation (Lf) unit per body of tetanus toxoid vaccine 2 weeks after the last challenge with FVIII. Plasma samples were obtained 3 weeks after the tetanus toxoid injection. Anti-tetanus toxoid antibody titers were measured by enzyme-linked immunosorbent assay as described in Materials and methods. Data are shown as the means ± standard deviations.



Effect of intrathymic administration of FVIII on anti-FVIII IgG formation in hemophilia A mice

We treated hemophilia A mice with repeated injections of human FVIII, and measured anti-FVIII IgG titers after the fifth injection at 16 weeks. IgG antibodies against FVIII were significantly inhibited in FVIII-IT mice as compared with non-IT mice (14.9 ± 11.2 μg mL^{-1} vs. 665.16 ± 225.4 μg mL^{-1}, P = 0.0038) (Fig. 3A). As these mice are on a B6 background, we used C57BL/6J mice as normal controls for experiments on the development of anti-FVIII IgG. The B6 control mice developed high titers of anti-FVIII inhibitory antibodies (101.6 ± 46.4 BU mL^{-1}; Fig. 2A). Levels of anti-FVIII IgG of FVIII-stimulated B6 control mice (409.9 ± 84.8 μg mL^{-1})

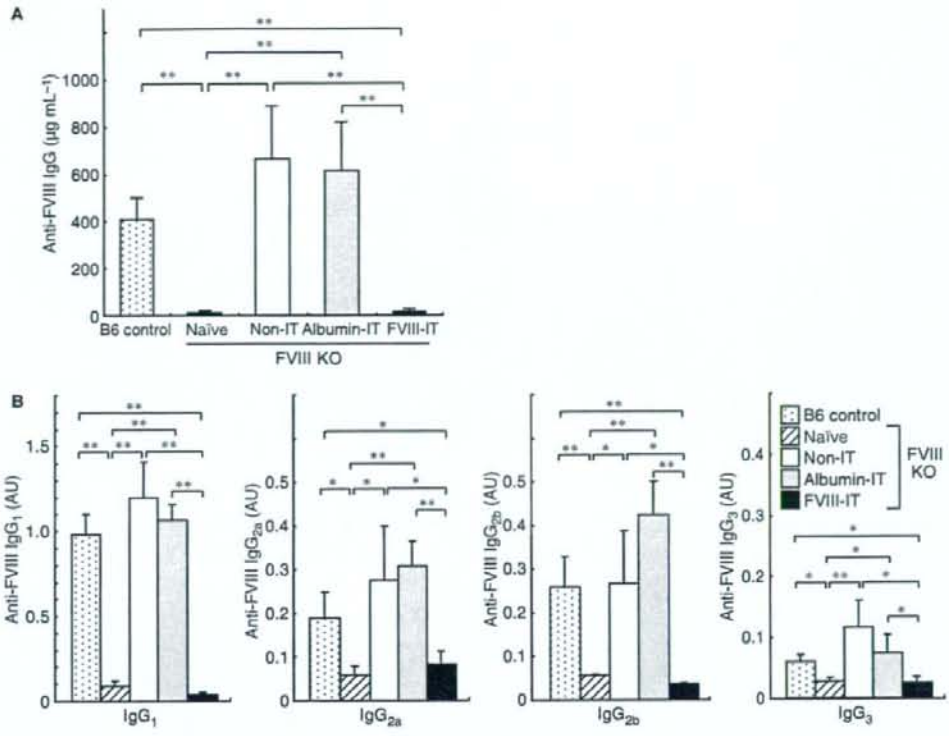


Fig. 3. Effect of intrathymic administration of FVIII on anti-FVIII IgG formation in hemophilia A mice. Normal B6 control mice (B6 control mice, $n = 5$), naïve FVIII-deficient mice (naïve mice, $n = 5$), FVIII-deficient mice without thymic treatment (non-IT mice, $n = 5$) and mice with prior thymic injection of human albumin (Albumin-IT mice, $n = 5$) or human FVIII (FVIII-IT mice, $n = 5$) were injected intravenously with human FVIII every 2 weeks. Each of the mice was bled on day 4 after the fifth stimulation, and total anti-human FVIII IgGs (A) and their titers of IgG subclasses (B) were measured by enzyme-linked immunosorbent assay as described in Materials and methods. The values [(A), μg mL^{-1}; (B), AU] represent the means ± standard deviations. $^{*}P < 0.05$; $^{**}P < 0.03$.

were significantly higher than those of FVIII-deficient naïve (5.0 ± 5.6 μg mL^{-1}, background values) or FVIII-IT mice. In addition, Alb-IT mice also produced significant amounts of anti-FVIII IgG antibodies (616.4 ± 207.9 μg mL^{-1}). All IgG isotypes of anti-FVIII antibodies in B6 control, Alb-IT and non-IT mice significantly increased as compared with those in FVIII-IT mice (Fig. 3B). These results suggest that intrathymic injection of FVIII efficiently suppressed the formation of antibodies against FVIII in hemophilia A mice.

The anti-FVIII specific CD4^{+} T-cell proliferative response is blocked by intrathymic administration of FVIII.

To evaluate whether intrathymic administration of FVIII had direct suppressive effect on FVIII-specific CD4^{+} T cells, we analyzed the CD4^{+} T-cell proliferative response to *in vitro* FVIII stimulation in the presence or absence of non-IT mouse-derived antigen-presenting cells (Fig. 4A). As shown in Fig. 4B, CD4^{+} T cells isolated from B6 control, naïve, non-IT, Alb-IT or FVIII-IT mice did not proliferate at any concentration of FVIII when they were cultured without antigen-presenting cells. Moreover, the antigen-presenting cells alone did not respond to the stimulation with FVIII. The CD4^{+} T cells of B6 control, non-IT and Alb-IT mice showed dose-dependent proliferation in response to FVIII when they were cocultured with the antigen-presenting cells (Fig. 4C). By contrast, the CD4^{+} T cells isolated from FVIII-IT mice did not show any proliferative response to FVIII, even if they were cocultured with antigen-presenting cells, indicating that the intrathymic administration of FVIII could be important for the prevention of an immune response to FVIII.

Cytokine responses are suppressed by intrathymic administration of FVIII.

The CD4^{+} T cells from B6 control (Fig. 5A), non-IT (Fig. 5C) and Alb-IT (Fig. 5D) mice produced significant amounts of IL-2, IL-12 and IFN-γ in response to FVIII stimulation. In contrast, the levels of IL-4 and IL-10 in these mice did not change even after addition of FVIII.

