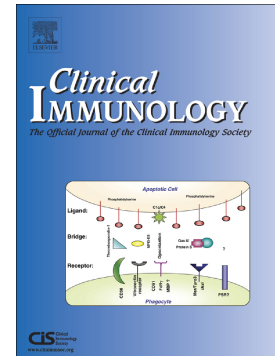


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**Identification of a potent regulatory T cell epitope in Factor V that modulates CD4+ and CD8+
Memory T cell responses**

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1. Introduction

Tregitope is a word that is used to describe natural regulatory T cell epitopes that are highly conserved and located in the sequence of human proteins such as immunoglobulin G (IgG). In addition to the IgG Tregitopes identified by De Groot since 2008 [1], the Edratide peptide of human IgG complementarity-determining region 1 (CDR1) [2], and the HSP70-derived peptide called B29 [3] might also be categorized as Tregitopes as they share key features with established Tregitopes such as promiscuous HLA DR binding, presence of the sequence in highly prevalent human proteins, and cross-conservation across species.

Regulatory T cells such as natural Tregs (also known as thymic-derived tTregs) and circulating “iTregs” or “pTregs” are considered to be important regulators of autoimmune disease [4]. Epitopes that activate these T cells may eventually be useful for treatment of autoimmunity. Edratide and HSP70 epitope (B29) have been shown to suppress inflammation and promote tolerance in murine models of autoimmune diseases including lupus [5], rheumatoid arthritis and collagen-induced arthritis [6]. Similarly, IgG-derived Tregitopes have induced antigen-specific tolerance or reduced the progression of disease in animal models of Type 1 diabetes [7,8], inflammatory bowel disease [9], multiple sclerosis (experimental autoimmune encephalomyelitis) [10], in gene therapy models [11] and allergy [12,13]. We have postulated that IgG Tregitopes induce epitope-specific natural Tregs (nTregs, now known as tTregs) that modulate T effector responses by inhibiting the activity of autoreactive effectors and/or by changing the phenotype of T_{eff} to induced Tregs (iTregs, also known as adaptive Tregs) as shown in tolerization to OVA in DO11.10 mice [8] and in HLA TCR-transgenic mice in the BM12 skin transplant model [14].

Here, we describe the discovery of a Tregitope-like peptide in Factor V, FV621. Considering the theory that Tregitopes might be present in other highly prevalent human serum proteins, the conserved structures of FVIII and FV, two highly homologous glycoproteins that are prevalent in serum, led us to search for Tregitopes that might be present in FV [15]. Indeed Factor V (FV)'s homology with FVIII is considerable: it shares a similar domain structure (A1-A2-B-A3-C1-C2) and ~35% sequence identity across the A and C domains [16]. Moreover, the amino acid sequence of FV also has considerable sequence similarity to that of ceruloplasmin, another highly prevalent human serum protein [17]. Therefore we applied immunoinformatics tools to identify sequences in FV that shared similar properties with previously identified and validated Tregitopes from our laboratory.

Using EpiMatrix, a T cell epitope prediction tool [18], we selected several peptides in FV that were likely to bind to multiple HLA DR alleles, thus meeting one of the key requirements for Tregitopes. We then applied JanusMatrix, an algorithm that searches for promiscuous HLA DR-binding peptides that have significant TCR-facing residue homology with epitopes found in other human proteins [19], to identify several FV HLA-DR-binding peptides with cross-conservation at TCR-facing residues to FVIII and (for certain peptides) also conserved with ceruloplasmin. These FV peptides were evaluated in HLA binding assays and an *in vitro* Tetanus Toxoid Bystander Suppression Assay (TTBSA), leading to the identification of a novel T regulatory cell epitope peptide now referred to as FV621.

