Antigen-specific immunotherapy with apitopes suppresses generation of FVIII inhibitor antibodies in HLA-transgenic mice

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Abstract:
Haemophilia A (HA) is a blood clotting disorder caused by various genetic deficiencies in the factor VIII (FVIII) encoding F8 gene. Patients receiving FVIII replacement therapy are at risk of developing neutralizing antibodies (FVIII inhibitors) rendering the FVIII replacement therapy ineffective. Immunological tolerance towards FVIII can be achieved through immune tolerance induction (ITI) protocols in some patients but this is a lengthy and costly desensitization programme. Long-term eradication of inhibitors in HA patients could be achieved by antigen-specific immunotherapy targeting CD4+ T cells since formation of FVIII inhibitors is T cell dependent. Here, we report a peptide-based, antigen-specific immunotherapy designed to specifically re-establish immune tolerance to FVIII through the development of antigen-processing-independent epitopes (apitopes). We identified two FVIII immunodominant peptides in immunised human leukocyte antigen (HLA) DRA*0101/DRB1*1501 transgenic (HLA-DR2tg) mice that were optimised for tolerogenicity. These modified peptide analogues were initially screened for recognition using FVIII-specific T cell hybridoma clones from FVIII-immunised HLA-DR2tg mice. The FVIII apitopes were promiscuous and bound common human HLA-DRB1*haplotypes. The combination of these two FVIII apitopes (ATX-F8-117), administered according to a dose escalation protocol, promoted T cell tolerance towards FVIII in HLA-DR2tg mice. Furthermore, treatment with ATX-F8-117 significantly reduced FVIII inhibitor formation. ATX-F8-117 regulates both anti-FVIII T cell and B cell responses, specifically the generation of FVIII inhibitors, revealing peptide-based antigen-specific immunotherapy as a promising approach to both suppress and treat inhibitor formation in susceptible HA patients.

Conflict of interest: COI declared - see note

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Antigen-specific immunotherapy with apitopes suppresses generation of FVIII inhibitor antibodies in HLA-transgenic mice

[running head] Tolerance promotion towards factor VIII

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Abstract

Haemophilia A (HA) is a blood clotting disorder caused by various genetic deficiencies in the factor VIII (FVIII) encoding F8 gene. Patients receiving FVIII replacement therapy are at risk of developing neutralizing antibodies (FVIII inhibitors) rendering the FVIII replacement therapy ineffective. Immunological tolerance towards FVIII can be achieved through immune tolerance induction (ITI) protocols in some patients but this is a lengthy and costly desensitization programme. Long-term eradication of inhibitors in HA patients could be achieved by antigen-specific immunotherapy targeting CD4+ T cells since formation of FVIII inhibitors is T cell dependent. Here, we report a peptide-based, antigen-specific immunotherapy designed to specifically re-establish immune tolerance to FVIII through the development of antigen-processing-independent epitopes (apitopes). We identified two FVIII immunodominant peptides in immunised human leukocyte antigen (HLA) DRA*0101/DRB1*1501 transgenic (HLA-DR2tg) mice that were optimised for tolerogenicity. These modified peptide analogues were initially screened for recognition using FVIII-specific T cell hybridoma clones from FVIII-immunised HLA-DR2tg mice. The FVIII apitopes were promiscuous and bound common human HLA-DRB1*haplotypes. The combination of these two FVIII apitopes (ATX-F8-117), administered according to a dose escalation protocol, promoted T cell tolerance towards FVIII in HLA-DR2tg mice. Furthermore, treatment with ATX-F8-117 significantly reduced FVIII inhibitor formation. ATX-F8-117 regulates both anti-FVIII T cell and B cell responses, specifically the generation of FVIII inhibitors, revealing peptide-based antigen-specific immunotherapy as a promising approach to both suppress and treat inhibitor formation in susceptible HA patients.

Key points

- ATX-F8-117 induces tolerance towards factor VIII thereby suppressing neutralising antibody formation
- ATX-F8-117 has potential to treat inhibitor formation in susceptible haemophilia A patients
Introduction

Haemophilia A (HA) is an X-linked inherited genetic disorder that results from a deficiency in the F8 gene encoding the blood clotting factor VIII (FVIII). Standard treatment of HA patients involves lifelong replacement therapy with human plasma-derived or recombinant FVIII (rFVIII) concentrates. However, approximately 30% of HA patients develop neutralizing antibodies (FVIII inhibitors) blocking the pro-coagulant function of the infused FVIII. Long-term success in eradicating the FVIII inhibitors can be achieved with Immune Tolerance Induction (ITI) therapy which adds, however, a heavy burden on both individual patients and health resources and, hence, alternative strategies are highly desirable.

The generation of FVIII inhibitors is CD4+ T-cell-dependent in both murine haemophilia models and HA patients. FVIII-specific immune activation occurs through antigen-presenting cells (APC) which internalize the FVIII protein, process and present antigenic peptides (epitopes) on major histocompatibility complex class II (MHCII) molecules to CD4+ T-cells in the presence of costimulatory signals. Studies have focused on prevention of FVIII inhibitor formation by decreasing the immunogenicity of the FVIII protein following the removal of promiscuous T-cell and/or B-cell epitopes without affecting FVIII coagulant activity. Other experimental approaches induce FVIII immune tolerance by using broadly acting immunosuppressive drugs, co-stimulatory pathway modulators, agents to selectively deplete B-cell subsets, antigen-coupled splenocytes, nanoparticles with encapsulated antigen and rapamycin, and others. Clearly, antigen-specific strategies that actively re-instate long-lasting immune tolerance to FVIII while maintaining the immuno-surveillance and anti-microbial immune responsiveness of HA patients will have major therapeutic benefit.