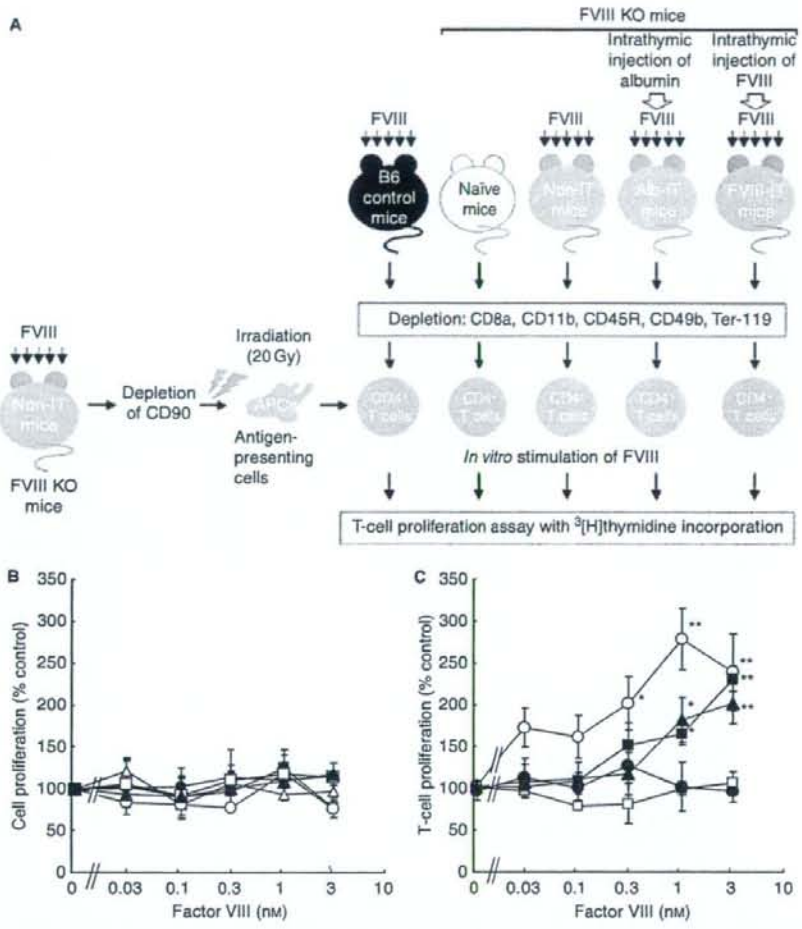


Fig. 4. The anti-FVIII specific T-cell proliferative response is blocked by intrathymic administration of FVIII. (A) B6 control mice, mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (Alb-IT mice) and mice that had been given intrathymic injections of FVIII (FVIII-IT) mice were intravenously injected with human FVIII every 2 weeks. The $CD4^+$ T cells were isolated from each of the mice, as well as from naïve FVIII-deficient mice, by depletion of CD8a, CD11b, CD45R, CD49b and Ter119 cells with a cell sorting system. The antigen-presenting cells were isolated from non-IT mice after repeated intravenous stimulation with FVIII with depletion of CD90 cells, followed by 20 Gy of irradiation. Each group of $CD4^+$ T cells was stimulated with FVIII in the absence or presence of the antigen-presenting cells. (B) The proliferation of $CD4^+$ T cells from B6 control mice (closed triangles, $n = 5$), naïve mice (open squares, $n = 5$), non-IT mice (open circles, $n = 7$), Alb-IT mice (closed squares, $n = 5$) and FVIII-IT mice (closed circles, $n = 7$) was analyzed under *in vitro* stimulation with FVIII (0–3 nmol L^{-1}) in the absence of antigen-presenting cells, as described in Materials and methods. The proliferation of antigen-presenting cells alone was also analyzed (open triangles, $n = 5$). (C) The proliferation of $CD4^+$ T cells from B6 control mice (closed triangles, $n = 5$), naïve mice (open squares, $n = 5$), non-IT mice (open circles, $n = 7$), Alb-IT mice (closed squares, $n = 5$), and FVIII-IT mice (closed circles, $n = 7$) was analyzed under *in vitro* stimulation with FVIII (0–3 nmol L^{-1}) in the presence of the non-IT mouse-derived antigen-presenting cells. Data are shown as the means ± standard deviations. $^*P < 0.05$, $^{**}P < 0.03$, when compared with the proliferation in the absence of FVIII.

FVIII-IT mice spontaneously produced higher amounts of IL-4 (71.2 ± 40.5 pg mL^{-1}; Fig. 5E) than non-IT mice (14.7 ± 6.8 pg mL^{-1}, $P = 0.016$; Fig. 5C) or Alb-IT mice (26.6 ± 2.9 pg mL^{-1}, $P = 0.035$; Fig. 5D). IL-4 has been shown to be the dominant cytokine required for the development of a Th2 phenotype from naïve $CD4^+$ T cells [22]. The population of $CD4^+$ T cells or antigen-presenting cells may be heterogeneous and contain a subpopulation of cells (such as Th3 cells) capable of producing IL-4 in our system [23]. Alternatively, IL-4, as a regulatory cytokine, might tend to suppress Th1 responses and enhance regulatory T-cell function in FVIII-IT mice [24]. In addition, the

FVIII-IT-mouse-derived $CD4^+$ T cells did not increase their production of the cytokines IL-2, IL-12, IFN-γ and IL-10 (Fig. 5E). These results suggest that the FVIII-specific Th1 cytokine response is suppressed by intrathymic administration of FVIII.

Intrathymic administration of FVIII induces antigen-specific regulatory T cells.

To evaluate the role of FVIII-specific regulatory T cells in the induction of thymic tolerance, we analyzed $CD4^+CD25^+$ T cells and $FoxP3^+$ cells after *in vitro* stimulation with FVIII

