Identification of a tolerogenic Factor V peptide and its potential role in Factor VIII tolerance induction

P.C4.03.05
ECI 2018

A. S. De Groot[1,2], A. Rosenberg[3], S. Lélias[1], G. Skowron[1], D. Medeiros[1], P. Hindocha[1], D. Kanakia[1], B. McGonnigal[1], F. Terry[1], B. Roberts[1], W. Martin[1]
1EpiVax, Inc., Providence, United States, 2University of Rhode Island, Providence, United States, 3Center for Drug Evaluation and Research, FDA, White Oak, United States.

Abstract

Inhibitory Anti-Drug Antibody (ADA) responses interfere with Factor VIII replacement efficacy in 25-30% of Hemophilia A (HA) cases, greatly increasing patient morbidity and treatment costs. As an extension of previous work on tolerance-inducing peptides in IgG (Tregitopes), we investigated whether there were peptides in other ubiquitous serum proteins that could have high homology to peptides found in Factor VIII. We hypothesized that tolerance to cross-conserved peptides in other prevalent proteins might explain why anti-FVIII antibodies fail to develop in some severely FVIII-deficient HA patients.

Using advanced computational modeling tools, we discovered in Factor V a potent regulatory peptide (FVP4) with an immunologic profile (HLA and TCR binding) that is homologous to a non-identical peptide in FVIII. We postulated that treatment with a FVP4-peptide containing biologic may be able to invoke tolerance to Factor VIII in patients who have anti-FVIII antibodies. In an ex vivo assay using human PBMCs, we found that the CD4 and CD8 T cell effector recall response was strongly inhibited by FVP4. However, other Factor V peptides with similar HLA-binding properties did not suppress the response. Using this assay, we are determining the evolution of markers on regulatory T cells, Antigen Presenting Cells, and effector T cells in order to identify specific parameters associated with cell populations and soluble factors mediating immune suppression in response to FVP4.

Introduction

- Hemophilia A is a genetic clotting disorder caused by absent or reduced levels of Factor VIII, affecting 1 in 4,000 to 1 in 5,000 males worldwide. Patients with HA produce truncated or mutated Factor VIII (FVIII) protein, or no protein at all.
- HA is treated by the periodic administration of natural or recombinant Factor VIII to restore a normal coagulation cascade. In the absence of a normally structured Factor VIII in the body during development, the natural process of immune tolerance induction to FVIII does not occur in the thymus. Thus, therapeutic FVIII is recognized as “foreign” by the immune system of HA patients resulting in the activation of FVIII-specific CD4 T cells and the development of anti-FVIII antibodies.
- Anti-drug antibody (ADA) response occurs in 25-30% of HA patients treated with recombinant FVIII, abrogating its effectiveness and greatly increasing patient morbidity and treatment costs (1). Notably, some severely FVIII-deficient HA patients do not develop anti-FVIII antibodies, suggesting that immune tolerance has developed in the absence of circulating native FVIII protein.
- We hypothesized that another endogenous protein with high homology to peptides found in Factor VIII may be responsible for this cross-tolerance.
- FVIII and Factor V are homologous glycoproteins that are cofactors for proteolytic activation in the coagulation cascade. They share a conserved domain structure (A1-A2-B-A3-C1-C2) and have 35% amino acid identity in the A and C domains (2).
- We have previously identified Tregitopes (**T** cell **reg**ulatory cell ep**itopes**) which are believed to be natural T cell epitopes derived from immunoglobulin G that stimulate T regulatory cells (Tregs) to modulate antigen-specific CD4 cells responses (3).
- Using a combination of computational modeling tools and in vitro functional assays, we discovered a potent regulatory peptide (FVP4) in human coagulation Factor V, with an immunologic profile (HLA binding and T cell regulatory effect) that is similar to a homologous peptide in FVIII.

Induction of antigen-specific tolerance

- Tolerance to self-proteins is induced primarily in the thymus (central tolerance), by a mechanism involving deletion of autoreactive T cells and generation of natural Tregs (nTregs).
- nTregs and/or tolerogenic APC can induce naïve CD4 T cells to acquire a suppressive phenotype (iTregs) in secondary lymph organs, or chronically inflamed tissues.
- Tregs recognize specific peptides in the context of MHC and respond suppressing peripheral effector T cells
- Co-presentation of effector and regulatory epitopes (costimulation) is a key component of Treg function.