Peptide immunotherapy using soluble CD4+ T-cell epitopes has been successfully used for the promotion of immune tolerance in experimental models and in clinical trials of hypersensitivity reactions and autoimmune disorders. T-cell tolerance mechanisms include deletion from the peripheral T-cell pool, anergy characterized by defective proliferation / IL-2 secretion, suppression through specialized regulatory T-cell subsets and immune deviation. In the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS), administration of a peptide analogue derived from myelin basic protein (MBP) induces tolerance through the generation of CD4+ T-cells with a regulatory phenotype. Such induced CD4+ regulatory type1 (Tr1) cells were anergic, produced the anti-inflammatory cytokine IL-10, suppressed naïve T-cell activation in vivo, and lacked the expression of the transcription factor FoxP3. Chronic antigen encounter leads to transcriptional re-programming within the cognate CD4+ T-cell dictating anergy and the regulatory
signature. Crucial for the generation of the IL-10+ regulatory CD4+ T-cell is the mapping of the immunodominant T-cell epitopes with a high affinity HLA-binding strength. By escalating the peptide dose, subcutaneous delivery of antigen induces effective and safe CD4+ T-cell tolerance even at high doses, a strategy also applied in allergen-specific immunotherapy (SIT) and our recent clinical trials. Despite compelling evidence that peptide immunotherapy shows great promise for establishing immunological tolerance, it remains to be determined whether T-cell epitope desensitization can be an effective means to block immune activation to human recombinant protein therapeutics and plasma-derived proteins.

We have previously demonstrated that T-cell epitopes able to induce CD4+ T-cell tolerance in vivo mimic naturally processed antigen but bind MHCII complexes on APC without the need for antigen processing. These Antigen Processing Independent epitOPES (apitopes) can be identified in vitro using APC lacking antigen processing functionality through chemical fixation. Numerous T-cell epitopes in human FVIII have been described; however, our approach is to identify the FVIII apitopes from immunodominant T-cell epitopes mapped by using haemophiliac mice humanized for human MHCII (human leukocyte antigen or HLA) molecules and T-cell hybridoma libraries. In this study, we use humanized transgenic mice expressing the human HLA-DRA*0101/HLA-DRB1*1501 allele (HLA-DR2tg) on a knockout background of murine MHCII molecules given that next to genetic and environmental factors, the HLA allele DRB1*1501 is reported as a risk factor for developing FVIII inhibitors.

We designed two peptide analogues from human FVIII immunodominant T-cell epitopes that mimicked the naturally-processed form of the antigen but did not require antigen processing for presentation on human HLA-DR2. A combination of the FVIII apitopes, ATX-F8-117, promoted FVIII-specific T-cell tolerance in vivo and suppressed FVIII inhibitor formation in a murine antibody model significantly, demonstrating their potential for immune intervention in FVIII inhibitor positive HA patients.
Material & Methods

Mice

Mice expressing the human HLA-DRA*0101/DRB1*1501 (HLA-DR2) allele were originally obtained from Lars Fugger and backcrossed into the IA-beta knockout C57BL/6 genetic background (H2-Aβ0 mice) lacking mouse endogenous MHC class II genes. The mice were bred under specific pathogen-free conditions at Charles River, UK and used in accordance with the guidelines of the local authorities.

Antigens

HLA-DRB1*1501-binding 15mer peptides from human FVIII were predicted using bioinformatics algorithms Propred, SYFPEITHI and the IEDB analysis resource consensus tool. For epitopes to induce tolerance, they must be soluble. When needed, N-terminal and C-terminal tags composed of glycine (G) and lysine (K) were added to optimize solubility of 15-mer peptides.

Peptides were synthesized using Fmoc chemistry with an N-terminal free amine and a C-terminal amide by GL Biochem (Shanghai) Ltd (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) for storage at -80°C. Alternatively, peptides P1 (KKGPRCLTRYSSFVNMEGKK) and P17 (KKGDNIMVTFRNQASRPYGKK) were synthesized by PolyPeptide Laboratories France (PPL; Strasbourg, France) as a non-GMP batch and stored at a stock solution in phosphate buffered saline (PBS) (Lonza, Basel, Switzerland) at -80°C. Recombinant human FVIII (rhFVIII) (Advate®, Baxter, Deerfield, USA) was purchased from the Bristol & South West Haemophilia Group, Bristol Haematology & Oncology Centre.

Mouse immunisation and T-cell proliferation assay

Mice were immunised subcutaneously at the base of the tail with 40µg rhFVIII or 100µg DNIMV and 100µg PRCLT emulsified in incomplete Freund’s adjuvants (IFA; Difco Laboratories, Michigan, USA) supplemented with 400µg heat-killed M. Tuberculosis H37Ra (Difco). Ten days after immunisation, draining lymph nodes and spleens were collected and single cell suspensions prepared. Cells were seeded in triplicate at 0.5x10^6 cells/well in X-vivo 15 medium supplemented with 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza) in 96-well flat bottom plates (Greiner Bio-One, Kremsmünster, Austria) with the indicated antigens. After 3 days, 60µl of supernatant was harvested and stored at -20°C. Cells were pulsed with [3H]-thymidine (Perkin Elmer, Waltham, USA) as described. Cytokine content of culture supernatants was analysed with the FlowCytomix Multiplex technology (eBioscience, San Diego, USA) according to the manufacturer’s instructions.
Fluorescence intensity was measured on BD accuri or BD FACScalibur flow cytometers (BD Biosciences, San Jose, USA).