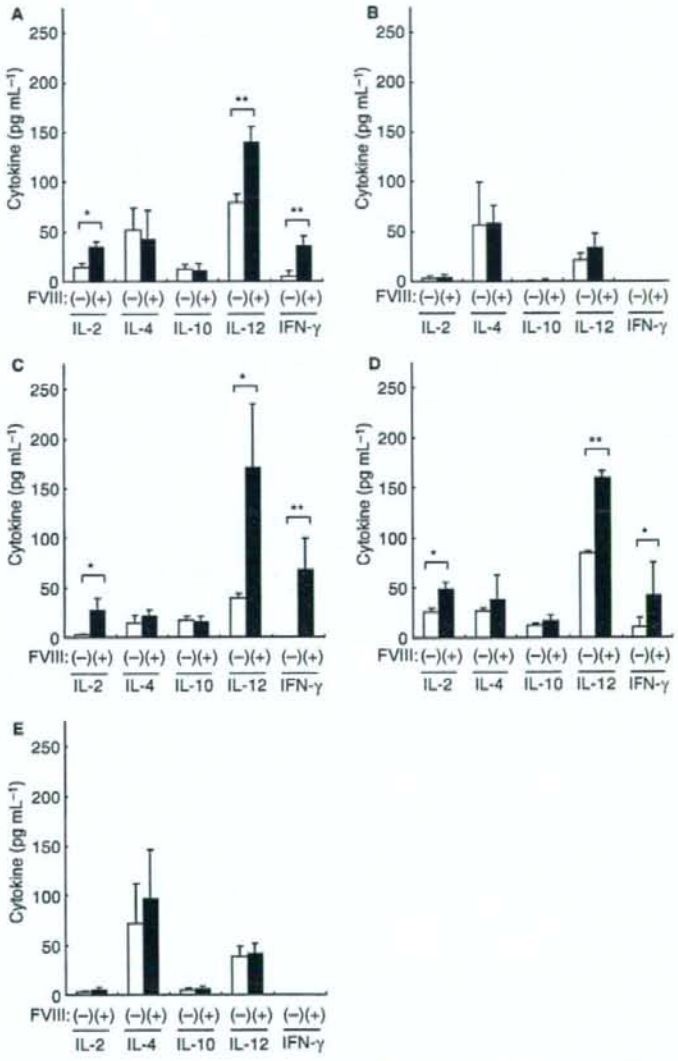


Fig. 5. The Th1 cytokine response is suppressed by intrathymic administration of FVIII. The $CD4^+$ T cells isolated from normal B6 control mice [(A), $n = 5$], FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice) [(C), $n = 6$], mice that had been given intrathymic injections of human albumin [(D), $n = 5$] and mice that had been given intrathymic injections of FVIII [(E), $n = 8$] after repeated intravenous stimulation with FVIII were mixed with the antigen-presenting cells derived from non-IT mice with FVIII stimulation. The $CD4^+$ T cells from FVIII-deficient naïve mice were also examined with the antigen-presenting cells [(B), $n = 5$]. Each group of cells was cultured in the absence (open bars) or presence of 3 nmol L^{-1} FVIII (closed bars), and its cytokine production [interleukin (IL)-2, IL-4, IL-10, IL-12, and interferon-γ (IFN-γ)] were analyzed by enzyme-linked immunosorbent assay as described in Materials and methods. The values (pg mL^{-1}) represent the means ± standard deviations. $^*P < 0.05$; $^{**}P < 0.03$.

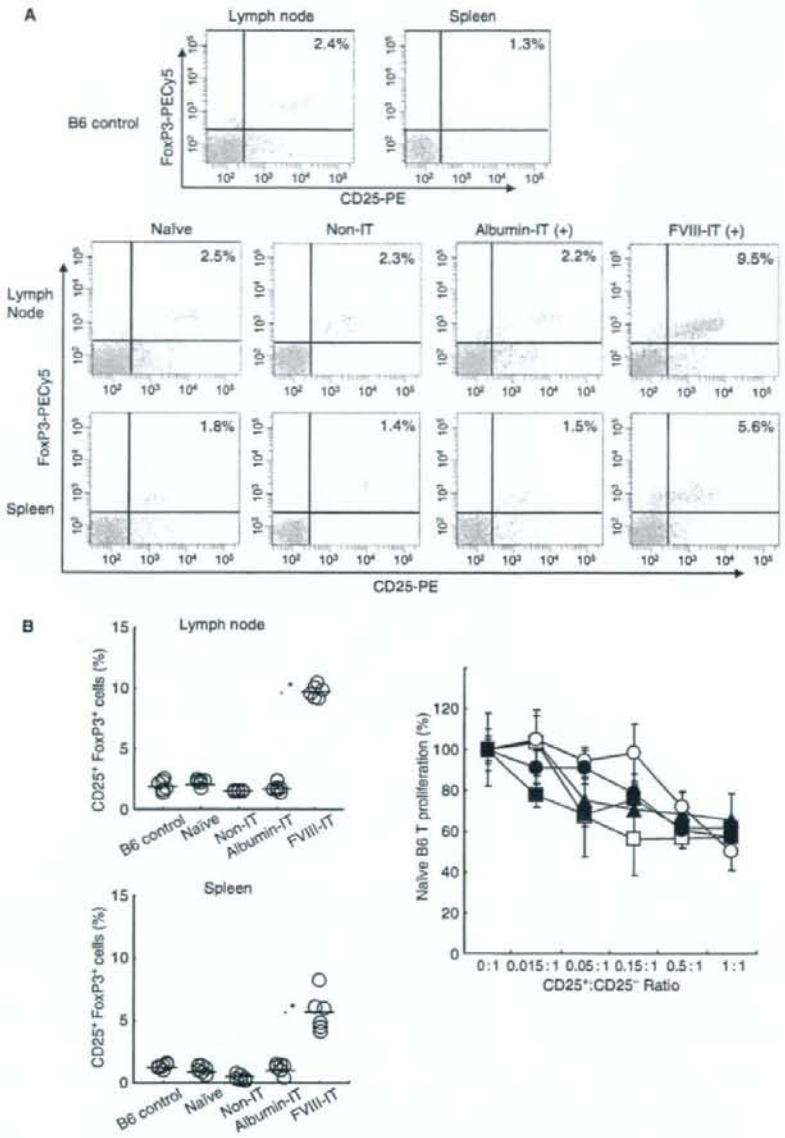


Fig. 6. Injection of FVIII into the thymus increases the numbers of FVIII-specific $CD25^+$ $FoxP3^+$ cells. (A) The lymph node and spleen-derived $CD4^+$ T cells of normal B6 control mice, FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (albumin-IT mice) or mice that had been given intrathymic injections of FVIII (FVIII-IT mice) with repeated intravenous FVIII stimulation were cultured with antigen-presenting cells from FVIII-stimulated non-IT mice in the presence of 3 nmol L^{-1} FVIII for 72 h. The $CD4^+$ T cells of FVIII-deficient naïve mice were also cultured with antigen-presenting cells and FVIII antigen. The percentage of $CD25^+$ $FoxP3^+$ cells among $CD4^+$ T cells was analyzed by flow cytometry as described in Materials and methods. (B) The frequencies of FVIII-specific-$CD25^+$ $FoxP3^+$ cells among $CD4^+$ T cells isolated from the lymph nodes and spleens of B6 control mice (n = 6), naïve mice (n = 6), non-IT mice (n = 6), albumin-IT mice (n = 6) or FVIII-IT mice (n = 6) were analyzed. $^{**}P < 0.03$ as compared with those of other groups. (C) The standard regulatory T-cell inhibition assay. Under stimulation by anti-mouse CD3 antibodies (BD Biosciences), the proliferation of naïve normal B6-derived $CD4^+$ $CD25^-$ T cells was analyzed in the presence of $CD4^+$ $CD25^+$ T cells from B6 control mice (closed triangles, n = 5), FVIII-deficient naïve mice (open squares, n = 5), non-IT mice (open circles, n = 5), albumin-IT mice (closed squares, n = 5), or FVIII-IT mice (closed circles, n = 5), at a variety of ratios. PE, phycoerythrin.