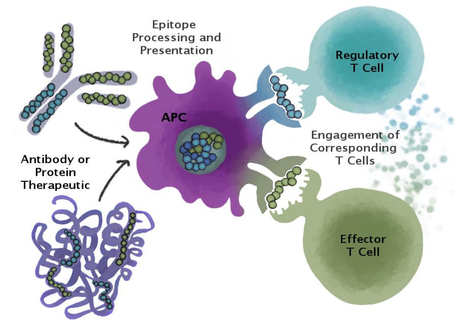


Figure 1: Schematic representation of antigen-specific tolerance induction

- T cell regulatory peptides typically bind multiple HLA alleles with high affinity and have TCR-facing residues highly conserved with similarly positioned residues in other human proteins (4).
- Regulatory peptides have been found in IgG (Tregitopes, ref. 3) and other proteins (5).

Tolerization to Factor VIII with Factor V peptides

- Effector epitopes present in therapeutic Factor VIII but absent in HA patients contribute to the generation of inhibitory FVIII antibodies (ADA). An additional factor might be the absence of FVIII tolerogenic epitopes.
- We hypothesized that HA patients have normal levels of Factor V and may have regulatory T cells (Tregs) with specificity for Factor V regulatory peptides.
- Some Factor V-derived regulatory peptides could have TCR-facing residues homologous to their FVIII counterparts.
- We hypothesized that these Factor V-specific Tregs could be used to invoke tolerance to FVIII, substituting for missing FVIII-specific Tregs in some HA patients.

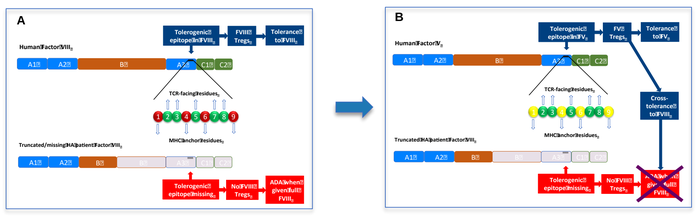


Figure 2: **Recruiting FV Tregs to tolerize against FVIII.** Stimulating natural Tregs reactive to Factor V tolerogenic peptides that are cross-reactive with FVIII could activate them and tolerize to Factor VIII. **A**, missing T cell epitopes in HA patients can lead to lack of tolerance to therapeutic FVIII and induction of ADA. **B**, Stimulation of Factor V Tregs with similar TCR binding characteristic than the missing FVIII Tregs could cross-inhibit FVIII effector T cells.

Selection of tolerogenic Factor V peptides

- Bioinformatics analysis was used to select Factor V-derived peptides presumed to be regulatory according to the following criteria:
  - ✓are homologous to sequences present in FVIII.
  - ✓are predicted to bind multiple HLA-DRB1 alleles.
  - ✓share at least four of the five T cell receptor contact residues (relative positions 2, 3, 5, 7, and 8) with their FVIII-derived homologues.
- Based on the criteria above, six Factor V-derived peptides were selected for in vitro screening.

Peptide Name	HLA-DR binding (IC50) *0101	*0301	*0401	*0701	*1101	*1301	*1501
Human FVP1	2170	78594	20114	7033	4361	2963	2695
Human FVP2	21020	27507	113296	1616	116754	306603	29801
Human FVP3	101393	80506	96423	2788	162410	Non-Binder	4218
Human FVP4	691	16	2422	251	1015	2885	104
Human FVP5	1915	Non-Binder	Non-Binder	105559	Non-Binder	Non-Binder	2024
Human FVP6	507	137077	30625	210	33700	844291	506

IC50 (nM)
Non-Binder
>100,000
10,000 -100,000
1,000 -10,000
100 -1,000
<100

Table 1: **FV peptides bind to multiple HLA-DRB1 alleles.** HLA-DRB1 binding of selected Factor V peptides was determined in vitro and IC_{50} values derived from the measurements. Five of the selected Factor V peptides bind to ≥80% of the alleles tested (DRB1*01:01,*03:01, *04:01,*07:01 ,*11:01, *13:01 and *15:01).

FVP4 modulates HLA-DR expression on APCs

PBMCs isolated from healthy donors were stimulated at 50 µg/mL with one of four selected FV-peptides, IgG-derived Tregitope 167 (positive control), Flu-HA 306-318 and Ova 323-339 (negative controls) and a buffer only (vehicle control), and incubated at 37°C. On day 7, cells were analyzed by flow cytometry for expression of CD11c and HLA-DR.

