Pharm Res (2013) 30:2855–2867
DOI 10.1007/s11095-013-1115-3

RESEARCH PAPER

Development of a Transgenic Mouse Model with Immune Tolerance for Human Coagulation Factor VIIa

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Received: 16 March 2013 / Accepted: 4 June 2013 / Published online: 18 June 2013

ABSTRACT

Purpose Human factor VIIa (FVIIa) is commonly used as bypassing therapy to treat bleeding episodes in hemophilia patients with neutralizing antibodies to factors VIII (FVIII) or IX (FIX). There is a need for a suitable animal model to assess the immunogenicity of new FVIIa products during preclinical development. The aim of this study was the design of a novel transgenic mouse model with immune tolerance to human FVIIa.

Methods The model was generated by transgenic expression of human *F7* cDNA. FVIIa-specific immune responses after treatment with human FVIIa were assessed by analyzing circulating antibodies, antibody producing plasma cells and $CD4^+$ T cells.

Results In contrast to wild-type mice, human FVII transgenic mice did not develop antibodies when treated with human FVIIa. The immune tolerance was specific and could be broken by application of human FVIIa together with a strong stimulus of the innate immune system. Break of tolerance was associated with increased numbers of pro-inflammatory FVIIa-specific $CD4^+$ T cells.

Conclusions The new mouse model is suitable to study the influence of the innate immune system on maintenance and break of immune tolerance against FVIIa and could be used to assess the immunogenicity of new FVIIa products during preclinical development.

KEY WORDS factor FVIIa · hemophilia · immunogenicity · therapeutic proteins · transgenic mouse models

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ABBREVIATIONS

ACTB	Beta-actin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
FIX	Coagulation factor IX
FVII	Coagulation factor VII
FVIIa	Coagulation factor VIIa
FVIII	Coagulation factor VIII
GMP	Good manufacturing practice
HPLC-SEC	High performance liquid chromatography-size exclusion chromatography
hprt	Hypoxanthine phosphoribosyltransferase
IFN-γ	Interferon gamma
Ig	Immunglobulin
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PMT	Photo Multiplier Tube
VWF	von Willebrand factor

INTRODUCTION

Patients with hemophilia A or hemophilia B receive replacement therapy with FVIII or FIX products, respectively. 20–30% of patients with severe hemophilia A, 3–15% with mild or moderate hemophilia A and 2–4% of patients with severe hemophilia B develop neutralizing antibodies against the coagulation factor products (1,2). These antibodies interfere with the functional activity of the coagulation factor which results in the reduction or complete loss of therapeutic efficacy. Patients who are refractory to further treatment with FVIII or FIX have to be treated with bypassing therapies that induce hemostasis independently of FVIII or FIX. Currently, FVIIa and activated prothrombin-complex concentrates are most widely used (3).

FVIIa has a short half-life which makes frequent treatment necessary. In order to improve the convenience for patients, current efforts focus on the extension of half-life (4,5), the enhancement of biological activity (6) and the application of FVIIa via alternative routes (7). These approaches include either new formulations or chemical or molecular modifications of the FVIIa protein. Most of these new approaches bear the risk of creating neo-epitopes for T cells or B cells or of creating structural alterations that stimulate innate immune cells. Consequently, such alterations could trigger the development of antibodies associated with critical clinical implications such as reduced efficacy, altered pharmacokinetics, hypersensitivity reactions, or break of immune tolerance to the endogenous counterpart.

Patients who have been treated with FVII- or FVIIa-containing products have very rarely been reported to develop neutralizing antibodies against these proteins. This indicates that products containing FVII or FVIIa are very safe and have a low probability of inducing unwanted immune responses. Astermark *et al.* reported antibodies against FVIIa in some patients suffering from hemophilia A and B, who had been treated with activated prothrombin complex concentrates and human FVIIa. These antibodies were not neutralizing in the hemophilia A patients but had some neutralizing effect in hemophilia B patients, most likely resulting from cross-reactivity of anti-FVIIa antibodies with FIX. FIX is structurally very similar to FVII (8). Delmer *et al.* (9) and Okajima *et al.* (10) described patients who presented with life-threatening bleeding disorders caused by autoantibody-induced FVII deficiency. In these cases, endogenous FVII levels were below 1% because of neutralization by antibodies. These rare examples of acquired FVII deficiencies indicate that the consequences of neutralizing antibodies against FVII or FVIIa could be dramatic for the patient. Recently, the phase III clinical trial of the FVIIa analogue vatreptacog alfa, containing three amino acid substitutions compared with native FVIIa, was discontinued because of the development of anti-drug antibodies in some patients (11). Another recombinant FVIIa analogue (BAY 86-6150) was recently terminated because of increased immunogenicity in a phase II/III clinical trial (12). BAY 86-6150 was a novel rFVIIa variant containing 6 amino acid changes compared to native FVIIa (5). These two examples illustrate that it is important to develop better tools that could help to assess the potential immunogenicity of new FVIIa candidates before they enter clinical development.

We developed a new transgenic mouse model which could help to identify candidates of new FVIIa preparations with high immunogenic risk before they enter clinical development. Human FVII transgenic mice are immunologically tolerant to human FVIIa because they express a human *F7* cDNA. The new model overcomes one of the major limitations of conventional mouse models which respond with antibodies to human FVIIa because of the xenogenic nature of the human protein. Similar mouse models were already described for human FVIII (13), human insulin (14), human tissue type-plasminogen activator variants (15), human FIX (16) and human interferon beta (17). In this study we describe the generation and characterization of the new transgenic mouse model and present the results of its immunological characterization.

MATERIALS AND METHODS

Generation of Human FVII-Transgenic Mice

Human FVII-transgenic mice were generated by Speedy Mouse® technology (Nucleis, France) to produce a single copy transgene insertion at the *hprt* locus as described (18). *F7* cDNA was isolated as described (19) and subsequently cloned into a pBluescript® II KS (+) vector (Stratagene). The vector was digested by EcoRI to extract the *F7* cDNA which was then cloned into a vector containing an albumin promoter, a chimeric rat/human growth hormone poly A signal (rGHhGH polyA) and an alpha-fetoprotein enhancer (pBS-Alb/αFeto) as described (20). The pBS-Alb/αFeto vector was digested by NotI and cloned into the polylinker of the Gateway® pENTR-1A vector (Invitrogen). The transgene was then transferred by the Gateway® technology into a destination vector pDEST-HPRT (Nucleis) carrying two homologous arms of the murine *hprt* gene and the human *hprt* promoter region including exon one of the human *hprt* gene. The vector was linearized using PvuI and electroporated into *hprt*-deficient BPES-embryonic stem (ES) cells by standard methods. The targeting construct contained the missing sequences in BPES-ES cells and two regions of homology of the *hprt* gene, which allowed insertion of the transgene at this locus (Fig. 1a). The targeted ES clones were selected in hypoxanthine-aminopterin-thymidine (HAT) medium (Sigma-Aldrich). Five HAT-resistant clones were injected into 129/Sv-derived blastocysts that were transplanted into recipient C57BL/6 females, one of which gave one chimeric male. The founder was crossed with C57BL/6 and seven female F1 mice were generated and tested for containing the transgene by PCR analysis. These mice were bred to homozygosity and backcrossed to C57BL/6. Experiments were conducted in fully backcrossed (>99.6% C57BL/6) male and female progeny.