in hemophilia A mice (Fig. 6A). The percentages of lymph node-derived $CD25^+FoxP3^+$ cells of FVIII-IT mice (9.6% ± 2.2%) were significantly greater than those of B6 control (2.0% ± 1.6%), naïve (2.2% ± 0.7%), non-IT (1.8% ± 0.2%) or Alb-IT (1.9% ± 0.8%) mice (Fig. 6B). Moreover, the numbers of spleen-derived $CD25^+FoxP3^+$ cells of FVIII-IT mice (6.5% ± 1.7%) were also augmented as compared with those of B6 control (1.3% ± 0.4%), naïve (1.0% ± 1.3%), non-IT (0.9% ± 1.5%) or Alb-IT (1.2% ± 1.6%) mice. We also performed standard regulatory T-cell inhibition experiments, stimulating normal B6 T cells with anti-CD3 antibodies and adding the presumptive regulatory T cells at a variety of ratios (Fig. 6C). The $CD4^+CD25^+$ T cells isolated from all of these mice similarly inhibited proliferation of normal B6 mouse-derived $CD4^+CD25^-$ T cells in a dose-dependent manner, suggesting that the thymic tolerance of our system might not only depend on the induction of regulatory T cells. Nevertheless, our results show that intrathymic administration of FVIII antigen leads to the augmentation of FVIII-specific regulatory T cells in mice that had never previously been exposed to the antigen.

$CD4^+CD25^+$ T cells isolated from mice with intrathymic injection of FVIII inhibit the FVIII-immunized T-cell proliferative response.

We evaluated the effect of $CD4^+CD25^+$ T cells on the proliferation of antigen-presenting cell-mediated $CD4^+$ T cells of non-IT mice with stimulation with FVIII (Fig. 7A). The $CD4^+CD25^+$ T cells derived from B6, naïve, non-IT or Alb-IT mice could not suppress $CD4^+$ T-cell proliferation (Fig. 7B). By contrast, the FVIII-IT-mouse derived $CD4^+CD25^+$ T cells significantly blocked the proliferation of the non-IT-mouse-derived $CD4^+$ T-cells. Interestingly, we could not find any inhibitory effect in a subset of $CD4^+CD25^-$ T cells of FVIII-IT mice, suggesting that this form of thymic tolerance may be dependent on the generation of FVIII-specific $CD4^+CD25^+$ T cells. We also examined whether $CD4^+CD25^+$ T cells from mice injected during the neonatal period could induce *in vivo* immune tolerance in naïve hemophilia A mice that were challenged with subsequent stimulation of FVIII and Freund's adjuvant. As shown in Fig. 8, adoptive transfer with 0.5×10^6 cells per body of $CD4^+CD25^+$ T cells isolated from FVIII-IT mice significantly blocked the development of anti-FVIII antibodies as compared with $CD4^+CD25^+$ T cells from control non-IT mice (6.9 ± 4.1, $n = 9$ vs. 54.4 ± 11.8 BU mL^{-1}, $n = 10$, respectively; $P = 0.038$). These data support a possible role of regulatory T cells in mediating immune tolerance induction *in vivo*.

Discussion

The development of FVIII inhibitory antibodies represents the major complication in the treatment of patients with hemophilia A with FVIII products [2,25. We used a murine hemophilia A model, because multiple intravenous injections of human FVIII into these mice resulted in high titers of anti-FVIII antibodies that have similar characteristics to those of FVIII inhibitors in clinical cases (Figs 2B and 3A) [3,19,26]. The thymus plays a crucial role in the development of the self-immune system [27]. The presentation of antigen to the thymus may allow for precise modification of the immune repertoire. In several experimental models of organ transplantation, the inoculation of donor antigens into the recipient thymus promoted donor-specific tolerance and achieved prolongation of xenograft survivals [14,15,16. We demonstrated that the accurate administration of FVIII into the thymus under a high-resolution ultrasound system (Fig. 1) resulted in the induction of unresponsiveness to FVIII antigen in hemophilia A mice (Fig. 2D,E). These thymic tolerant mice, immunized with TT, developed high anti-TT antibody titers (Fig. 2H). Thus, our results indicate that the tolerance to FVIII exposure by direct thymic injection is antigen-specific, and that the ability to mount a humoral immune response to other T-cell-dependent antigens remains intact in mice with direct exposure to FVIII in the thymus.

The induction of FVIII-specific tolerance may require the $CD4^+$ T-cell subsets that promote FVIII inhibitors [28]. In addition, the IgG subclass of the antibody directed against an antigen is a good indicator of the $CD4^+$ subset contribution to the antibody response to the specific antigen. We showed that the IgG subclasses of anti-FVIII antibodies were mainly IgG_1, $IgG2_a$ and $IgG2_b$ in mice without thymic treatment; however, the production of each IgG subclass was significantly suppressed in hemophilia A mice with FVIII thymic injection (Fig. 3B). The Th1 immune response is known to be predominant in patients with inhibitors in the long term [29], and was also the predominant response in mice that developed antibodies after challenge in adulthood [30,31]. Furthermore, FVIII-deficient mice injected with FVIII into the thymus failed to develop T-cell proliferative response to FVIII antigen (Fig. 4C), and these $CD4^+$ T cells did not significantly increase their production of IL-2, IL-12 or IFN-γ in response to FVIII stimulation (Fig. 5E). Our observation is consistent with the finding of acquired thymic tolerance in experimental autoimmune encephalomyelitis, where intrathymic injection of myelin basic protein or its encephalitogenic peptide induced Th1 cell unresponsiveness and prevented peripheral expansion of antigen-specific $CD4^+$ T cells [32]. Thus, the thymic-treated mice showed reduced amounts of Th1 cytokines, which are important for antibody production. Intrathymic inoculation with antigen may provide a unique opportunity to study the generation of immunoregulatory T cells.