**T-cell hybridoma cell culture**

T-cell hybridomas were generated as described. Briefly, lymph node cells or CD4+ T-cells purified from immunised mice (immunised with rhFVIII (Advate®)), were co-cultured in the presence of rhFVIII and irradiated (30Gy) syngeneic splenocytes for 3-10 days at 37°C 5%CO₂ in supplemented X-vivo 15 medium. Lymphoblasts were fused with a TCRα-β- BW5147 fusion partner cells using polyethylene glycol (PEG; Sigma-Aldrich). T-cell hybridoma growth was selected upon addition of hypoxanthine-aminopterin-thymidine (HAT) medium (Sigma-Aldrich). Clones were tested for antigen-specificity by culturing hybridoma cells with a lymphoblastoid cell line homozygous for HLA-DRB1*1501 (MGAR; International Histocompatibility Working group, Seattle, USA) as antigen-presenting cells and 10-20µg/ml antigen. After 48 hours, IL-2 production in supernatants was assessed by enzyme-linked immunosorbent assay (ELISA) (eBioscience). An antigen presentation assay was performed by co-culturing 5x10⁴ hybridoma clones with 5x10⁴ MGAR cells, either left untreated or previously fixed with 1% paraformaldehyde (Sigma-Aldrich), in the presence of antigens for 48 hours followed by analysis of the IL-2 cytokine content of supernatant.

**MHC class II peptide binding competition assay**

Relative affinity binding of peptides to common human HLA-DRB1 haplotypes was determined with ProlImmune’s REVEAL® in vitro HLA binding assay (www.proimmune.com, ProlImmune, Oxford, UK).

**Human PBMC assay**

Peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors (NHS National Blood Service, Bristol, UK) or from patients with congenital HA, with a current negative FVIII inhibitor status, at the Haemophilia Treatment Center at Radboud University Medical Center (Nijmegen, The Netherlands) as previously described. All studies were performed with approval of the local ethical committees and after informed consent of the patients and healthy volunteers was obtained. PBMC were stimulated with 5-10µg/ml rhFVIII, 20-40µg/ml peptides or 20µg/ml Tuberculin purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark) as a positive control and proliferation was measured as previously described.

**FVIII antibody model**

HLA-DR2tg mice (FVIII sufficient) were prophylactically treated subcutaneously with equivalent doses of peptides P1 and P17 (ATX-F8-117) by dose escalation (0.1-1-10-100-100-100µg of each
peptide) in 100μl PBS over the 2 weeks preceding immunization. Control animals were treated with a DR2-binding prostatic acid phosphatase (PAP) 133-152 control peptide in 100μl PBS. Animals were immunised subcutaneously weekly with 1μg rhFVIII in 100μl sterile water for injection as provided by the manufacturer. Plasma samples were taken biweekly and collected by mixing whole blood in sodium citrate buffer (Merck, Darmstadt, Germany) followed by 10min centrifugation at 2500g. For a therapeutic treatment regime, immunisation with rhFVIII was started before the start of peptide treatment with ATX-F8-117 by dose escalation (0.1-1-10-100-100μg of each peptide). For detailed description of numbers of mice, timing of peptide administration, FVIII immunisation and bleeding, see legends of Figure 3 and 4.

**Anti-FVIII antibody titer determination**

96 well half-area plates (Corning) were coated overnight with 1μg/mL rhFVIII in carbonate coating buffer at 4°C. Plates were blocked with 10% Fetal Bovine Serum (FBS, Life Technologies, Carlsbad, USA) in PBS. Plasma samples were loaded as serial dilutions in 10%FBS/PBS. Anti-FVIII immunoglobulin (IgG) antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Abcam, Cambridge, UK) followed by 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific, Waltham, USA). Absorbance was read at 450 nm (Tecan, Maennedorf, Switzerland). Antibody titer was expressed as the highest dilution of plasma sample showing a positive result (optical density > cut-off point). The cut-off point was set at 10x average of blank controls analysed in one assay. Alternatively, concentration of anti-FVIII specific antibodies was estimated from a standard curve obtained using serial dilutions of anti-human FVIII IgG2a (Thermo Fisher Scientific) starting at 50ng/ml.

**Anti-FVIII IgG isotype ELISA**

IgG subclass of anti-FVIII antibodies was assessed using an in-house established ELISA assay. Briefly, 96 well plates (Corning) were coated overnight with 1μg/mL of rhFVIII in PBS at room temperature. Plates were blocked using 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) in PBS. Standards or plasma samples were diluted in 1%BSA in PBS. Anti-FVIII IgG isotype-specific antibodies were detected by anti-mouse HRP-conjugated IgG1 (Southern Biotech, Alabama, USA), IgG2a (Southern Biotech), IgG2b (Abcam) or IgG2c (Abcam) and TMB substrate solution (Perbio Science). Absorbance was detected at 450 nm using an ELISA reader (Tecan). Concentration of anti-FVIII isotype specific antibodies was estimated from a standard curve obtained using serial dilutions of mouse anti-human FVIII IgG1 (Merck Millipore), anti-human FVIII IgG2a (Thermo Fisher Scientific) starting at 50ng/ml or mouse anti-FVIII reference plasma starting at 50 arbitrary units (AU)/ml.
**FVIII inhibitor assay**

Anti-FVIII inhibitor antibodies were determined by a chromogenic method using the chromogenic Coatest SP4 FVIII assay (Diapharma, West Chester, USA). Briefly, plasma samples were inactivated by incubation at 56°C for 30 minutes and mixed with human pooled plasma (1IU/ml FVIII) at a 1:1 ratio for incubation at 2 hours at 37°C after which manufacturer’s instructions were followed. The % residual FVIII activity of the test sample was calculated compared to the reference samples and FVIII inhibitor titres were expressed in Bethesda Units (BU)/ml.