Since the publication of studies showing that IgG-derived Tregitopes engage natural Tregs and induce the conversion of conventional T cells to induced Tregs (iTregs) via cytokine-dependent and other mechanisms that have been referred to as 'infectious tolerance', we

evaluated whether FV621 had the same effect in *in vitro* assays [19]. The studies presented here further characterize the suppressive and modulatory effects of the FV621 peptide in a set of *in vitro* assays and also describe their effect on dendritic cells. Additionally, we bioconjugated the FV621 peptide to human serum albumin (HSA) and evaluated the bioconjugated product in the same assays. Theoretically, this bioconjugated product could be designed to co-deliver Tregitopes with an immunogenic protein, driving antigen-specific adaptive tolerance (**Fig. 1**).

2. Materials & Methods

2.1. Bioinformatics screening and selection of Factor V peptides

Immunoinformatics analysis using the EpiMatrix and JanusMatrix algorithms [18,19] was used to select Factor V-derived peptides predicted to be regulatory according to the following criteria: the peptides are predicted to bind four or more HLA-DRB1 alleles; and their TCR facing residues share a high degree of homology to sequences with similar HLA-profile in FVIII and/or to other prevalent proteins. Six Factor V-derived peptides that were promiscuous and highly conserved in the human proteome were selected for *in vitro* screening (HLA binding and T reg assays). And although information on MHC elution and identification by mass spectrometry of these peptides was not a criteria for their selection, subsequently, HLA-DR-restricted sequences that overlap with the more HLA-DR-promiscuous FV621 sequence been reported to be eluted from HLA DR [20,21].

In addition, previously described IgG-derived Tregitopes 167 and 289 that are located in the Fc domain and Tregitope 84 located in the kappa domain of IgG [1,8,10,22], were used as positive control peptides in the HLA binding and T reg assays described below.

2.2. Peptide synthesis

The peptides used in these studies were synthesized by 21st Century Biochemicals (Marlborough, MA). Molecular weight accuracy was verified by mass spectrometry and all peptides were determined to be more than 90% pure by HPLC. Amino acid analysis was performed on all peptide samples so as to normalize net peptide content across assays. A list of the peptides used in the studies is provided in Supplementary Table 1.

2.3. HLA Binding Assays

The six FV peptides were tested for *in vitro* binding to HLA-D1.B1*0101, *0301, *0401, *0701, *1101, *1301 and *1501 alleles. HLA binding assay results for IgG Fc derived Tregitope peptides have been previously reported [23], and were repeated for comparison with FV621. The HLA-DRB1 alleles selected for the HLA binding assays represent a wide array of class II alleles, known as “supertype alleles” that share similar binding peptide side-chain preferences for their binding pockets [24].

The HLA binding assay used for these studies was originally described by Steere et al [25], and has been standardized by Fr.Vax to validate *in silico* binding predictions. This assay has been described in greater detail in previous publications [25–27].

2.4. Human Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs from healthy donors were isolated from leukocyte reduction filters purchased from the Rhode Island Blood Center (RIBC) in Providence, RI. High resolution HLA class II haplotyping of donor PBMCs was performed at the Transplant Immunology Laboratory at Hartford Hospital in Hartford, CT. The HLA alleles possessed by individual donors included in this panel represented HLA-DR alleles covering 95% of the allele-specific MHC-binding preferences

reported for human populations [28,29]. HLA distribution and gender/age distribution of donors are provided in **Supplemental Table 2**.

For the T reg assays, PBMCs were thawed according standard procedure, labeled with carboxyfluorescein succinimidyl ester (CFSE), and rested overnight in 96 well culture plate prior to use in *in vitro* assays. To evaluate the dose response of FV621 on the viability, cells were incubated with FV621 for 2, 3, 5 and 7 days in culture and live cells evaluated by flow cytometry using a live-dead viability dye and by counting in a cellometer (Nexcelom) using trypan blue exclusion. All assays were performed in RPMI complete medium RPMI-1640 + GlutaMax (Life Technologies) containing 10mM HEPES buffer (Life Technologies), 2mM L-glutamine (Life Technologies), 50µg/ml Gentamicin (Life Technologies), 10% Human AB serum (Sigma), MEM Non-essential amino acids (Gibco) and 55µM β-mercaptoethanol (Gibco).