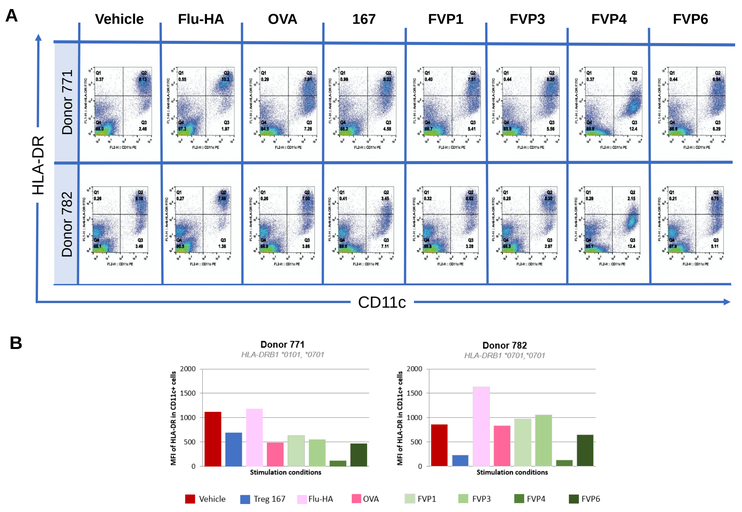


Figure 3: **FVP4 down-regulates the expression of HLA-DR surface on antigen presenting cells (APC).** A) Plots of CD11c+ vs HLA-DR reveal an observed shift in the distribution of HLA-DR for each stimulation condition with a decrease in HLA-DR observed for Tregitope 167 and even more notably for FVP4. B) To evaluate the relative expression of HLA-DR on all CD11c+ cells, the mean fluorescence intensity (MFI) of HLA-DR expression for the CD11c-high segments (Q2+Q3) of each dot-plot was calculated for donors 771 and 782.

Inhibition of CD4 T cells proliferation by FVP4

CD4 T cells displaying antigen-specific recall response are inhibited by FVP4 peptide. In the example in. Figure 4, a population of proliferating CD4 T cells responding to TT stimulation – most also showing activation of CD25 (IL2RA) – is strongly inhibited by increasing concentrations of FVP4 peptide but not of FVP1 (control).

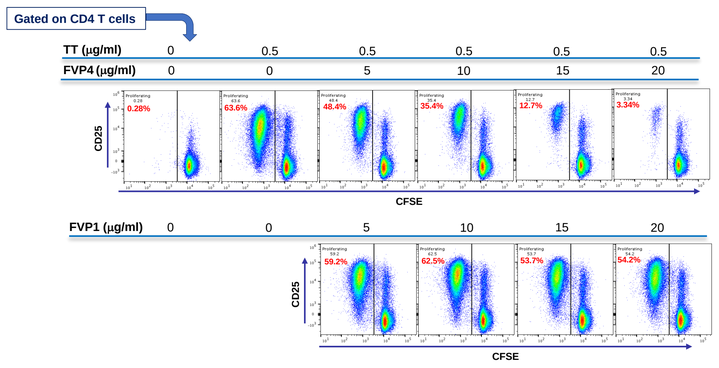


Figure 4: FVP4 Peptide reduces T effector cell response to TT. PBMCs from a healthy donor (donor 135 in Figure 7) were stimulated with 0.5 mg/ml of TT with or without the addition of the indicated concentrations of FVP4 peptide or FVP1 (control). Proliferation and activation cell markers were measured six days post-stimulation by flow cytometry.

Memory response of Tetanus Toxoid on CD4 T cells

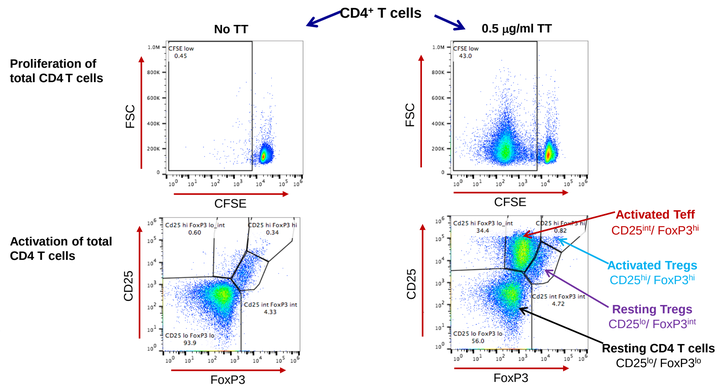


Figure 5: TT-specific effector response in the “Four Quadrant” and CFSE assay. Flow analysis of response to Tetanus Toxoid (TT) in human PBMCs shows both proliferation (top panel) and activation (bottom panel) of memory CD4 T cell populations. The example typifies a trend observed across donors immunized against Tetanus. The CD4 T cell activation response illustrations four distinct populations as shown in the CD25 vs FoxP3 dot plot (bottom right) with an increase in the CD25int/FoxP3 population “Activated T effectors”.
This memory CD4 T cell response is the basis of the TT bystander suppression assay (TTBSA), whereby a regulatory peptide is added along with TT and evaluated for its inhibitory impact on CD4 T cell proliferation and activation.