Genomic DNA Preparation, RNA Preparation and cDNA Synthesis

Genomic DNA from ear punches or tail cuts was isolated using the Nexttec Genomic DNA Isolation Kit (Nexttec) following the protocols provided by the manufacturer. Organs (heart, liver, abdominal lymph nodes, lung, spleen, reproductive organs, kidneys, thymus, femoral muscle, bone marrow, brain) obtained from 8 week old male and female human FVII

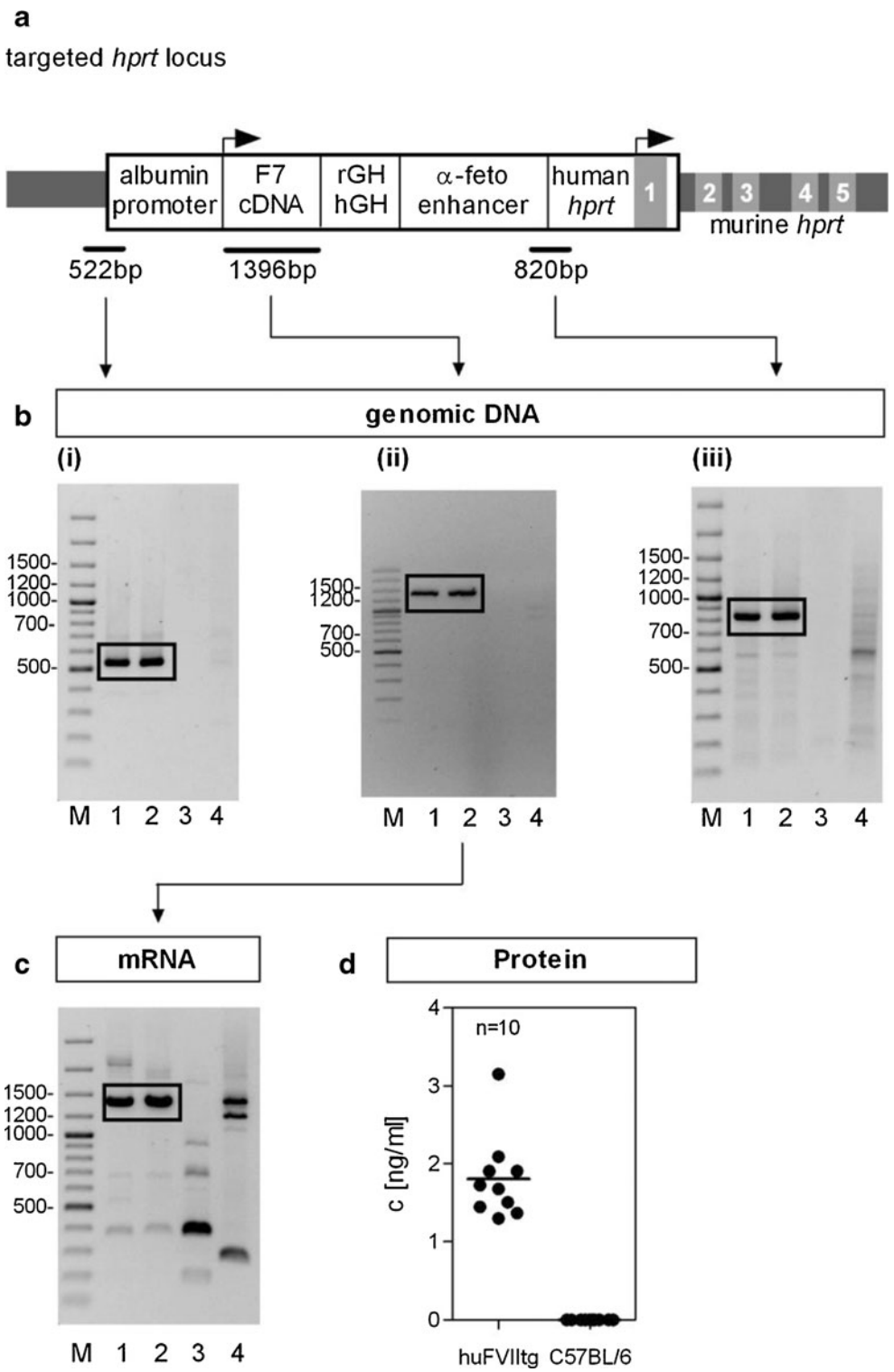

Fig. 1 Human *F7* cDNA is correctly integrated into the murine *hprt* locus. (**a**) Schematic representation of the integrated human *F7* construct consisting of the murine albumin promoter, human *F7* cDNA, a 2.1 kb fragment of a chimeric rat–human growth hormone gene (rGHhGH), the murine alpha-fetoprotein (α-feto) enhancer, and parts of the *hprt* locus to target for homologous recombination at the murine *hprt* locus. Transcriptional start sites are indicated by *arrows*. Exons of the *hprt* gene are indicated by *numbered boxes*. Positions and expected product lengths of confirmatory PCRs are designated. (**b**) Confirmatory PCRs on the gDNA level. PCR products of gDNA obtained from human FVII-transgenic mice (*1*—male, *2*—female), a male C57BL/6 control (*3*) and a human control DNA (*4*) were analyzed. The molecular size (in bp) is indicated in the first lanes (*M*). Panel (*i*): Murine *hprt* to albumin promoter (552 bp). Panel (*ii*): *F7* cDNA (1,396 bp). Panel (*iii*): α-feto enhancer to human *hprt* (820 bp). (**c**) Confirmatory PCR on the mRNA level. Liver cDNA of human FVII-transgenic mice and C57BL/6 mice was analysed. The PCR setup shown in 1b, panel (*ii*) was repeated (lanes M; 1–4 denoted as in 1b). (**d**) Detection of human FVII protein in plasma samples of human FVII-transgenic mice (huFVIItg) and C57BL/6 mice. (Each point represents an individual mouse, *lines* show mean values. Human FVII levels in plasma samples of C57BL/6 mice were below the detection limit of the assay).

transgenic mice and neonatal thymi (taken 1 day after birth) from male and female human FVII transgenic mice were used for RNA extraction. Wildtype C57BL/6 mice were included as controls (data not shown). Total RNA of the organs was preserved with RNALater reagent (Qiagen) and the RNeasy procedure (Qiagen) was applied, following the protocols provided by the manufacturer. Purified total RNA was deprived of genomic DNA using gDNA Wipeout buffer (component of the QuantiTect Reverse Transcription kit, Quiagen) and subsequently reversely transcribed into cDNA by using the QuantiTect Reverse Transcription kit.