2.5. Tetanus Toxoid Bystander Suppression (T reg) Assay

To evaluate the regulatory potential of the established and predicted Tregitope peptides and peptide controls, we adapted a previously published Tetanus Toxoid Bystander Suppression Assay (TTBSA) that measures the inhibitory capacity of potential regulatory peptides on the recall response of human CD4 T cells to the tetanus toxoid (TT) antigen [30]. TT vaccination is a routine, nearly universal immunization, such that anonymous blood bank blood donor PBMCs can be used for *in vitro* assays that require TT-specific memory T cells.

The modified TTBSA is performed as follows: PBMCs were labeled with CFSE (eBioscience) cell proliferation dye (2.5 µM) and then plated at 3×10^5 cells per well on U-bottom 96-wells plates (Falcon) in RPMI complete media. Labeled cells are rested overnight at 37°C, 5% CO₂ and the following day, the cultures are stimulated with candidate Tregitope peptides or control

peptides. Test peptides were solubilized in a minimal amount of DMSO (<1%) and added to the culture medium at a range of concentrations (8, 16, 24, or 5, 10, 15, 20µg/mL). Tetanus toxoid (TT) (Astarte Biologics, cat no. 1002) at 0.5 µg/ml was then added to all wells including positive control wells without Tregitope peptides. The cells are cultured for 6 days, harvested at day 7 and stained for expression of cell surface and intracellular markers and analyzed by flow cytometry. In addition to testing the effect of FV621 on TT, a whole antigen, we also tested whether the immune response to CEFT peptides (obtained from Panatecs, PA-CEFT-001) would be modified by FV621. This CEFT suppression assay is performed using the same timeline and reagents as the TTBSA, by simply replacing TT with CEFT peptides at 0.5 µg/ml.

2.6. HSA-FV621 peptide conjugate in tetanus toxoid bystander suppression assay

As peptide drugs are known to have an extremely short half-life in humans, we explored whether human serum albumin (HSA)-conjugated FV621 could prolong the serum half-life of FV621 while also targeting the delivery of the peptide to APCs, with the aim of translating the conjugated product to *in vivo* pre-clinical studies. The conjugation process involves a maleimide linkage, a process that has been utilized for drug delivery in previous clinical trials [31,32]. This maleimide-based chemistry can be used to covalently link a payload (here, Tregitope peptide FV621) to HSA in a 1:1 stoichiometry. This concept evolved from our previous studies of Tregitope-Albumin fusions in T1D [31]. Maleimidopropionamido (MPA) forms a stable thiolester conjugate with the available free Cys34 in HSA. We used recombinant HSA (kindly provided by Albumedix, Nottingham UK) to conjugate FV621 and tested inhibitory capacity comparing to unconjugated FV621 in TTBSA.

2.7. CD8+ T cell Bystander Assay

To assess whether FV621 also suppressed CD8+ T cell recall responses, we co-incubated PBMCs from healthy donors cultured as described above (in the TTBSA) but substituted a mixture of class I peptides derived from Cytomegalovirus, Epstein-Barr virus and Influenza virus (CEF peptide pool, available from Mabtech, cat no. 3615-1), for Tetanus Toxoid. The cells were incubated with CEF alone or with the addition of FV621 peptide. In a comparable experiment performed with IgG Tregitopes instead of FV621 peptide [22], MHC class I-restricted CD8+ T cell responses were measured in the presence or absence of IgG Tregitopes after six days of incubation (and epitope-specific CD8+ T cell responses were suppressed). These types of assays serve to demonstrate that the effect of Tregitopes (IgG-derived or FV621) is not due to competition at the class II HLA DR peptide binding groove by similar-binding peptides.