Inhibition of activated CD4 T cells by FVP4

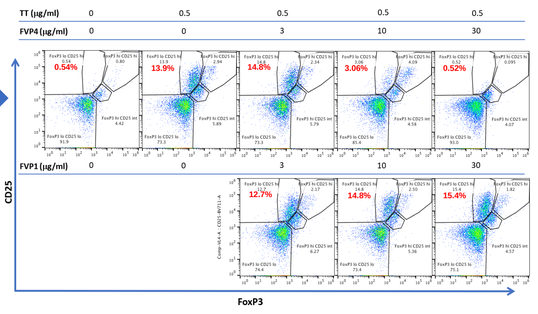
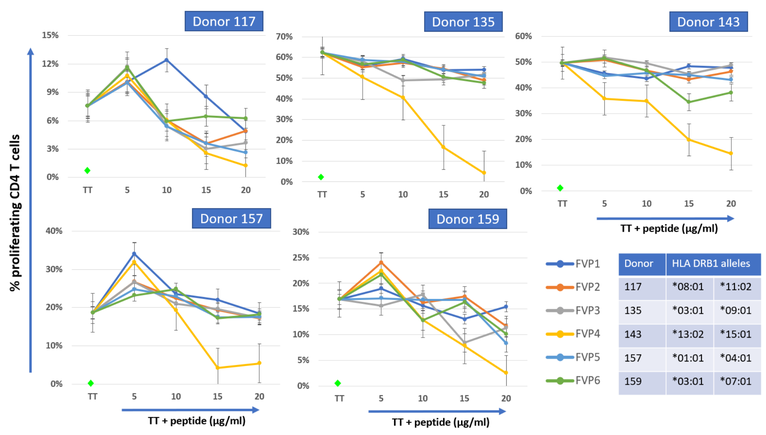


Figure 6: **Inhibition of activated Teff by FVP4 peptide in the TT Bystander Suppression Assay.** Inhibition by FVP4 turns most of the TT activated T cells ($CD25^{hi}$) to an inactive phenotype. PBMCs from a healthy donor were stimulated with 0.5 µg/ml of TT with or without the addition of the indicated concentrations of FVP4 peptide or FVP1 (control). Proliferation and activation cell markers were measured six days post-stimulation by flow cytometry.

Tetanus Toxoid bystander suppression assay



Donor	HLA DRB1 alleles	
117	*08:01	*11:02
135	*03:01	*09:01
143	*13:02	*15:01
157	*01:01	*04:01
159	*03:01	*07:01

Figure 7: FV peptides impact TT-induced proliferation of CD4 T effector cells in the TT bystander suppression assay. PBMCs from each donor were incubated in the standard TTBSA alone, with 0.5 µg/ml of TT, or with TT plus the indicates concentration of FV peptides. Green diamonds represent background proliferation with no TT or peptides added. Percentage of proliferating CD4 cells (as indicated by CFSA dilution) is plotted for each condition. The insert shows the HLA-DRB1 haplotype of each donor.

FVP4 impacts Granzyme B expressing Teff & Tregs

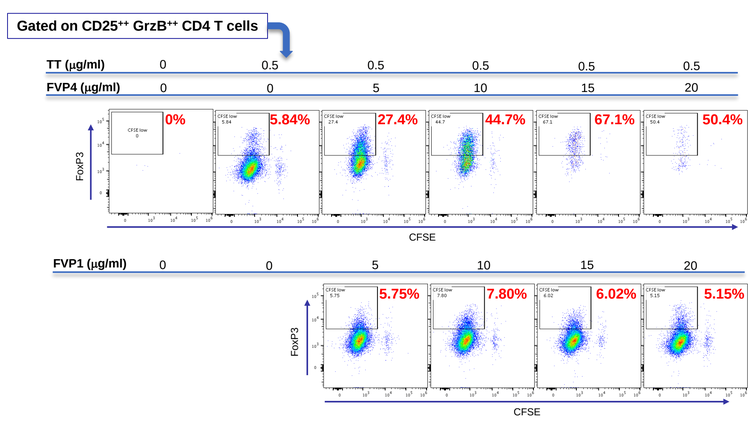


Figure 8: FVP4 differentially impacts activated CD4 T cell populations. To investigate the effect of FVP4 on highly activated, proliferating and cytolytically active CD4 T cells, we first gated on the CD3+ CD4+ T cell population, and then identified a population of highly activated, granzyme B-expressing CD4 T cells (CD25++ Granzyme B++) which includes both Teff + Tregs. The addition of FVP4, but not the control peptide FVP1, to the Tetanus Toxoid Suppression Bystander Assay markedly reduced the population of TT-responsive, proliferating (CFSElo), activated, GrB+ Teffs (FoxP3int) in a dose-dependent manner. The smaller population of proliferating, activated, GrB+ Tregs (FoxP3hi) were also reduced, but their relative proportion increased with higher FVP4 concentrations.
.