Genotyping of Human FVII-Transgenic Mice

Genomic DNA, RNA and cDNA were prepared by standard methods. PCR was used to confirm complete and correct transgene insertion as illustrated in Fig. 1b. DNA amplification was done using GoTaq polymerase (Promega). A forward primer from the murine *hprt* gene (5′-CAGGAACCTATT ATGCTGGC-3′) and a reverse primer form the albumin promoter (5′-GCAGAAGCTTAGGAAGATGG-3′) were used to amplify the upstream region of the transgene. The expected size of the corresponding PCR product is 522 base pairs (bp). A forward primer from the alpha-fetoprotein enhancer (5′-GAATAGTCAAGGGTAGAGCC-3′) and a reverse primer (5′-GATAGGTCAGGTAAGCAAGCAAC-3′) from the human *hprt* promoter were used to amplify the downstream region of the transgene. The expected size of the corresponding PCR product is 820 bp. Insertion of the complete *F7* sequence was confirmed by using primers spanning the whole *F7* region. This reaction was done both on the gDNA and the mRNA level. The forward primer was 5′-TAGGGAAATGGGGCTCGCAGG-3′ and the reverse primer was 5′-TCTCCCAGGCCC TCAGGCT-3′. The expected size of the corresponding PCR product is 1,396 bp. The PCR reactions were analyzed on 1.5% agarose gels (Nusieve 3:1, Lonza), stained with SYBR Gold (Invitrogen) and visualized on a Gel Doc XR imager (Bio-Rad).

Reverse-Transcription Quantitative Real-Time-PCR (RT qPCR)

Quantitative determination of gene expression levels on the cDNA samples was done using an ABI Prism 7500 Fast PCR system (Applied Biosystems). Commercially available TaqMan Gene Expression kits (Applied Biosystems) for human *F7*, murine *F7* and murine *beta-actin* (*ACTB*) were used. All TaqMan Gene Expression kits were tested for specificity and efficiency. Relative gene expression levels were determined according to the manufacturer's ΔCt method (Applied Biosystems).

Animal Studies and Blood Sampling

Mice were male or female and aged 8 to 12 weeks at the beginning of the treatment studies. A total of 471 FVII transgenic mice and a total of 128 C57BL/6 wildtype control mice were included in the different studies. All studies were carried out in accordance with Austrian federal law (Act BG 501/1989) regulating animal experimentation and were approved by the institutional animal care and use committee. All invasive procedures were carried out under anesthesia with xylazin (Xylasol; Graeub) and ketamine (Ketasol; Graeub). Blood samples were obtained by cardiac puncture or retro-orbital sampling. The samples obtained from individual mice were added to 0.1 M sodium citrate (Gespag) at a 4:1 (vol/vol) ratio. Plasma was separated by centrifugation and stored at −20°C until further analysis.

Determination of Human FVII Levels

The FVII-EIA Matched Antibody Set (CoaChrom Diagnostica) was used to determine human FVII levels according to the manufacturers' protocol in an enzyme-linked immunosorbent assay (ELISA) setting as described (21). A standard dilution series of human FVIIa was used to determine the amount of human FVII contained in plasma samples of human FVII-transgenic mice and C57BL/6 wildtype mice. Plasma from a healthy human donor was used as an assay positive control. In parallel to plasma obtained from C57BL/6 wildtype mice, plasma from Balb/c wildtype mice was used as an additional negative control.

Treatment with Human FVIIa and Human VWF

Treatment with Human FVIIa

Mice received eight weekly intravenous (i.v.) or subcutaneous (s.c.) doses of human FVIIa. Doses of 2.5 μg per dose (corresponding to about 100 μg/kg), 10 μg per dose (corresponding to about 400 μg/kg), 50 μg per dose (corresponding to about 2,000 μg/kg) and 100 μg per dose (corresponding to about

Table I Specifications of Recombinant Human FVIIa After Reconstitution of Lyophilized Powder

Specification	Result
pH value	5.6
Endotoxin/LAL	<1.5 EU/ml
Sterility	Sterile
Total protein (UV280)	693 μg/ml
FVIIa activity (clotting assay)	23,225 U/ml
Specific FVIIa clotting activity	33,514 U/mg
Dimers and aggregates (SE-HPLC)	Dimers: 4.0%, aggregates: 3.6%

4,000 μg/kg) were applied in 200 μL human FVIIa-buffer (Baxter BioScience). Animal groups ranged from 5 to 10 animals per group. In some studies mice received eight i.v. doses of 10 μg human FVIIa prior to or together with lipopolysaccharide (LPS, E. coli 0111:B4, Invivogen) at the doses indicated. LPS is known to induce the innate immune system via triggering of Toll-like receptor (TLR) 4 (22).

The human FVIIa used throughout the studies was bulk material produced under GMP conditions, free of any additional proteins, obtained from Baxter BioScience. The specifications of the human FVIIa are given in Table I.

Treatment with Human VWF

Mice received eight weekly i.v. doses of human VWF (20 μg per dose diluted in 200 μL DPBS, Sigma-Aldrich). The VWF used was albumin-free bulk material obtained from Baxter BioScience.

Detection of Anti-FVIIa Antibodies in Blood Plasma

Titers of total anti-FVIIa antibodies, titers of FVIIa-specific IgG subclasses and titers of FVIIa-specific IgA in blood plasma were analyzed by ELISA as previously described for FVIII (23). Human FVIIa at a concentration of 1 μg/mL was used as coating antigen.

Preparation of Spleen Cells, Bone Marrow Cells and Lymph Node Cells

Spleen Cells

Spleens were obtained at 3 days after the last immunization, finely minced and passed through a 70-μm nylon cell strainer (Beckton Dickinson). Spleen cells were collected in RPMI 1640 (Life Technologies) supplemented with 10% preselected fetal calf serum (FCS), 2 mM L-glutamine (both from Hyclone), 100 U/mL penicillin, 100 mg/mL streptomycin (both from Life Technologies), and 5×10^{-5} M beta-mercaptoethanol (Sigma-Aldrich). Resulting single-cell suspensions were cleared of erythrocytes by hemolysis using a hypotonic buffer (pH 7.2) composed of 0.15 M ammonium chloride, 10 mM potassium bicarbonate (both from Merck), and 0.1 mM ethylenediaminetetraacetic acid (Life Technologies).

Bone Marrow Cells

Bone marrow obtained at 3 days after the last immunization was extracted from the femur, tibia and humerus. Cells were passed through a 70-μM nylon cell strainer and collected in RPMI 1640 supplemented with 10% FCS. The medium was identical to that described for spleen cells (see above). Single-cell suspensions were cleared of erythrocytes by hemolysis using the same hypotonic buffer as described for spleen cells.

Lymph Node Cells

Peritoneal, cervical, axillary, brachial and mesenteric lymph nodes were collected at 3 days after the last immunization, finely minced, passed through a 70-μm nylon cell strainer and collected in RPMI 1640 supplemented with 10% FCS as described for spleen cells (see above).

Analysis of FVIIa-Specific Antibody Secreting Cells (ASC) in Spleen, Bone Marrow and Lymph Nodes

Spleen, bone marrow and lymph node cells were prepared using standard procedures. FVIIa-specific ASC in spleen, bone marrow and lymph nodes were analyzed by enzyme-linked immunospot assays (ELISPOT) as described for FVIII (23). Human FVIIa at a concentration of 10 μg/mL was used as a coating antigen.

Analytical HPLC-SEC for Analysis of Human FVIIa Aggregates

The analytical HPLC-SEC analysis was done using an Agilent 1200 series system (Santa Clara, CA, USA) equipped with a vacuum degasser, a binary pump, a thermostatically controlled autosampler and a fluorescence detector. A TOSOH TSKgel G3000PWxl column (7.8×300 mm) with a pore size of 200 Å and 7.0 μm particles was used. The mobile phase consisted of 50 mM Trizma Hydrochloride; 450 mM Sodium Chloride; 5 mM CaCl2 with pH 7.0±0.2. The flow rate was set to 0.5 ml/min and detection was achieved with a fluorescence detector using excitation at 280 nm and emission at 340 nm. PMT was set to 11. Percentages of aggregates were calculated as a ratio of fraction peak areas to total peak area.