Several investigators showed that clonal deletion of antigen-reactive T cells might be predominantly responsible after thymic injection of antigen [33–35. Others showed that induction of acquired thymic tolerance occurred through the indirect recognition of donor peptides inoculated into the thymus [36,32,37. $CD4^+CD25^+$ T cells are known to be weakly reactive to antigenic stimulation and to be able to mediate suppression of $CD25^-$ naïve T cells [38,39]. We demonstrated that intrathymic administration of FVIII resulted in an enrichment of $CD4^+CD25^+FoxP3^+$ T cells

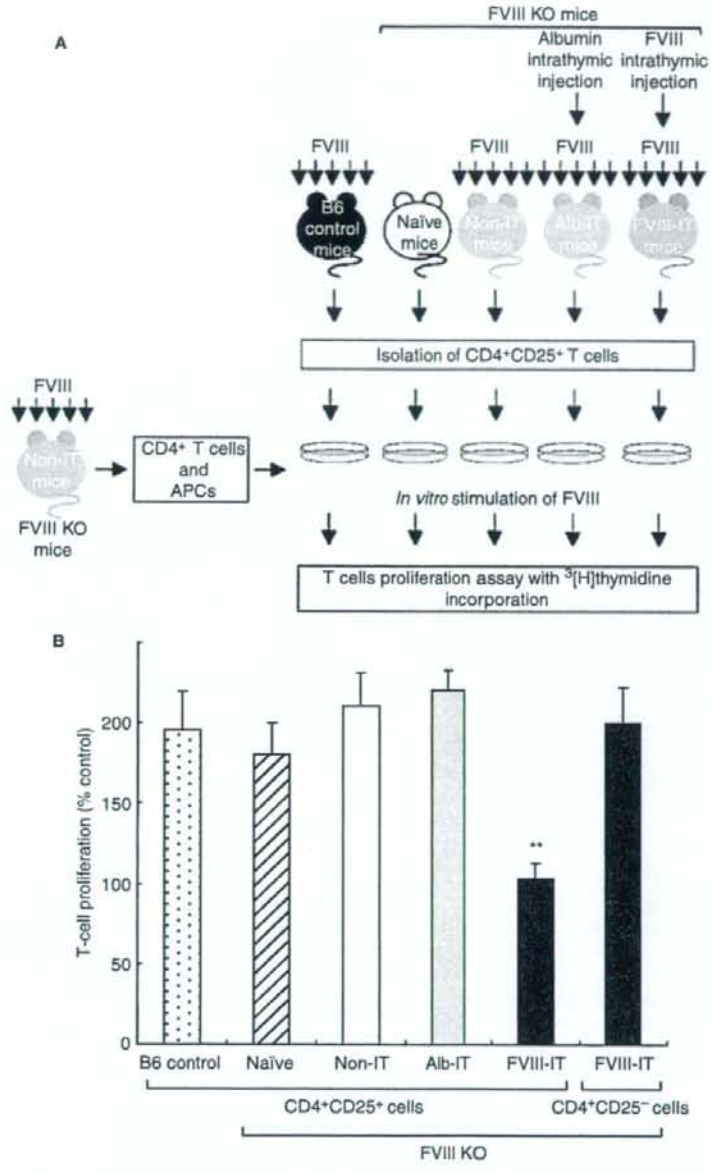


Fig. 7. $CD4^+CD25^+$ T cells isolated from thymus-injected mice inhibit the FVIII-immunized $CD4^+$ T-cell proliferative response. (A) $CD4^+CD25^+$ T cells were isolated from spleens of B6 control mice, FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (Alb-IT mice), and mice that had been given intrathymic injections of FVIII (FVIII-IT mice), each of which had been stimulated with intravenous injections of FVIII, by cell sorting, as described in Materials and methods. The $CD4^+CD25^+$ T cells were also isolated from FVIII-deficient naïve mice. The $CD4^+$ T cells and antigen-presentingt cells (APCs) from non-IT mice with repeated intravenous stimulation with FVIII were separated by cell sorting, as described previously. The isolated cells were used for the T-cell proliferation assay with *in vitro* stimulation with FVIII. (B) The IT-mouse-derived $CD4^+$ T cells and APCs were cultured with $CD4^+CD25^+$ T cells isolated from B6 control mice ($n = 5$), naïve mice ($n = 5$), non-IT mice ($n = 6$), Alb-IT mice ($n = 5$) or FVIII-IT mice ($n = 6$) in the presence of 3 nmol L^{-1} FVIII for 72 h. The amounts of $[^3H]$thymidine incorporation were determined by scintillation counting. Data are shown as means ± standard deviations. $^{**}P < 0.03$ as compared with other groups.

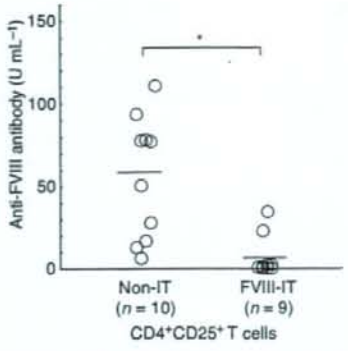


Fig. 8. Adoptive transfer of $CD4^+CD25^+$ regulatory T cells into syngeneic hemophilia A mice. The $CD4^+CD25^+$ T cells were prepared from the spleens of mice without intrathymic administration of FVIII (non-IT mice) or mice that had been given intrathymic injections of FVIII (FVIII-IT mice). A total of 0.5×10^6 $CD4^+CD25^+$ T cells per body in 100 μL of phosphate-buffered saline (PBS) was injected into naïve hemophilia A mice via the jugular vein. Each mouse was challenged with repeated intravenous stimulation of 0.05 u g^{-1} body weight FVIII every 2 weeks, and inhibitory antibody titers were measured after the fifth injection by Bethesda assay.

in peripheral lymphoid organs (Fig. 6A,B), suggesting that increased frequencies of antigen-specific regulatory T cells may be in favor of positive selection as the likely mechanism contributing to increased frequencies of antigen-specific $CD4^+CD25^+$ T cells upon recognition of the antigen in the thymus [40,41]. FoxP3 is thought to positively control the function of regulatory T cells in a binary fashion, as FoxP3 expression is sufficient to specify immune-suppressive activities in conventional T cells, and it is critically important for the development and function of regulatory T cells [42,43]. The results obtained with *ex vivo* expanded regulatory T cells in experimental models are worth noting, because infusion with *ex vivo* activated and expanded regulatory T cells significantly inhibited lethal graft-vs.-host disease in several murine models [44–46]. It is known that $CD4^+CD25^+$ T cells are not the only regulatory lymphocytes that have been found. $CD25^-$ regulatory T cells include the Th3 cells identified in the mucosal immune system and the Tr1 cells characterized *in vitro*. In particular, Tr1 cells could be generated after stimulation with a high concentration of IL-10 [47,48]. However, we could not find any inhibitory effect in a subset of $CD4^+CD25^-$ T cells (Fig. 7B). In addition, $CD4^+$ T cells isolated from FVIII-IT mice produced small amounts of IL-10 (Fig. 5E). Although Tr1 cells might not play an important role in the immune tolerance induction by thymic injection of FVIII antigen in our system, our study is limited to the role of $CD4^+CD25^+FoxP3^+$ regulatory T cells in inducing immune tolerance with thymic injection of specific antigens. Consequently, the antigen-specific regulatory T cells developed by inthrathymic injection of FVIII may efficiently prevent the formation of antibody against FVIII in our murine models.