FVP4 inhibits activated effector CD8 T cells

FVP4 peptide also showed inhibition of the antigen-specific recall response of CD8 T cells. Figure 9 shows that a population of proliferating CD8 T cells responding to CEF peptide mix stimulation – most also showing activation of CD25 ($IL2R\alpha$), is strongly inhibited by increasing concentrations of FVP4 peptide but not of FVP1 (control). In the same experiment, the response of CD4 T cells showed little change.

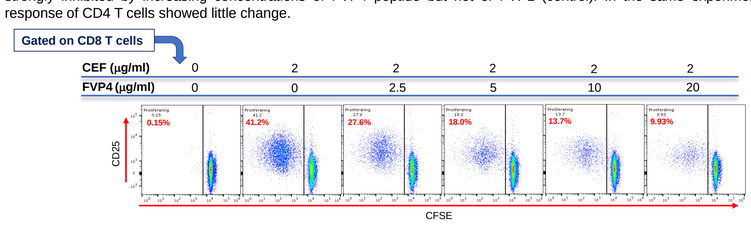


Figure 9: FVP4 reduces CD8 T cell proliferation. CD8 T cells cells activated by CEF (Cytomegalovirus, Epstein-Barr virus and Influenza virus) pool of Class I peptides are sensitive to the inhibitory effect of FVP4 peptide (donor 121).

Figure 10: **FVP4 inhibits CEF- activated T cell response.** CD8 T cells cells activated by CEF respond strongly to inhibition by FVP4 (left panel), while CD4 cells are largely unaffected (right panel). PBMCs from two healthy donors were incubated at increasing concentrations of CEF peptides mix, with or without FVP4 peptide as indicated. Response was measured analyzing CFSE dilution and other activation markers (not shown) by flow cytometry.

Conclusions

- **The purpose of the present work was to test the hypothesis that there might be Factor V regulatory peptides able to down-modulate the ADA response to FVIII in HA patients.**
- **Using EpiVax’s proprietary tools, Factor V was screened for regulatory peptide candidates. A human Factor V-derived peptide (FVP4) was found to consistently inhibit ex vivo CD4 and CD8 T cell effector responses in donors covering the nine major HLA-DRB1 supertypes.**
- **Preliminary data on the mechanism of action points to the involvement of cytotoxic Tregs in the regulatory process.**
- **These data support our hypothesis that HA patients with normal levels of Factor V may have Tregs with specificity for FVP4 which could be used to invoke tolerance to Factor VIII.**
- **Peptide FVP4 might be useful in the development of biotherapeutics able to stimulate regulatory T cells that will cross-inhibit an ADA response to FVIII.**

References

1. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. J Thromb Haemost. 2009; 7:1446–56
2. Pipe S. W., Eickhorst A. N., McKinley S. H., Saenko E. L. & Kaufman R. J.Mild hemophilia A caused by increased rate of factor VIII A2 subunit dissociation: evidence for nonproteolytic inactivation of factor VIIIa *in vivo*. Blood. 1999; 93, 176–183
3. De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, et al. Activation of natural regulatory T cells by IgG Fc-derived peptide “Tregitopes”. Blood. 2008; 112:3303–11
4. Moise L, Gutierrez AH, Bailey-Kellogg C, Terry F, Leng Q, Abdel Hady KM, VerBerkmoes NC, Sztein MB, Losikoff PT, Martin WD, Rothman AL, De Groot AS. The two-faced T cell epitope: examining the host-microbe interface with JanusMatrix. Hum Vaccin Immunother. 2013; 9(7):1577-86
5. Boswell S1, Sharif S, Alisa A, Pereira SP, Williams R, Behboudi S. Induction of latency-associated peptide (transforming growth factor-β(1)) expression on CD4+ T cells reduces Toll-like receptor 4 ligand-induced tumor necrosis factor-α production in a transforming growth factor-β-dependent manner. Immunology. 2011 Jul; 133(3):278-87

For more information, please contact: Katie Porter at 401-272-2123, ext. 115; or at info@epivax.com *www.epivax.com*