Analysis of FVIIa-Specific $CD4^+$ T Cells

FVIIa-specific $CD4^+$ T cells were detected by expression of CD154 after *in vitro* restimulation with human FVIIa according to Frentsch *et al.* (24). Mice were treated with eight weekly doses of human FVIIa. Three days after the last dose, spleens were obtained and spleen cells were prepared as described above. Spleen cells were *in vitro* re-stimulated for 16 h at 37°C and 5% CO2 with either human FVIIa (100-μg/mL) or culture medium (negative control) as indicated. After 1 h of restimulation, 1 μg/mL Brefeldin A (BD Pharmingen) was added to the cultures to inhibit intracellular protein transport. After incubation, a live/dead cell marker (LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, Invitrogen, Life Technologies) was used according to

the manufacturer's protocol. Afterwards, cells were washed and nonspecific binding sites were blocked by a mixture of anti-CD16 and anti-CD32 antibodies (Fc Block, BD Pharmingen). Next, cells were stained with a peridinin-chlorophyll protein complex (PerCP)-labeled antibody against extracellular CD4 (clone RM4-5, BD Pharmingen) and subsequently permeabilized for intracellular staining using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's instructions. Finally, cells were stained with an allophycocyanin (APC)-labeled antibody against intracellular CD154 (clone MR1, eBioscience) and a phycoerythrin-cyanine 7 (PE-Cy7)-labeled antibody against interacellular interferon-gamma (IFN-γ). Frequencies of CD4+CD154+ double positive cells (Fig. 7a) producing IFN-γ (Fig. 7b) were analyzed using a BD LSR Fortessa flow cytometer (BD Bioscience) and FlowJo software (Version 7.6.3., Tree Star).

Statistical Analysis

Statistical analysis was done using GraphPad Prism (Version 5.01, GraphPad Softaware, Inc.). The data shown in Figs. 3b and 7b were checked for normal distribution using the Kolmogorov–Smirnov test. The data shown in Fig. 3b (C57BL/6) was analyzed for differences between the s.c. and i.v. group using the nonparametric Mann Whitney test. P less than 0.05 was considered statistically significant. The data shown in Fig. 7b was analyzed using the unpaired two-tailed Student's t test for comparison of means between groups. P less than 0.05 was considered statistically significant.

RESULTS

Generation of a Mouse Line that Carries a Human *F7* cDNA as a Transgene

We aimed at creating a mouse model that is immunologically tolerant towards native human FVIIa. For this purpose, we expressed a human *F7* cDNA as a transgene which was integrated at the murine *hprt* locus (Fig. 1a). Resulting human FVII-transgenic mice produce litters of normal size and sex ratio and have the expected longevity. The mice show normal growth characteristics and do not have a bleeding phenotype (as determined in tail bleed studies, data not shown). All organs appear of regular size and shape.

We validated the correct insertion of the transgene by PCR of the critical transitional sequences into and out of the *hprt* locus (Fig. 1a) and gained the expected PCR products from genomic DNA (gDNA) samples of male and female human FVII-transgenic mice. By comparison, we did not see any PCR products when amplifying gDNA obtained from C57BL/6 wildtype mice or when amplifying human control gDNA (Fig. 1b).

Next, we asked if the human *F7* cDNA was completely integrated into the *hprt* locus. Using primers spanning the entire *F7* cDNA region, we could confirm the complete integration of the transgene (Fig. 1b). Based on these data we then asked if the *F7* transgene was completely transcribed into mRNA. Total RNA of liver samples was reversely transcribed and analyzed for the expression of *F7*. RNA isolated from male wildtype C57BL/6 control mice and commercially available human cDNA were used as positive and negative controls, respectively. Human *F7* mRNA of the correct size could be detected in RNA samples obtained from both sexes of human FVII transgenic mice but not in RNA samples obtained from wildtype C57BL/6 control mice (Fig. 1c). Subsequent sequence analysis of the PCR products confirmed the correct sequence of the transgene (data not shown). The commercially available human cDNA control gave rise to two *F7*-specific bands corresponding to the two existing *F7* isoforms (25) (Fig. 1c).

Subsequently, we asked if the human FVII protein can be detected in the circulation of transgenic mice. We analyzed plasma samples by ELISA and detected approximately 1.8-ng/mL human FVII. By comparison, levels of human FVII in wildtype C57BL/6 control mice were below the limit of detection (0.7 ng/mL) of the assay (Fig. 1d).

Based on these data we conclude that human FVII-transgenic mice express and transcribe the complete sequence of the human *F7* cDNA. Furthermore, transgenic mice express small amounts of human FVII protein in the circulation.

Human FVII-Transgenic Mice Express Human *F7* mRNA Mainly in the Liver and in the Neonatal Thymus

Next, we assessed the transcription of human *F7* cDNA in different tissues including liver, lymph nodes, lung, spleen, reproductive organs, kidney, heart, thymus, muscle, bone marrow and brain. We normalized the expression of human *F7* to the expression of the murine *ACTB* gene. Human *F7* is highly expressed in the liver of human FVII-transgenic mice, other organs show very low expression levels (Fig. 2a). Organs from C57BL/6 mice which were included as negative controls did not generate any signal specific for human *F7* (data not shown). We then asked how the expression levels of human and murine *F7* compare in the liver. We analyzed mRNA levels for both human and murine *F7* in the same sample and normalized them to the expression of murine *ACTB*. Our results indicate that human F7 mRNA levels in the liver are about five times lower than murine F7 mRNA levels (Fig. 2b).

The presentation of protein antigen in the thymus is important for generating immune tolerance during fetal development (26). Therefore, we analyzed mRNA levels of

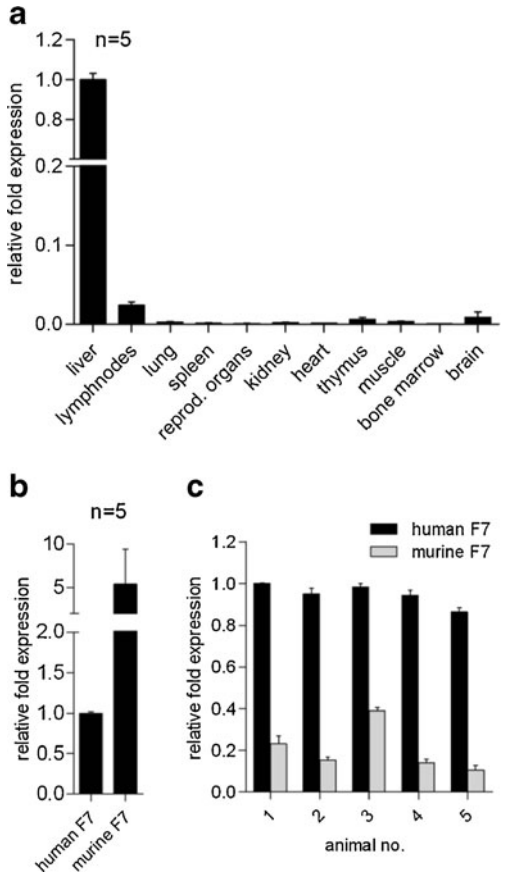

Fig. 2 Human *F7* is mainly expressed in the liver of adult animals and shows high expression levels in the neonatal thymus of human FVII-transgenic mice. (**a**) Two-step reverse transcription PCR analysis of human *F7* mRNA expression in several organs of adult mice. Total RNA was isolated from the organs and reversely transcribed into cDNA. The fold expression of human *F7* over a murine *ACBT* reference was determined for each mouse in the individual organs and subsequently normalized to the mean liver expression. (**b**) Comparison of expression levels of human *F7* and murine *F7* mRNA levels in adult liver in relation to murine *ACTB*. (**a** and **b**) Mean values ± SEM of five different animals. (**c**) Comparison of expression levels of human *F7* and murine *F7* mRNA in neonatal thymus in relation to murine *ACTB*. Data were normalized to the mean human *F7* expression of animal no. 1. Mean of triplicate measurements ± SD of five different animals.