**Statistics**

Statistical significance was determined using GraphPad Prism software with tests indicated in each section.

**Data sharing statement**

For original data, please contact d.wraith@bham.ac.uk.
Results

Two major human FVIII T-cell epitopes P1 and P17 identified using humanized HLA-DR2 mice

A set of fifteen-mer peptides of hFVIII were selected (Table 1) based on the core residues of high-affinity HLA-DRB1*1501 binding peptides predicted by multiple binding prediction algorithms. The immunodominant peptides were identified by T-cell hybridoma technology using rhFVIII-immunised FVIII-sufficient (FVIII+/+) mice expressing the human HLA-DR2 molecule. T cell hybridoma’s were derived from mice immunized with rhFVIII (Advate®) and all responded to the FVIII protein. The majority of T-cell hybridoma clones responded to the peptides with the sequence DNIMVTFRNQASRPY (designated DNIMV) and PRCLTRYSSFVNME (designated PRCLT). Respectively 40.7% and 11.1% of hybridoma’s responded. T-cell hybridoma clones were also produced from FVIII−/− HLA-DR2tg mice (Supplementary Table 1) with similar results. Overall, these data indicate that these two peptides constitute two immunodominant T-cell epitopes in the HLA-DR2-restricted CD4+ T-cell response to hFVIII in HLA-DR2tg mice, confirming data by Steinitz et al. who identified T-cell epitopes using a similar set-up and of which 2 epitopes overlap with our peptides38.

Peptide characteristics of PRCLT and DNIMV were optimized for solubility35 by adding N-terminal and C-terminal tags composed of the linker glycine (G) and charged amino acid lysine (K) outside of the core binding residues to generate the modified peptides P1 (KKG-PRCLT-GKK) and P17 (KKG-DNIMV-GKK). We previously demonstrated that therapeutic peptides should mimic the HLA-binding of the naturally-processed epitopes of the target antigen by acting as apitopes37. Therefore, we addressed whether FVIII-specific T-cell hybridoma clones from HLA-DR2tg mice responded to the modified peptides P1 and P17 on paraformaldehyde-fixed APC. PRCLT-specific clones produced IL-2 in response to PRCLT and the modified P1 peptide on both fresh and fixed APC, but recognized FVIII on fresh APC only (figure 1A). Similarly, DNIMV-specific clones were activated by incubation with peptides P17 or DNIMV on both fresh and fixed APCs (Figure 1B), whereas no IL-2 was measured in the presence of FVIII and fixed APC.

In sum, peptides P1 and P17 were designed from the human FVIII immunodominant HLA-DR2 binding peptides PRCLT and DNIMV respectively and behave as apitopes.

Human FVIII epitopes P1 and P17 are HLA pan-DR binding peptides

Since apitopes P1 and P17 were identified using T-cell clones from HLA-DR2tg mice, an in vitro MHC binding competition assay was performed to study the apitope binding affinities to common human HLA-DRB1 haplotypes. The Prolmmune REVEAL® binding assay detects peptide binding to recombinant MHCII molecules based on peptide induced stabilisation of the MHC molecule.
Apitopes P1 and P17 bind with a higher relative affinity to HLA-DRA*0101; DRB1*15:01 complexes than the control peptide as expected (IC50 < 0.1µM, Table 2). Interestingly, both P1 and P17 peptides bind HLA-DRA*0101; DRB1*11:01 and HLA-DRA*0101; DRB1*04:01 complexes with intermediate to high efficiency compared to the control peptide (Table 2) suggesting that P1 and P17 bind common human HLA-DRB1 molecules. Of note, since a different assay is performed for every HLA-DRB1 molecule, IC50 values can only be compared between the 2 peptides tested for one particular HLA-DRB1 molecule. Hence, the results of this assay can only be relative. IC50 values cannot be compared between different HLA-DRB1 molecules.

Next, we addressed whether P1 and P17 induced proliferation in PBMC from healthy subjects (FVIII+/+) and HA (FVIII−/) patients expressing a variety of HLA-DRB1*haplotypes. PBMC from healthy individuals commonly recognized recombinant FVIII and at least one apitope. Interestingly, PBMC from 14 out of 23 HA patients with a current negative inhibitor status responded to recombinant FVIII by proliferation, and 9 out of 14 recognized one or both apitopes, whereas 9/23 HA patients did not respond to recombinant FVIII at all (Table 3 and supplementary table S2). Overall, this implies that P1 and P17 are major human FVIII epitopes in HA patients, and both healthy donors and HA patients expressing various HLA haplotypes frequently respond to the peptides.

**Peptide cocktail ATX-F8-117 induces tolerance among FVIII-specific T-cells**

To determine whether P1 and P17 were capable of regulating T-cell responses to hFVIII, HLA-DR2tg mice were treated with a combination of P1 and P17 (ATX-F8-117) according to a dose escalation strategy followed by immunisation (Figure 2A).

Spleens from mice that received control treatment with PBS proliferated vigorously upon recall with rhFVIII in vitro (Figure 2B). Subcutaneous treatment with ATX-F8-117, however, completely abrogated the proliferative response of splenocyte cultures upon re-challenge with rhFVIII (Figure 2B). Of note, splenocytes from both treatment groups showed a comparable recall response to PPD as an immunisation control (Supplementary Figure S1). Furthermore, spleens from mice treated with PBS secreted high levels of IFN-γ, IL-2 and IL-17 in response to antigen re-stimulation in vitro (Figure 2C). Repetitive treatment with ATX-F8-117 reduced splenic cytokine production of IFN-γ, IL-2 and IL-17 significantly when re-stimulated with rhFVIII. Together, ATX-F8-117 induces antigen-specific T-cell tolerance upon subcutaneous administration in humanised HLA-DR2tg mice.