The $CD4^+CD25^+$ regulatory T cells may be responsible for the translation of tolerance from an antigen-inoculated thymus to a mature but naïve peripheral immune system [38,39]. We showed that only $CD4^+CD25^+$ T cells from mice intrathmically treated with FVIII suppressed the antigen-presenting cell-mediated proliferative response of $CD4^+$ T cells under *in vitro* FVIII stimulation (Fig. 7B), and that the *in vivo* adoptive transfers with $CD4^+CD25^+$ T cells isolated from FVIII-IT mice blocked the development of anti-FVIII antibodies in naïve hemophilia A mice (Fig. 8). Several experiments involving the T-cell compartment have suggested that receptor editing and clonal anergy are involved in tolerance induction [49,50]. Although intrinsic processes of these cells are essential for survival of the organism, they are imperfect at times, and autoreactive T cells can be found even in the peripheral blood of immunologically competent animals and humans [51]. The $CD4^+CD25^+$ regulatory T cells, after thymic selection, are able to suppress proliferation of these autoreactive T cells [39]. Although the exact nature of the mechanisms driving regulatory T-cell generation in the thymus is the subject of intense scrutiny [52], our results delineate a simple means to generate immunoregulatory T cells in hemophilia A mice by central tolerance induction, and provide a relevant assay for their function *ex vitro*. The immune system of neonatal mice, which is more immature than that of newborns or infants, resembles more closely that of human fetuses, and the conclusions drawn from our study may not necessarily apply to patients suffering from hemophilia A. Further study of the precise mechanism of action and the events that determine whether a developing T cell will undergo deletion or will assume an anergic regulatory cell role will have the potential to define new strategies to induce stable tolerance in hemophilia A.

In conclusion, an understanding of the underlying mechanisms of T-cell tolerance induced by intrathymic inoculation of FVIII is essential for the development of this novel strategy for hemophilia A patients with inhibitors.

Addendum

S. Madoiwa designed and performed the research, analyzed data, and wrote the paper; T. Yamauchi, E. Kobayashi, Y. Hakamata, M. Dokai, N. Makino, Y. Kashiwakura and A. Ishiwata performed experiments; T Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

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Disclosure of Conflict of Interests

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References

1 Hoyer LW. Hemophilia A. *N Engl J Med* 1994; **330**: 38–47.
2 Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients – a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia* 1999; **5**: 145–54.
3 Madoiwa S, Yamauchi T, Hakamata Y, Kobayashi E, Arai M, Sugo T, Mimuro J, Sakata Y. Induction of immune tolerance by neonatal intravenous injection of human factor VIII in murine hemophilia A. *J Thromb Haemost* 2004; **2**: 754–62.
4 Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; **22**: 531–62.
5 Kyewski B, Klein L. A central role for central tolerance. *Annu Rev Immunol* 2006; **24**: 571–606.
6 Kishimoto H, Surh CD, Sprent J. A role for Fas in negative selection of thymocytes in vivo. *J Exp Med* 1998; **187**: 1427–38.
7 von Boehmer H, Aifantis I, Gounari F, Azogui O, Haughn L, Apostolou I, Jaeckel E, Grassi F, Klein L. Thymic selection revisited: how essential is it? *Immunol Rev* 2003; **191**: 62–78.
8 Blais ME, Louis I, Perreault C. T-cell development: an extrathymic perspective. *Immunol Rev* 2006; **209**: 103–14.
9 Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998; **10**: 1969–80.
10 Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287–96.
11 Anderson G, Jenkinson WE, Jones T, Parnell SM, Kinsella FA, White AJ, Pongrac'z JE, Rossi SW, Jenkinson EJ. Establishment and functioning of intrathymic microenvironments. *Immunol Rev* 2006; **209**: 10–27.
12 Darrasse-Jeze G, Marodon G, Salomon BL, Catala M, Klatzmann D. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood* 2005; **105**: 4715–21.
13 Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345–52.
14 Posselt AM, Barker CF, Tomaszewski JE, Markmann JF, Choti MA, Naji A. Induction of donor-specific unresponsiveness by intrathymic islet transplantation. *Science (NY)* 1990; **249**: 1293–5.
15 Sayegh MH, Perico N, Gallon L, Imberti O, Hancock WW, Remuzzi G, Carpenter CB. Mechanisms of acquired thymic unresponsiveness to renal allografts. Thymic recognition of immunodominant allo-MHC peptides induces peripheral T cell anergy. *Transplantation* 1994; **58**: 125–32.
16 Jonker M, van den Hout Y, Noort RC, Versteeg-van der Voort Maarschalk MF, Claas FH, Woude FJ, Hollander D, Perico N, Remuzzi G. Immunomodulation by intrathymic injection of donor leukocytes in rhesus monkeys. *Transplantation* 2001; **72**: 1432–6.
17 Posselt AM, Barker CF, Friedman AL, Naji A. Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. *Science (NY)* 1992; **256**: 1321–4.
18 Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995; **10**: 119–21.
19 Qian J, Borovok M, Bi L, Kazazian HH Jr, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost* 1999; **81**: 240–4.
20 Oluwole SF, Jin MX, Chowdhury NC, Ohajekwe OA. Effectiveness of intrathymic inoculation of soluble antigens in the induction of specific unresponsiveness to rat islet allografts without transient recipient immunosuppression. *Transplantation* 1994; **58**: 1077–81.
21 Zhang M, Vacchio MS, Vistica BP, Lesage S, Egwuagu CE, Yu CR, Gelderman MP, Kennedy MC, Wawrousek EF, Gery I. T cell tolerance to a neo-self antigen expressed by thymic epithelial cells: the soluble form is more effective than the membrane-bound form. *J Immunol* 2003; **170**: 3954–62.
22 Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev* 2002; **2**: 933–44.
23 Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med* 1995; **182**: 1579–84.
24 Anastasi E, Campese AF, Bellavia D, Bulotta A, Balestri A, Pascucci M, Checquolo S, Gradini R, Lendahl U, Frati L, Gulino A, Di Mario U, Screpanti I. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. *J Immunol* 2003; **171**: 4504–11.
25 Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992; **339**: 594–8.
26 Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *Thromb Haemost* 2000; **84**: 826–32.
27 Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000; **101**: 455–8.
28 Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; **383**: 787–93.
29 Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost* 2002; **88**: 568–75.
30 Wu H, Reding M, Qian J, Okita DK, Parker E, Lollar P, Hoyer LW, Conti-Fine BM. Mechanism of the immune response to human factor VIII in murine hemophilia A. *Thromb Haemost* 2001; **85**: 125–33.
31 Sasgary M, Ahmad RU, Schwarz HP, Turecek PL, Reipert BM. Single cell analysis of factor VIII-specific T cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 266–72.
32 Chen W, Sayegh MH, Khoury SJ. Mechanisms of acquired thymic tolerance in vivo: intrathymic injection of antigen induces apoptosis of thymocytes and peripheral T cell anergy. *J Immunol* 1998; **160**: 1504–8.
33 Markmann JF, Odorico JS, Bassiri H, Desai N, Kim JI, Barker CF. Deletion of donor-reactive T lymphocytes in adult mice after intrathymic inoculation with lymphoid cells. *Transplantation* 1993; **55**: 871–6.
34 Khoury SJ, Sayegh MH, Hancock WW, Gallon L, Carpenter CB, Weiner HL. Acquired tolerance to experimental autoimmune encephalomyelitis by intrathymic injection of myelin basic protein or its major encephalitogenic peptide. *J Exp Med* 1993; **178**: 559–66.
35 Gillanders WE, Arima T, Tu F, Hansen TH, Flye MW. Evidence for clonal deletion and clonal anergy after intrathymic antigen injection in a transplantation model. *Transplantation* 1997; **64**: 1159–66.
36 Chowdhury NC, Murphy B, Sayegh MH, Jin MX, Roy DK, Hardy MA, Oluwole SF. Acquired systemic tolerance to rat cardiac allografts induced by intrathymic inoculation of synthetic polymorphic MHC class I allopeptides. *Transplantation* 1996; **62**: 1878–82.
37 Oluwole SF, Chowdhury NC, Ingram M, Garrovillo M, Jin MX, Agrawal S. Mechanism of acquired thymic tolerance induced by a single major histocompatibility complex class I peptide with the dominant epitope: differential analysis of regulatory cytokines in the lymphoid and intragraft compartments. *Transplantation* 1999; **68**: 418–29.
38 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor

alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151–64.

39 Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999; **162**: 5317–26.

40 Saborio DV, Chowdhury NC, Jin MX, Chandraker A, Sayegh MH, Oluwole SF. Regulatory T cells maintain peripheral tolerance to islet allografts induced by intrathymic injection of MHC class I allopeptides. *Cell Transplant* 1999; **8**: 375–81.

41 Marodon G, Fisson S, Levacher B, Fabre M, Salomon BL, Klatzmann D. Induction of antigen-specific tolerance by intrathymic injection of lentiviral vectors. *Blood* 2006; **108**: 2972–8.

42 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330–6.

43 Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol* 2006; **24**: 209–26.

44 Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 2002; **99**: 3493–9.

45 Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J Exp Med* 2002; **196**: 401–6.

46 Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, Cohen JL. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest* 2003; **112**: 1688–96.

47 Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25– T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004; **172**: 5149–53.

48 Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 2006; **212**: 28–50.

49 Van Parijs L, Abbas AK. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science (NY)* 1998; **280**: 243–8.

50 O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004; **10**: 801–5.

51 Le NT, Chao N. Regulating regulatory T cells. *Bone Marrow Transplant* 2007; **39**: 1–9.

52 Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med* 2004; **200**: 1039–49.

Phenotypic Correction of Hemophilia A by Ectopic Expression of Activated Factor VII in Platelets

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Platelets are receiving much attention as novel target cells to secrete a coagulation factor for hemophilia gene therapy. In order to extend the application of platelet-directed gene therapy, we examined whether ectopic expression of activated factor VII (FVIIa) in platelets would result in an efficient bypass therapy to induce sufficient thrombin generation on platelet surfaces in mice with hemophilia A. Transduction of bone marrow cells with a simian immunodeficiency virus (SIV)-based lentiviral vector harboring the platelet-specific *GPIbα* promoter resulted in efficient transgene expression in platelets. FVIIa antigen was expressed in platelets by this SIV system; FVII transgene products were found to localize in the cytoplasm and translocate toward the sub-membrane zone and cell surface after activation. Although FVII antigen levels in platelets did not reach the therapeutic levels seen with FVIIa infusion therapy, whole-blood coagulation, as assessed by thromboelastography, was significantly improved in mice with hemophilia A. Further, we observed correction of the bleeding phenotype in mice with hemophilia A after transplantation, even in the presence of FVIII-neutralizing antibodies. Our results demonstrate that FVIIa-expressing platelets can strengthen hemostatic function and may be useful in treating hemophilia and other inherited bleeding disorders. These findings are comparable to the proven therapeutic effects of FVIIa infusion.

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INTRODUCTION

Platelets are differentiated anucleate cells whose functions are essential for hemostasis. Because platelets can circulate throughout the body, release a number of mediators on demand, and provide a scaffold for the coagulation cascade, the targeting of platelets as a circulating delivery system would seem a reasonable approach to genetic modification of hemostasis. The feasibility of such a platelet-directed approach was originally demonstrated by Poncz *et al.* in transgenic mice.[1] Platelet expression of urokinase-type plasminogen activator, using a platelet-specific *platelet factor-4* promoter, enabled urokinase-type plasminogen activator to be stored in platelets and then released within developing thrombi when the platelets became activated.[1] Further, platelet-specific expression of factor VIII (FVIII) can be achieved in a transgenic setting, with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma.[2] In addition, Shi *et al.* have demonstrated that ectopically expressed FVIII in platelets can be used in the treatment of hemophilia with or without FVIII-neutralizing antibodies, and that targeted FVIII expression in platelets continues to support hemostasis even in the presence of high titers of FVIII-neutralizing antibodies.[3] We and others have applied this approach to gene therapy, and have demonstrated that transplantation of hematopoietic stem cells (HSCs) transduced with a lentiviral vector containing *human FVIII* driven by a platelet-specific promoter improves the hemostatic function of mice with FVIII-deficient hemophilia A, despite the levels of FVIII in their plasma being scant or undetectable.[4,5]

In order to further extend the application of platelet-directed gene therapy, we focused our attention on the extrinsic pathway initiated by tissue factor (TF). Assembly of TF and activated Factor VII (FVIIa) complexes on anionic phospholipids expressed on activated cell membranes is the most important initiation mechanism for blood coagulation.[6] Recently, recombinant human FVIIa (rhFVIIa: NovoSeven) has proven to be a highly successful alternative treatment for hemophilia patients.[7] Patients with a variety of other coagulation deficiencies that are characterized by impaired thrombin generation have been successfully treated with rhFVIIa.[8] In addition, liver-directed gene therapy with an adeno-associated virus vector equipped with hFVIIa achieved therapeutic plasma hFVIIa levels in a mouse model of hemophilia B, and phenotypic correction was observed when a murine FVIIa (mFVIIa) homolog was used.[9] In addition, it is possible that platelets that stably express FVIIa can efficiently induce hemostasis at the site of vascular injury in a variety of hemorrhagic disorders. In this study, we used gene therapy to examine whether platelet-specific FVIIa expression would result in an efficient bypass therapy for

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factor X activation, thereby generating sufficient thrombin on platelet surfaces in FVIII-deficient mice.