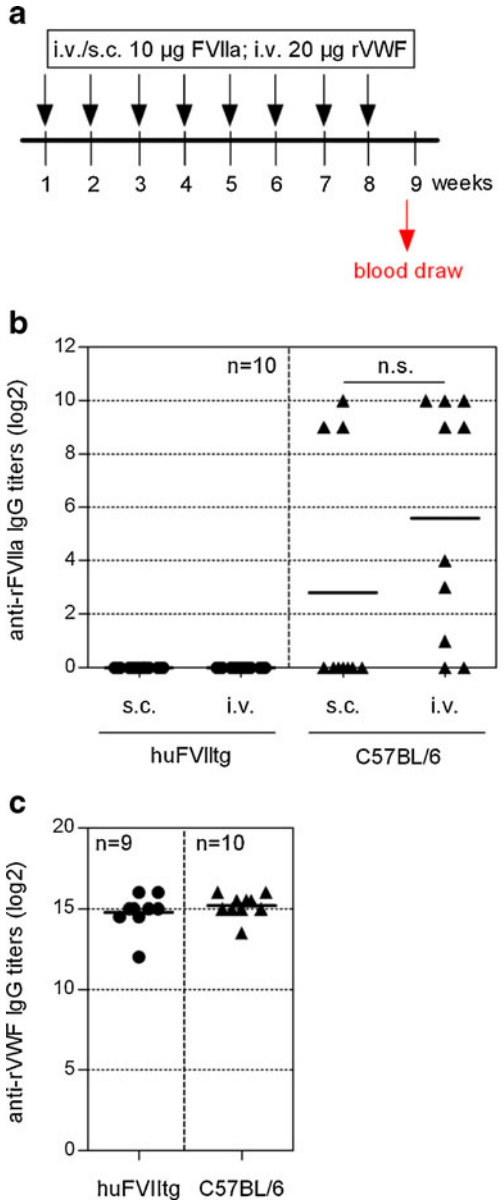

Fig. 3 Human FVII-transgenic mice are immune tolerant to human FVIIa and otherwise fully immune competent. (**a**) Human FVII-transgenic (huFVIItg) mice (*Black circle*) and C57BL/6 control mice (*Black triangle*) were treated with eight weekly doses of i.v. or s.c. human FVIIa or i.v. human von Willebrand Factor (rVWF). (**b**, **c**) Plasma samples were analyzed 1 week after the last immunization. Titers of anti-human FVIIa IgG antibodies (**b**), and titers od anti-rVWF IgG antibodies (**c**) were determined by ELISA (each point represents an individual mouse, *lines* show mean values; results are representative for three experiments). *n.s.* not significant.

human and murine *F7* in neonatal thymi of FVII transgenic mice which were obtained 1 day after birth. Our results indicate expression levels of human *F7* mRNA five-fold higher than those of murine *F7* mRNA (Fig. 2c).

We conclude that the human *F7* transgene in the new human FVII transgenic mouse model is mainly expressed in the liver and in the neonatal thymus.

Human FVII-Transgenic Mice Express Specific Immune Tolerance to Human FVIIa

After confirming expression of the transgene we asked if transgenic mice develop specific immune tolerance against native human FVIIa. We treated mice with eight i.v. or subcutaneous (s.c.) doses of human FVIIa in weekly intervals and analyzed specific antibody responses against FVIIa after the last dose (Fig. 3a). Human FVII-transgenic mice did not develop detectable antibodies against human FVIIa while a proportion of C57BL/6 wildtype control mice developed high titers of anti-FVIIa IgG antibodies after i.v. and after

s.c. treatment (Fig. 3b). In order to exclude the possibility that the human *F7* cDNA transgene resulted in some form of general immune suppression, we asked if human FVII-transgenic mice could still respond to an unrelated human protein. We treated mice with eight weekly i.v. doses of human VWF which was shown to induce high titer antibodies in mice (14). All human FVII-transgenic and C57BL/6 wildtype control mice developed high titers of VWF-specific antibodies (Fig. 3c).

Based on these data we conclude that human FVII transgenic mice express specific immune tolerance towards native human FVIIa.

Transgenic Mice Develop Antibodies When Treated with Human FVIIa Associated with a Strong Stimulation of the Innate Immune System

After confirming specific immune tolerance against human FVIIa, we asked if transgenic mice would still be able to develop antibodies against human FVIIa when challenged under conditions that favor immune responses against self-proteins. Several animal studies demonstrated that the presentation of a self-antigen to the immune system together with a strong stimulation of the innate immune system can trigger autoimmune responses against the respective self-antigen (27–29). Therefore, we asked if co-administration of human FVIIa together with a strong stimulus of the innate immune system might break immune tolerance and induce autoantibodies to the human FVIIa which is recognized as self-antigen in FVII transgenic mice (Fig. 4a). We used LPS as a model to stimulate the innate immune system. LPS triggers the innate immune receptor TLR4 (22). Our results indicate that co-administration of human FVIIa with 2 µg of LPS caused a break of immune tolerance to human FVIIa, resulting in the formation of antibodies in all mice included in the study (Fig. 4b). The antibody response was associated with the formation of FVIIa-specific ASC that could be detected in spleen, bone marrow and lymph nodes (Fig. 4c). Maximal antibody response was seen after administration of 10 µg human FVIIa together with 2 µg LPS. Further increases in the LPS dose did not have any additional effect on antibody responses (Fig. 4d). As expected, LPS also induced an amplification of antibody responses in C57BL/6 wildtype mice that were included as controls (Fig. 4b).

The analysis of IgG antibody subclasses induced by co-administration of human FVIIa and LPS revealed the presence of IgG1, IgG2a, IgG2b and IgG2c antibodies. In addition, some animals developed low titer IgG3 antibodies and one animal low titer IgA antibodies (Fig. 5a). The lack of subclass restriction of IgG antibodies was confirmed by the presence of FVIIa-specific antibody-secreting cells producing IgG1, IgG2a, IgG2b and IgG2c (Fig. 5b).