When performing the same experiment in FVIII−/− HLA-DR2tg mice, a reduction of the proliferative response towards rhFVIII was observed in splenocyte cultures from ATX-F8-117-treated mice as compared to controls (Supplementary Figure S2).
ATX-F8-117 suppresses FVIII inhibitor formation in HLA-DR2tg mice

Since ATX-F8-117 can regulate the anti-FVIII T-cell response, we evaluated the efficacy of the peptide cocktail to modulate anti-FVIII humoral immune responses in a murine FVIII antibody model. As outlined in figure 3A, HLA-DR2tg mice were pre-treated with ATX-F8-117 or PAP 133-152 (control)⁶¹, according to a dose escalation regimen, followed by repeated immunisation with rhFVIII. Peptide treatment was continued at the top dose and administered 3 days after each rhFVIII immunisation.

In both groups, mice developed detectable levels of anti-FVIII IgG antibodies after four subcutaneous injections with rhFVIII (day 28, Figure 3B) and recurrent rhFVIII immunisations further elevated total anti-FVIII IgG titers up to day 56 in both groups (Figure 3B). However, pre-treatment with ATX-F8-117 successfully decreased total anti-FVIII IgG levels compared to controls (Figure 3B). Similarly, HLA-DR2tg mice started to develop FVIII inhibitors after four subcutaneous immunisations with rhFVIII (day 28, data not shown) and subsequent rhFVIII injections lead to a strong increase in the FVIII inhibitor levels in control animals (to 56.6 BU/ml on day 56, Figure 3C). In contrast, pre-treatment with ATX-F8-117 significantly reduced the FVIII inhibitor levels to 50% at day 56 (26.9 BU/ml). For longitudinal antibody data of individual mice of both anti-FVIII IgG and FVIII inhibitors, see Supplementary Table 3). The IgG subclass distribution of anti-FVIII antibodies of control treated animals was predominately IgG1 (Figure 3D). Lower proportions belonged to the IgG2b subclass and very low levels of anti-FVIII IgG2a isotype antibodies could be detected at day 56. Upon prophylactic treatment with ATX-F8-117, the anti-FVIII IgG1 subclass antibodies were significantly reduced compared to control mice (Figure 3D). Interestingly, correlation analysis between FVIII inhibitors and anti-FVIII IgG1 subclass antibodies but not anti-FVIII IgG2a and IgG2b antibodies reached statistical significance (Figure 3E and data not shown).

Thus, pre-treatment with ATX-F8-117 is highly efficacious in suppressing FVIII inhibitor formation in a murine FVIII antibody model developing predominately anti-FVIII IgG1 subclass antibodies.

**ATX-F8-117 suppresses new FVIII inhibitor formation in HLA-DR2tg mice with an ongoing anti-FVIII immune response**

To demonstrate the efficacy of ATX-F8-117 to modulate the anti-FVIII immune responses in animals with pre-existing FVIII inhibitory antibodies, HLA-DR2tg mice were immunised repetitively subcutaneously with rhFVIII during which peptide treatment was initiated after the tenth immunisation with rhFVIII (day 65) as outlined in figure 4A.

Prior to the start of peptide therapy (day 65), all mice developed high levels of anti-FVIII IgG antibodies (day 56, Figure 4B). Following control treatment, plasma levels of anti-FVIII IgG antibodies
increased with recurrent rhFVIII immunisations up to day 105-106 after a small initial decline in antibody levels due to a break in the rhFVIII immunisation regime at day 70 (Figure 4B). Therapeutic intervention with ATX-F8-117 significantly reduced total anti-FVIII IgG levels from day 98 onwards compared to controls (Figure 4B). Similarly, mice developed high FVIII inhibitory antibodies prior to peptide treatment start (day 56, Figure 4C). FVIII inhibitors increased remarkably in the control group following repeated rhFVIII injections (Figure 4C) from 39.0 BU/ml on day 56 to 93.8 BU/ml on day 105-106. Treatment with ATX-F8-117 successfully reduced plasma levels of FVIII inhibitors up to 58% at day 105-106 (39.6 BU/ml) compared to control animals (Figure 4C). For longitudinal antibody data of individual mice of both anti-FVIII IgG and FVIII inhibitors, see Supplementary Table S4).

The IgG subclass distribution of anti-FVIII antibodies from control animals was again predominately IgG1 (Figure 4D). Also, fewer anti-FVIII antibodies belonged to the IgG2b subclass family and no anti-FVIII IgG2a isotype antibodies could be detected in plasma from controls (Figure 4D). Upon therapeutic treatment with ATX-F8-117, lower anti-FVIII IgG1 subclass antibodies were detected compared to the control group (Figure 4D). Correlation analysis between FVIII inhibitors and the anti-FVIII IgG1 subclass antibodies reached statistical significance in the control group (Figure 4E, left panel).