RESULTS

Enhanced green fluorescent protein expression in platelets after transplantation of HSCs transduced with a simian immunodeficiency virus lentiviral vector harboring the platelet *GPIbα* promoter

We have previously shown that transplantation of c-kit$^+$, sca-1$^+$, and lineage$^-$ (KSL) murine HSCs that are transduced with an simian immunodeficiency virus (SIV)-based lentiviral vector carrying enhanced green fluorescent protein (*eGFP*), driven by a platelet-specific *GPIbα* promoter, enables efficient expression of eGFP in platelets.[4] Because transplantation of KSL cells requires nontransduced bone marrow cells, engraftment by the transduced cells is no >40–55% after transplantation.[4] In order to obviate the need for competitor cells, we validated the transplantation procedure using unfractionated bone marrow cells. As shown in Figure 1, transplantation using unfractionated bone marrow cells resulted in more efficient gene targeting to platelets. eGFP expression in platelets was sustained for at least 3 months after transplantation (Figure 1b), and we found that 0.60–2.78 vector copies/genome had integrated into the cells of the mice that had received the transplants (Figure 1c).

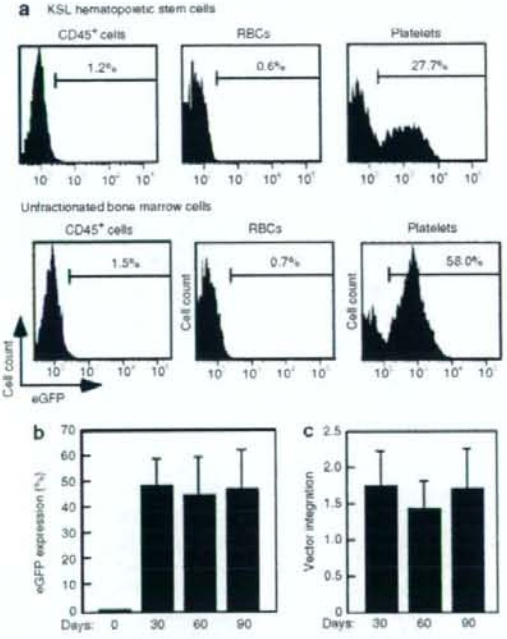

Figure 1 Effects of different stem cell sources on enhanced green fluorescent protein (eGFP) expression in platelets *in vivo*. KSL cells or whole bone marrow cells obtained from Ly5.1 mice were transduced with SIV-GPIbα-eGFP at a multiplicity of infection of 30. Irradiated Ly5.2 mice received either transduced KSL cells (1×10^4) together with competitor cells (2×10^5), or transduced unfractionated bone marrow cells (2×10^6). (**a**) Representative flow cytometry analysis of eGFP-positive cells among CD45$^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood 30 days after transplantation. (**b**) Percentages of eGFP-positive platelets at 30, 60, and 90 days after transplantation. Columns and error bars represent the mean ± SD ($n = 7$). (**c**) Proviral integration into the genomic DNA of bone marrow cells was quantified at 30, 60, and 90 days after transplantation by real-time quantitative PCR. Columns and error bars represent the mean ± SD ($n = 7$). SIV, simian immunodeficiency virus.

hFVII-2RKR expression in platelets induced by platelet-directed gene transduction

The rhFVIIa product currently in clinical use is produced in a single-chain form and activated to the two-chain form during protein purification.[7] Approximately 1% of circulating hFVII in healthy individuals is in the activated form, and the amount of hFVIIa required for bypassing is much larger than the physiological concentration.[7] In order to secrete the activated form of hFVII from transduced cells, we inserted into the factor X activation–cleavage site two arginine/lysine/arginine (RKR) sequences recognized by an intracellular paired basic amino-acid cleaving enzyme/furin type protease, resulting in the secretion of the two-chain molecule with a structure similar to hFVIIa (hFVII-2RKR; Figure 2a).[8]

We first examined whether functional FVIIa was produced in megakaryocytes. After transduction with SIV vector containing *hFVII-2RKR* driven by cytomegalovirus promoter, hFVII antigen in the supernatant from the megakaryoblastic cell line UT-7/TPO was detected and found to have activity similar to that from HEP-G2 cells (Figure 3a and c). The FVII activity of FVII-2RKR was much higher than that of plasma-derived hFVII (Figure 3c), thereby suggesting that FVII-2RKR could be cleaved into two chains. In addition, mRNA expression of γ-glutamyl carboxylase, which post-translationally modifies glutamyl residues into γ-carboxyglutamyl residues of vitamin K–dependent coagulation

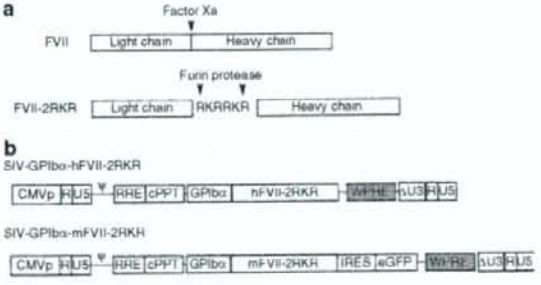

Figure 2 Schematic representation of factor VII (FVII) and simian immunodeficiency virus (SIV) lentiviral vector. (**a**) FVII and engineered activated FVII (FVII-2RKR) construct showing the light and heavy chains. Arrows indicate the recognition sites of physiological factor X activation (FXa) and the intracellular paired basic amino-acid cleaving enzyme/furin type protease. (**b**) The SIV lentiviral vector for platelet-specific gene expression consisted of a cytomegalovirus (CMV)/long-terminal repeat (LTR) chimeric promoter followed by a packaging signal (ψ), a rev-binding element (RRE) for cytoplasmic export of the RNA, the transgene expression region consisting of an internal promoter (*GPIbα*) and the transgene (*hFVII-2RKR* or *mFVII-2RKR-IRES-eGFP*), woodchuck hepatitis virus post regulatory element (WPRE), and a 3′-self-inactivating LTR. cPPT, central polypurine tract; eGFP, enhanced green fluorescent protein; hFVII, human FVII; IRES, internal ribosomal entry site; mFVII, murine FVII.