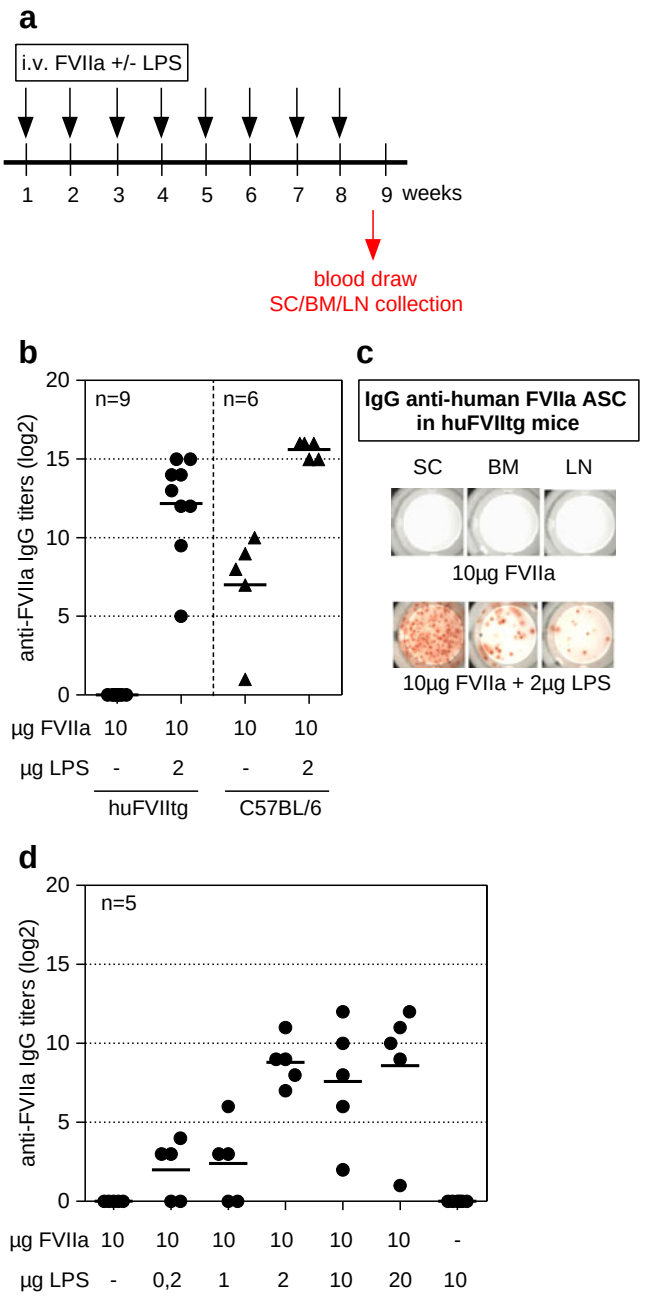

Fig. 4 Immune tolerance to human FVIIa in human FVII-transgenic mice breaks down when human FVIIa is given with a concomitant stimulation of the innate immune system. (**a**) Human FVII-transgenic mice and C57BL/6 wildtype control mice were treated with eight weekly i.v. doses of human FVIIa with or without co-administration of LPS. Amounts of human FVIIa and LPS were administered as indicated in (**b** and **d**). Spleen cells (*SC*), bone marrow (*BM*), lymph nodes (*LN*) and plasma for analysis were drawn 1 week after the last immunization. (**b**) Titers of anti-FVIIa IgG antibodies were determined by ELISA (each point represents an individual mouse, *lines* show mean values; results are representative for nine experiments). (**c**) Anti-FVIIa ASC distribution in different lymphoid organs was analyzed by ELISPOT. Each spot represents one ASC. Three human FVII-transgenic mice receiving human FVIIa with or without LPS were pooled for analysis; results are representative for five experiments. (**d**) Titers of anti-FVIIa IgG antibodies were determined by ELISA (each point represents an individual mouse, *lines* show mean values; results are representative for three experiments).

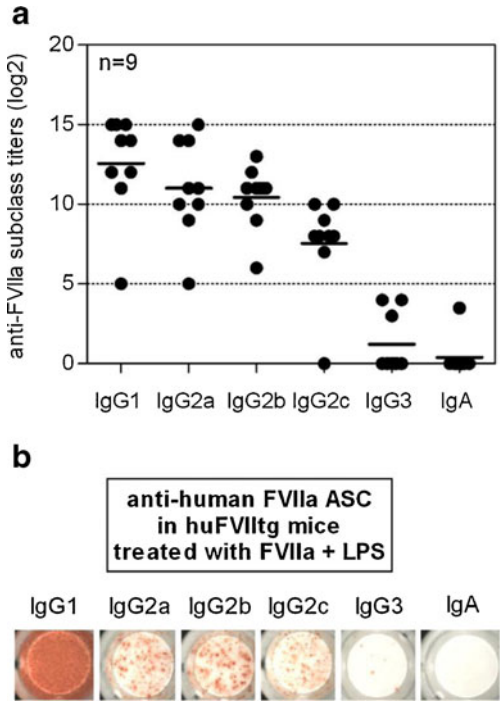

Fig. 5 Break of immune tolerance against human FVIIa in human FVII-transgenic mice is not isotypically restricted. Human FVII-transgenic mice were treated with a mixture of 10 μg human FVIIa and 2 μg of LPS as described for Fig. 4a. (**a**) Titers of anti-human FVIIa IgG subclasses and IgA were analyzed by ELISA (each point represents an individual mouse, *lines* show mean values; results are representative for three experiments). (**b**) The distribution of anti-human FVIIa IgG and IgA ASC was analyzed by ELISPOT. Each spot represents one ASC. Three animals were pooled for analysis; results are representative for three experiments.

Next, we asked if the stimulation of the innate immune system by LPS was sufficient to break immune tolerance to FVIIa or if mixing of LPS with FVIIa might induce structural alterations in the FVIIa protein which could contribute to the break of immune tolerance. Analysis by HPLC-SEC did not reveal any differences between FVIIa and mixtures of FVIIa and LPS with regard to the proportion of monomers, oligomers and aggregates in the preparation (Fig. 6a and b). Furthermore, separate injections of LPS and FVIIa were sufficient to break immune tolerance when LPS was given 1 h prior to FVIIa (Fig. 6c).

Based on these data we conclude that the application of FVIIa in association with a strong stimulus of the innate immune system caused a break of immune tolerance against human FVIIa.

Break of Immune Tolerance is Associated with the Activation of Human FVIIa-Specific Memory CD4$^+$ T Cells Expressing a Pro-Inflammatory Phenotype

After we had shown that co-administration of LPS and FVIIa caused a break of immune tolerance to FVIIa in human FVII-transgenic mice, we asked if this is associated with the activation of FVIIa-specific CD4$^+$ T cells. We