These results show that therapeutic intervention with ATX-F8-117 is efficacious in preventing new FVIII inhibitor formation in HLA-DR2tg mice with circulating anti-FVIII inhibitory antibodies.
Discussion

There is a clear need for improved approaches for induction of immunological tolerance to FVIII in haemophilia A. Long-term success in eradicating the FVIII inhibitors can be achieved with Immune Tolerance Induction (ITI) therapy. This involves the frequent administration of high doses of FVIII and is based on high dose tolerance, first described by Mitchison in the 1960s. Unfortunately, however, this approach is generally more effective in those with low levels of inhibitors and can fail in individuals with higher levels. Furthermore, the approach is highly expensive and requires frequent invasive treatment thereby placing a heavy burden on both individual patients and health resources: alternative strategies are highly desirable. One such approach has been tested in vitro involved transduction of human Treg cells with cloned T cell receptor genes from a haemophiliac patient’s FVIII specific T-cell. The transduced cells suppressed T-cell proliferation in vitro and most importantly reduced antibody production by spleen cells from an immunised HLA-DR transgenic mouse. This provides evidence that Treg cells can modulate the immune response to FVIII and also demonstrates that epitope specific Treg cells mediate linked suppression of the response to other epitopes within FVIII. This approach would be difficult to adopt for the general haemophiliac population since it would be highly patient or at least HLA-DR specific and would probably require frequent infusions of Treg cells. An alternative approach would be to promote the generation of a regulatory phenotype in vivo through the administration of pan-DR binding T-cell epitopes derived from FVIII. Our analysis reveals that two CD4+ T-cell epitopes constitute the dominant HLA-DR2 restricted human FVIII epitopes in humanized FVIII-sufficient and haemophiliac transgenic mice. This confirms previous observations using similar technology whereby Steinitz revealed that two of three dominant epitopes (3 dominant epitopes identified from 8 immunogenic regions) overlap with the DNIMV and PRCTL peptides described here and showed that they bind common HLA-DRB1*haplotypes. Apitopes P1 and P17 induced proliferation in PBMC cultures from healthy and haemophiliac blood donors expressing various HLA-DRB1*haplotypes. PBMC from HA patients responding to FVIII recognized at least one of the two epitopes. Furthermore, Van Haren and colleagues revealed that few core peptides were presented by HLA-DR molecules of FVIII-pulsed donor-derived human dendritic cells (DC) with diverse HLA-DRB1*haplotypes. Interestingly, one core peptide sequence FRNQASRPY, part of the P17 sequence, was a promiscous, pan-DR, HLA-DR binding peptide. These data underline the relevance and potential of the P1 and P17 epitopes for the treatment of inhibitor positive HA patients.

Apitopes P1 and P17 can bind MHCII molecules on APC rendered incapable of antigen processing by chemical fixation i.e. they are antigen processing independent. Why is it important for peptides to be designed as apitopes? Tolerogenic peptides must be designed to mimic the naturally processed
epitope in order to induce tolerance\textsuperscript{37}. Recently, we have shown that apitopes selectively bind steady-state DC in lymphoid organs and do not bind B-cells or monocytes \textit{in vivo}\textsuperscript{65}. Empty or peptide-receptive MHCII molecules are abundantly expressed on the surface of steady-state and immature DC in particular\textsuperscript{66,67} and we know that these cells induce tolerance \textit{in vivo}\textsuperscript{68}.

Apitope cocktail ATX-F8-117 promoted antigen-specific T-cell tolerance \textit{in vivo}, following a dose escalation strategy, indicating that apitope immunotherapy is an effective means of tolerance promotion to protein therapeutics. Both the proliferative capacity and IL-2 production of CD4\textsuperscript{+} T-cells was abrogated in splenocyte cultures in recall to FVIII but not to PPD, showing that apitope-mediated tolerance is antigen-specific. In the absence of overt infection, both immature and intermediate, steady-state DC maturation stages are prevalent, these steady-state DC present self-peptides for the induction of CD4+ T-cell tolerance\textsuperscript{69}.

Since ATX-F8-117 is designed to re-instate tolerance to FVIII replacement therapy rather than to control bleeding episodes in HA patients, FVIII-sufficient HLA-DR2tg mice were used as a FVIII antibody model. The use of FVIII-sufficient mice as a model for FVIII inhibitor treatment reflects the clinical scenario of HA patients which have residual dysfunctional FVIII circulating but are at risk of developing immune responses to the replacement therapy. Reipert and colleagues have demonstrated that while the rate of anti-FVIII titer development differs between FVIII-sufficient and FVIII-knockout animals, similar total anti-FVIII antibody levels appear after recurrent rhFVIII injections via the subcutaneous route\textsuperscript{70}. Here, HLA-DR2tg animals developed high levels of anti-FVIII IgG following eight weekly subcutaneous immunisations with rhFVIII; nevertheless, the rise in inhibitor antibodies could still be reversed by apitope treatment.

Previous reports have shown that the most abundant IgG subclass of anti-FVIII antibodies in HA patients with inhibitors were IgG1 and IgG4\textsuperscript{71}. The appearance of FVIII inhibitors in experimental haemophiliac mouse models can be attributed to the IgG1 and IgG2a subclasses depending on the genetic background and immunisation strategy\textsuperscript{70,72}. In the model described here, HLA-DR2tg mice received recurrent rhFVIII immunisation in the absence of microbial stimuli which initiated predominately Th2-driven anti-FVIII IgG1 isotype development. Additionally, correlation analysis indicated that there was a direct association with FVIII inhibitors and IgG1 subclass antibodies in control animals. Hence, our data are consistent with the association of FVIII inhibitors and IgG1 subclass described in haemophiliac mouse models and patients\textsuperscript{70,71,73}.

Data highlight the efficacy of ATX-F8-117 to suppress formation of new FVIII inhibitors in naïve and FVIII-primed HLA-DR2 humanized animals. A potential mode of action is thought to occur through the lack of the CD4+ T-cell help required for B-cells to transform to an antibody-producing plasma
cell. However, accumulating evidence indicates that regulatory T-cells can directly control B-cell antibody production in vitro\textsuperscript{74,75}. Based on these data, it will be interesting to see how the anergic CD4+ T-cells induced by ATX-F8-117 are able to control the anti-FVIII antibody response. Indications that apitope treatment can indeed reduce the levels of circulating antibodies in humans, come from a phase I clinical trial performed in patient suffering from Graves’ hyperthyroidism\textsuperscript{23}.