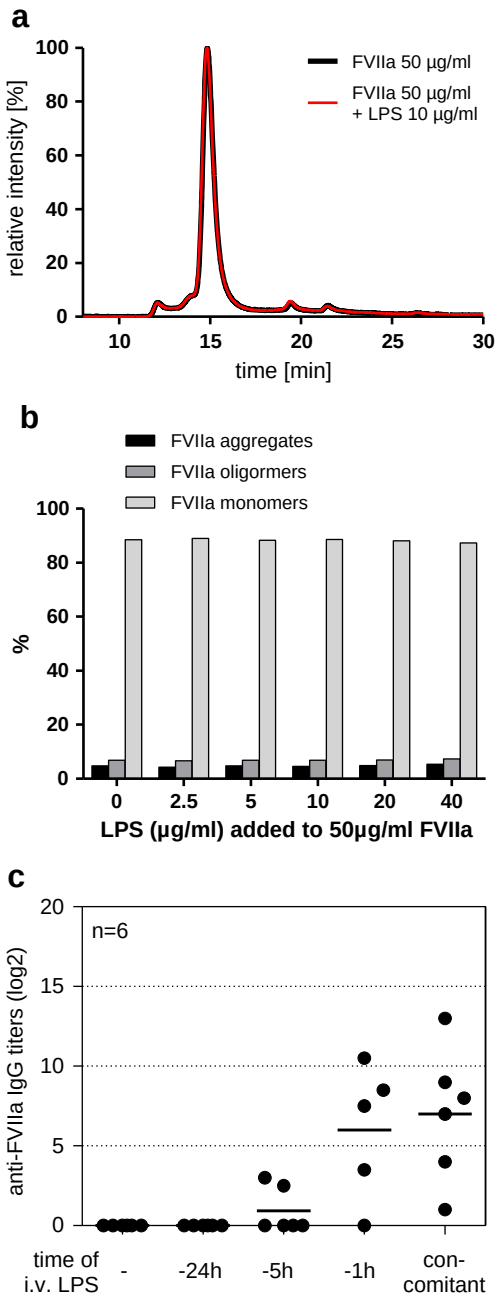

Fig. 6 Break of immune tolerance to human FVIIa in human FVII-transgenic mice is not due to structural alterations of FVIIa induced by mixing with LPS. (**a**) Human FVIIa HPLC-SEC analysis was done with human FVIIa only (50 μg/ml, *black line*) or with a mixture of human FVIIa (50 μg/ml) and LPS (10 μg/ml, red line). (**b**) Human FVIIa (50 μg/ml) was mixed with different amounts of LPS as indicated and analyzed by HPLC-SEC. Percentages of human FVIIa aggregates, oligomers and monomers were deducted from fraction peaks, calculated as a ratio of fraction peak area to total peak area. (**c**) Human FVII-transgenic mice were treated with eight weekly i.v. doses of 10 μg human FVIIa with or without additional administration of 2 μg of LPS at the indicated time points. Titers of anti-FVIIa IgG antibodies were determined by ELISA (each point represents an individual mouse, *lines* show mean values; results are representative for two experiments).

assessed the frequency of FVIIa-specific CD4$^+$ T cells in human FVII-transgenic mice that were treated with eight

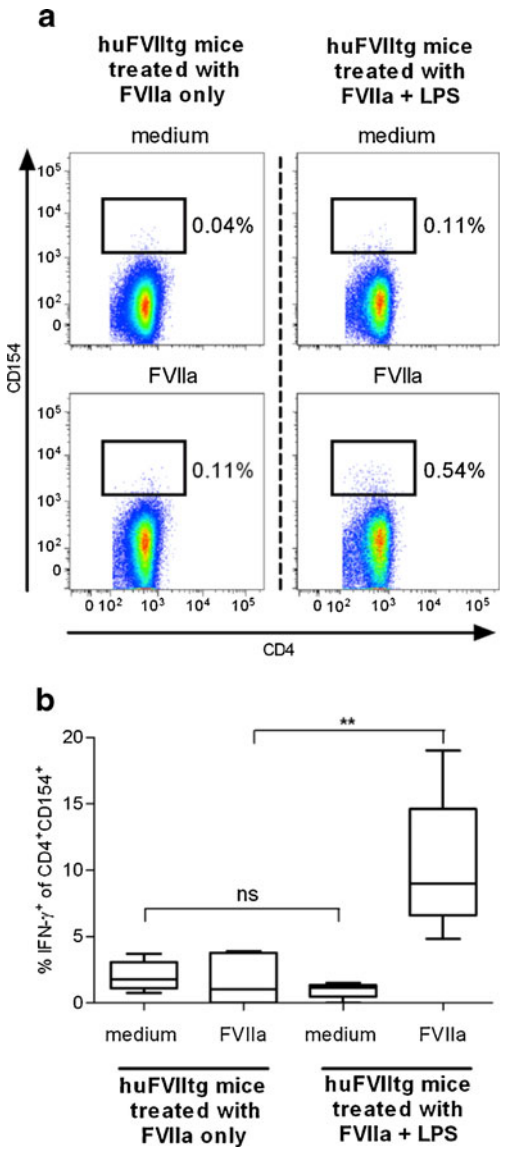

Fig. 7 Increased numbers of pro-inflammatory IFN-γ producing human FVIIa-specific $CD4^+$ T cells in human FVII-transgenic mice after treatment with human FVIIa and LPS. Human FVII-transgenic mice were treated with eight weekly i.v. doses of 10 μg human FVIIa without LPS (no break of tolerance) or with 2 μg LPS (break of tolerance). (**a**) Spleen cells were collected 3 days after the last immunization, *in vitro* re-stimulated with medium or human FVIIa and subsequently evaluated for the presence of $CD4^+$ $CD154^+$ FVIIa-specific T cells. One representative experiment of five is shown. (**b**) $CD4^+$ $CD154^+$ T cells were further analyzed for the production of IFN-γ. The *box and whisker* plot summarizes the frequencies of $CD4^+$ $CD154^+$ IFN-γ^+ T cells of five independent experiments (*box* = quartiles, *whiskers* = min/max; ***P* value = 0.0092; determined by Student's t-test).

i.v. doses of either human FVIIa only (maintenance of tolerance) or human FVIIa co-administered with LPS (break of tolerance). Spleen cells obtained from both groups of mice were *in vitro* re-stimulated with either human FVIIa or with medium (negative control) and subsequently stained for CD4 and CD154, a marker of activated $CD4^+$ T cells (24,30) (Fig. 7a). The frequency of FVIIa-specific $CD4^+$ T cells in spleen cells obtained from mice treated with human FVIIa only was found to be about 0.11% of all $CD4^+$ T cells. The frequency of FVIIa-specific $CD4^+$ T cells in spleen cells obtained from mice treated with a co-administration of human FVIIa and LPS was found to be about 0.53% of all $CD4^+$ T cells which accounts for an approximately five-fold increase compared with the medium control (0.11%, Fig. 7a). When we further analyzed $CD154^+CD4^+$ T cells for the production of IFN-γ, a well-known pro-inflammatory cytokine, we found FVIIa-specific $CD4^+$ T cells producing IFN-γ only in spleen cells obtained from mice treated with the combination of human FVIIa and LPS (Fig. 7b).

Based on these data we conclude that the break of immune tolerance to human FVIIa in human FVII transgenic mice is supported by the activation of FVIIa-specific pro-inflammatory $CD4^+$ T cells that produce IFN-γ.

DISCUSSION

Until recently, the assessment of the immunogenic potential of human proteins in preclinical animal models was difficult because of the xenogenic nature of human proteins in animals. However, new transgenic mouse models have been described that express the human protein of interest as a transgene and consequently do not develop antibodies against this protein (13–17). The immune system in these models recognizes the human protein as a self-protein and only develops antibodies if immune tolerance breaks down after the application of highly immunogenic variants of the protein (13,31) or the application of the protein in the presence of a strong stimulus of the innate immune system (32).