Multiple inhibitory B-cell epitopes have been mapped to the A2 and C2 domain of the hFVIII protein which are essential for its pro-coagulant activity, but non-neutralizing antibodies may bind to multiple other FVIII surfaces, and some inhibitory antibodies also bind to other domains and may affect VWF binding or FVIII clearance\textsuperscript{10}. Interestingly, the FVIII immunodominant T-cell epitopes, as identified in this study, were mapped to FVIII A2 domain (PRCLT) and A3 domain (DNIMV) and not to the C2 domain, a finding confirmed elsewhere\textsuperscript{38}. Peptide treatment with a single epitope can result in suppression of other epitopes within the same protein through linked suppression\textsuperscript{76,77}. Interestingly, administration of each separate apitope P1 or P17 in HLA-DR2tg mice followed by immunisation with full-length rhFVIII in CFA reduced cytokine production and proliferation in secondary lymphoid organs to rhFVIII re-stimulation \textit{ex vivo}. Moreover, no anti-apitope antibodies were detected in the plasma of our experimental FVIII antibody model following rhFVIII immunisation excluding that the inhibitory FVIII antibodies decrease through direct absorption from the circulation upon apitope treatment. It should be noted that while most FVIII B-cell epitopes are conformational epitopes\textsuperscript{10}, a screening of HA patients for the presence of anti-apitope antibodies prior to treatment with ATX-F8-117 remains vitally important to prevent peptide-induced anaphylaxis\textsuperscript{78}. The promising results of recent clinical trials of apitope therapy in antibody mediated Graves’ disease support the clinical development of ATX-F8-117 for suppression of inhibitor formation in people with haemophilia A and the use of apitopes for other anti-drug antibody complications.
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Authorship Contributions

K.P., L.J., H.S., E.S. and D.C.W.: study design, interpretation of the data and manuscript preparation
K.P., K.S.N., H.S. and W.S.: performed research and collected data.

Conflict of Interest Disclosures

K.P., K.S.N, W.S., E.S. and L.J. were employees of Apitope International NV at the time he/she contributed to this study. D.C.W. serves as Chief Scientific Officer for Apitope International NV on a consultative basis. Other authors declare no conflict of interest.

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Tables

Table 1. Human FVIII HLA-DRB1*1501 restricted T-cell binding epitopes. Fifteen-mer high-affinity binding MHC class II epitopes of the human FVIII protein were predicted for human HLA-DRB1*1501 allele using bioinformatics tools. Peptides with amino acids highlighted in bold are designated by the first 5 N-terminal amino acids in their sequence.

| Epitope sequence | At position |
|------------------|-------------|
| TQTLHKFILLFAVFD  | 208-222     |
| MHTVNGYVNRLPGL   | 250-264     |
| LGQFLFFCHISSHQH   | 322-336     |
| DTLILFKNQASRPY   | 478-492     |
| PRCLTRYSSFVNME   | 545-559     |
| TENIQROFLNPAGVO   | 607-621     |
| DNIMVTFRQASRPY   | 1788-1802   |
| SLYISQFIIMYSLDG   | 2114-2128   |
| GTLMVFQGNVDSSGI   | 2140-2154   |
| PPIARYIRLHPTHY   | 2161-2175   |
| PLLTRYLRHIPOSW   | 2318-2332   |
| RYLRIHPOSWVHQIA  | 2322-2336   |
Table 2. Apitopes P1 and P17 bind common human HLA-DRB1*haplotypes. Affinity binding of apitopes P1 and P17 shown as IC50 values (in µM) to the indicated MHC alleles as determined with an in vitro MHC binding assay (ProImmune REVEAL® assay) studying displacement of a control peptide by apitopes P1 and P17.

| HLA-DRA*01:01; DRB1*15:01 | HLA-DRA*01:01; DRB1*03:01 | HLA-DRA*01:01; DRB1*04:01 | HLA-DRA*01:01; DRB1*11:01 |
|---------------------------|---------------------------|---------------------------|---------------------------|
| P1                        | 0.06                      | 32.21                     | 0.40                      | 1.28                      |
| P17                       | 0.04                      | 21.90                     | 5.09                      | 4.19                      |
Table 3. Apitopes P1 and P17 induce proliferation in human PBMC from healthy blood donors and HA patients. PBMC isolated from peripheral blood of healthy blood donors or HA patients were cultured with graded concentrations of rhFVIII or peptides P1 and P17. PBMC proliferation was assessed at day 6 to day 8 of culture by [3H]-thymidine incorporation and expressed as the stimulation index (SI). A positive response to peptides P1 or P17 and rhFVIII was defined as SI > 2. Data is summarized and presented as percentage positive responding of total healthy blood donors or haemophilia patients analysed.

|                    | Response to rhFVIII and one or both apitopes | Response to one or both apitopes (from FVIII responders) | No response to rhFVIII |
|--------------------|---------------------------------------------|--------------------------------------------------------|-----------------------|
| FVIII⁺ healthy donors | 11/15 (73%)                              | 9/11 (82%)                                             | 4/15 (27%)            |
| FVIII⁻ patients     | 14/23 (61%)                               | 9/14 (64%)                                             | 9/23 (39%)            |
Figure Legends

**Figure 1. Peptides P1 and P17 are HLA-DRB1*1501 restricted FVIII apitopes.**

T-cell hybridoma clones derived from PRCLT- or DNIMV-immunised HLA-DR2tg mice were co-cultured with an EBV-transformed human HLA-DRB1*1501 expressing B cell line MGAR. The MGAR cells were either left untreated (fresh) or fixed with 0.5% paraformaldehyde (fixed) to prevent antigen processing. Recognition of peptides P1, P17, PRCLT and DNIMV or recombinant hFVIII by two clones specific for PRCLT (A) and two clones specific for DNIMV (B) was addressed after 48 hours by analysing IL-2 cytokine secretion in the supernatants by ELISA.

**Figure 2. ATX-F8-117 induces FVIII-specific T-cell unresponsiveness in humanized HLA-DR2tg mice**

(A) Groups of 8-10 HLA-DR2tg mice (FVIII sufficient) received pre-treatment with a combination of peptides P1 and P17 (ATX-F8-117) according to the dose escalation scheme (0.1-1-10-100-100-100µg of each peptide) or PBS as described. Following pre-treatment, mice were immunised with peptides PRCLT and DNIMV in complete Freund’s adjuvant (CFA) containing 400µg heat-killed Mycobacterium tuberculosis. (B) T-cell activation was assessed 10 days after the immunisation by determining the proliferation of splenocytes upon re-stimulation with rhFVIII. Data is expressed as stimulation indices (SI). Graphs show mean data of each treatment group (mean +/- SEM). Statistical significance: * = p<0.05 and ** = p<0.01, by repeated measures 2-way ANOVA of log-transformed data. (C) Cytokine content of the cell culture supernatants re-stimulated with rhFVIII was analysed using cytokine bead array technology. Graphs show mean data of each treatment group (mean +/- SEM). Statistical significance: * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001, by repeated measures 2-way ANOVA and Bonferroni post-test.

**Figure 3. Preventive treatment with ATX-F8-117 reduces FVIII inhibitor formation in a FVIII antibody model**

(A) Groups of 18-19 HLA-DR2tg mice (FVIII sufficient) were treated by dose-escalation with ATX-F8-117 (○ symbols, PolyPeptide Laboratories) or prostatic acid phosphatase (PAP) 133-152 (● symbols) as a peptide control. Four days after the peptide pre-treatment, mice were primed eight times via subcutaneous flank injections with 1µg rhFVIII at weekly intervals. Treatment with ATX-F8-117 or PAP 133-152 control was continued once weekly for additional eight times starting three days after the initial FVIII priming. Plasma samples were collected from both treatment groups as indicated (B = bleed). (B) Plasma was collected at day 28, day 42 and day 56 and total anti-FVIII IgG levels determined by ELISA. Graphs show endpoint anti-FVIII IgG titers on a log-scale and mean +/- SEM. Each dot represents data from one mouse. (C) FVIII inhibitors from plasma collected at indicated
timepoints were analysed by a modified Bethesda assay. Graphs show mean data of each treatment group (mean +/- SEM) and each dot represents data from one mouse. (D) Plasma was collected at day 56 and anti-FVIII IgG subclass distribution determined by an ELISA assay. Each dot represents data from one mouse and mean data (mean +/- SEM) is shown of each treatment group. Statistical significance: * = p<0.05, ** = p<0.01, *** = p<0.001 and ns = not significant, two tailed Mann-Whitney U test. (E) Graphs show correlation between anti-FVIII IgG1 subclass antibodies and FVIII inhibitors in plasma samples from PAP 133-152 (upper panel) or ATX-F8-117 (lower panel) treated animals collected at day 56 using the 2-tailed nonparametric Spearman correlation analysis. The correlation coefficient (r) is shown together with the 95% confidence intervals. Statistical significance: *** = p<0.001 and **** = p<0.0001. Data representative of 2 experiments performed.

Figure 4. Therapeutic application of ATX-F8-117 reduces the formation of new FVIII inhibitor antibodies in a primed FVIII antibody model

(A) Groups of 20 HLA-DR2tg mice (FVIII sufficient) were primed with ten weekly rhFVIII immunisations followed by treatment by dose-escalation with ATX-F8-117 (○ symbols, PolyPeptide Laboratories) or prostatic acid phosphatase (PAP) 133-152 (● symbols) via subcutaneous flank injections. Three days after the dose escalating peptide treatment, mice were primed for an additional four times via subcutaneous flank injections with 1µg rhFVIII at weekly intervals. Treatment with ATX-F8-117 or PAP 133-152 control was continued twice weekly starting two days after the FVIII priming. Plasma samples were collected from both treatment groups as indicated (B = bleed). (B) Plasma was collected at indicated time points and total anti-FVIII IgG levels determined by ELISA. Graphs show mean data of each treatment group (mean +/- SEM). (C) FVIII inhibitors from plasma collected at indicated timepoints were analysed by a modified Bethesda assay. Graphs show mean data of each treatment group (mean +/- SEM). (D) Plasma was collected at day 105-106 and anti-FVIII IgG subclass distribution determined by ELISA. Each dot represents data from one mouse and mean data (mean +/- SEM) is shown of each treatment group. Statistical significance: * = p<0.05, ** = p<0.01, and ns = not significant, robust regression model using M estimation, Huber weighting, using the default parameter c=1.345 with treatment as a factor and day 56 data as a covariate. IgG2a data analysed by exact Wilcoxon rank sum test. (E) Graphs show correlation between anti-FVIII IgG1 subclass antibodies and FVIII inhibitors in plasma samples from PAP 133-152 (left panel) or ATX-F8-117 (right panel) treated animals collected at day 105-106 using the 2-tailed nonparametric Spearman correlation analysis. The correlation coefficient (r) is shown together with the 95% confidence intervals. Statistical significance: *** = p<0.001, and ns = not significant.
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