Along these lines, we report the development of a new mouse line that expresses human *F7* cDNA as a transgene. We integrated the transgenic construct containing the human *F7* cDNA into the murine *hprt* locus. This locus was chosen because it was shown to have a permissive chromatin environment allowing promoter-dependent transcription (18). We used a liver-specific albumin promoter to direct the expression of the human FVII to the liver, the natural place for the synthesis of murine FVII. We did not knock out the murine *F7* gene because doing so could result in perinatal lethality (33). In contrast to murine FVIIa, human FVIIa does not efficiently interact with murine tissue factor; an interaction which is essential for the initiation of the extrinsic pathway of blood coagulation (21).

We were able to show that the human *F7* cDNA transgene was correctly integrated into the *hprt* locus and completely transcribed and expressed. Furthermore, we could detect small amounts of human FVII protein in the circulation of transgenic mice. However, the concentration of circulating human FVII (1.8 ng/mL) is low compared with FVII levels found in human plasma (350–450 ng/mL) (34). The low protein concentration could have several reasons. The integration site and the promoter

of the transgene differ from those of its endogenous counterpart murine FVII. This could influence the function of the transgene and might suppress its effective transcription and translation (35). Furthermore, we used a *F7* cDNA construct that lacks gene regulatory elements, which are known to be important for optimal gene expression (36,37). The absence of introns in the transgenic sequence can result in disturbances of the nucleosome alignment, which is important for the transcription of the transgene. Therefore, the expression of cDNA constructs is usually rather low (37,38). There are several other examples of transgenic mouse lines reported which are immunologically tolerant to specific human proteins while expressing little or no detectable transgenic human protein in their circulation (13,14,17).

Mice of the new human FVII transgenic line do not develop antibodies against native human FVIIa. The non-responsiveness is because of specific immune tolerance as mice are still able to respond normally to an unrelated human protein. The development of specific immune tolerance is most likely caused by the deletion of high-avidity autoreactive $CD4^+$ T cells during the time when the T-cell repertoire in the thymus is shaped and central immune tolerance is established (39). In addition, peripheral tolerance mechanisms like anergy, clonal deletion or active suppression by regulatory T cells (40,41) could contribute to specific immune tolerance. Both pathways of tolerance induction require the presentation of peptides by MHC class II expressed on antigen presenting cells (APCs). The expression of human *F7* mRNA in the neonatal thymus of transgenic mice suggests that human FVII protein is synthesized during embryonic development and that human FVII peptides are presented to the immune system which would be required for the induction of immune tolerance. Some T cells recognizing human FVII might have escaped clonal deletion in the thymus and would need to be regulated by peripheral tolerance mechanisms. The presence of human FVII protein in the circulation of transgenic mice could provide the autoantigen required for maintaining peripheral FVII-specific T cells in a regulatory state.

An important objective in the development of the human FVII-transgenic mouse line was the requirement that mice should still be able to develop antibodies against human FVIIa when challenged with a FVIIa protein preparation that expresses increased immunogenicity. We generated a highly immunogenic FVIIa preparation by mixing FVIIa with LPS, a well-characterized stimulator of the innate immune system. LPS triggers TLR4 expressed by a number of immune cells, thereby triggering signal-transduction pathways that lead to the expression of pro-inflammatory cytokines (42). Importantly, LPS triggers TLR4 expressed on dendritic cells, thereby inducing a pro-inflammatory state of dendritic cells (43). Such pro-inflammatory dendritic cells can trigger autoreactive CD4+ T cells that have the capacity to help autoreactive B cells to differentiate into autoantibody producing plasma cells (22). Eriksson *et al.* provided evidence that dendritic cells pulsed with a heart muscle-specific α-myosin peptide MYHC-α (614–629) and activated by LPS and anti-CD40 antibodies were able to induce autoimmune heart failure in mice, indicating that the activation state of dendritic cells presenting self-antigens regulates maintenance or break of immune tolerance against self-antigens (28). Furthermore, Marty *et al.* presented evidence that MyD88 signalling is essential to stimulate self-antigen-presenting dendritic cells to induce heart-specific CD4+ T-cell responses in the peripheral compartments in mice. MyD88 is an essential adaptor molecule that mediates complex proinflammatory pathways involving several toll-like receptors and IL-1 receptor type 1 activation (43). It is well established that LPS binds to TLR4, thereby inducing pro-inflammatory signal transduction pathways via MyD88 (44).

Based on the data published by Eriksson and Marty, we believe that break of immune tolerance against FVIIa in human FVII transgenic mice might be caused by proinflammatory dendritic cells presenting FVIIa peptides to the immune system. These pro-inflammatory dendritic cells could trigger autoreactive CD4+ T cells that have the capacity to help autoreactive B cells to differentiate into autoantibody producing plasma cells. A T cell-dependent mechanism for the break of immune tolerance against human FVIIa is supported by the lack of IgG subclass restriction of antibodies against FVIIa and by the detection of increased numbers of $CD154^+$ FVIIa-specific $CD4^+$ T cells after *in vitro* restimulation of spleen cells obtained from mice immunized with a mixture of human FVIIa and LPS.

Break of immune tolerance against FVIIa was also seen when LPS and FVIIa were injected separated from each other. These results indicate that break of immune tolerance was not caused by structural alterations in the FVIIa protein which could have potentially been induced by mixing it with LPS.

It is important to note that we chose LPS as an experimental model to provide evidence that the application of human FVIIa together with a strong stimulation of the innate immune system can break tolerance to human FVIIa which is recognized as a self-proteins. The LPS doses used in our studies (2 μg/dose corresponding to about 80 μg/kg) are not clinically relevant. A similar approach was chosen by Bril *et al.* (45) who demonstrated that co-administration of Freund's adjuvant and human factor VIII was able to break immune tolerance to human factor VIII in a human factor VIII transgenic mouse model. Both our results and the results presented by Bril *et al.* indicate that application of therapeutic proteins associated with a strong stimulus of the innate immune system can result in break of immune tolerance to the human protein. Previously it was shown that certain structural alterations of proteins and peptides, e.g. the formation of cross-beta structures (amyloid-like properties), can result in the activation of innate immune cells, in particular the induction of a pro-inflammatory phenotype in human dendritic cells (46).

Previous evidence suggests that the pattern of cross-beta structures is recognized by CD14, the LPS receptor (47). Cross-beta sheet structures (amyloid-like properties) were shown to be associated with protein missfolding and with increased immunogenicity of therapeutic proteins (48).

In summary, we created a new transgenic mouse line that is specifically immune tolerant towards native human FVIIa. Tolerance is achieved by transgenic expression of the human *F7* cDNA. Immune tolerance breaks down when mice are challenged with human FVIIa associated with a strong stimulation of the innate immune system. The new mouse line could provide a useful tool to assess the immunogenicity of new FVIIa product candidates before they enter clinical development. Furthermore, the new mouse model provides a valuable tool for studying the mechanisms of immune tolerance regulation against therapeutic proteins.

ACKNOWLEDGMENTS AND DISCLOSURES

We are grateful to Nicole Pfeffer, Thomas Wurz, Fatima Al-Awadi, Nidha Abrar, Monika Grewal, Lydia Süly, Barbara Hammer, Markus Pasztorek, Christian Lubich and Thomas Prenninger for technical assistance. We also thank Elise Langdon-Neuner for editing the manuscript. This work was supported by Baxter BioScience, Vienna.

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