2.8. Flow Cytometry and gating strategy

Cells were washed with 1XPBS + 5% fetal bovine serum (FBS) and stained with Live/Dead Violet fixable stain (Life Technologies) in 1XPBS (Gibco) at 4°C, washed and stained with surface receptor antibodies in 1XPBS + 5%FBS in the dark at 4°C. Cells were fixed with Fixation/Permeabilization Buffer (eBioscience) in the dark at room temperature for 30 min and stained for intracellular markers in Permeabilization Buffer (eBioscience) at 4°C. Stained cells were acquired and data collected on an Attune NxT Cytometer with three laser capacity (violet-405nm, blue-488nm, and red-637nm) (Life Technologies) and analyzed using FlowJo software (Treestar, Inc). For gating, after exclusion of doublets, lymphocytes were first identified by a low forward scatter (FSC) and low side scatter (SSC) gate. Dead cells were excluded by Live-Dead staining. The following antibodies were used in this assay: CD3 (OKT3)-PECy7, CD4 (OKT4)-

BV410, CD8 (SK1)-APC, CD25 (BC96)-AF700, CD127-PE from Biolegend. Granzyme B (GB11)-AF647, HLA-DR (L243)-FITC, FoxP3 (PCH101)-PECy5.5, CD11c-PE (3.9) and carboxyfluorescein (CFSE) were purchased from eBiosciences.

Proliferation of CD4⁺ T cells were evaluated by dilution of CFSE with the proliferating population identified as CFSE^{low}. The gating strategy for proliferation of CD4 T cells is illustrated in Supplementary Figure 1A. CD4⁺ T effector cells were identified by the expression of CD25^{hi} and FoxP3^{int} and CD4⁺ T regulatory cells were identified by the expression of CD127^{low}CD25^{hi} and FoxP3^{hi}. Antigen presenting cells (APCs) were identified by the expression of CD11c and HLA-DR. Additional information on gating strategies for cell surface markers (CD8 T cells, CD4 T cells, Tregs, APC) and proliferation are illustrated in the corresponding result sections.

2.9. OVA immunogenicity mouse model

To demonstrate the *in vivo* effect of FV621, we used a well-established OVA (ovalbumin) immunogenicity model established in C57BL/6 mice. Mice were sensitized with OVA emulsified in CFA and injected IM at day 0 and re-sensitized with OVA emulsified in CFA and injected IM at day 14 (50µg OVA/mouse). Mice in the Tregitope co-treatment arm received FV621 (25µg/mouse) emulsified in CFA and delivered with OVA at day 0 (only) to evaluate the inhibitory effect of FV621 on the immune response to OVA. Serum was collected at day 17 and anti-OVA antibody titers in serum were analyzed by sandwich ELISA. Briefly, a 96-well plate (Immulon 2HB, ThermoFisher) was coated with 100 µl of OVA (egg albumin (Sigma, cat# A5503) at 10mg/ml protein in PBS and incubated O/N at 4°C. After washing with 1XPBS-Tween, wells were blocked with ELISA blocking buffer (1XPBS-Tween-20 (0.05%) + 5%FBS for 1h at 4°C. A seven point serial dilution of anti-OVA Monoclonal IgG1 Ab (Chondrex, cat# 3008) was used to prepare the standard curve. Mouse serum samples were serially diluted and plated in the ELISA wells to determine the concentration of anti-OVA antibody.

In addition, spleens from OVA and OVA+Tregitope-treated mice were collected at day 17 and spleen mononuclear cells were prepared by dissection and straining. Red blood cells were lysed with

1X RBC lysis buffer (ThermoFisher) and cellular IFN γ production in response to OVA stimulation (100 μ l/well) in vitro was measured by ELISpot assay using Mabtech kit (3321-4APW-10). Spleen cells were plated in triplicate in IFN γ pre-coated Mabtech plates and stimulated with OVA (100 μ g/ml) for O/N at 37 $^{\circ}$ C; IFN γ production was detected by fluorescence-conjugated detection reagent. IFN γ -producing cell spots were counted using an automated ELISPOT reader system from Zeiss at Zellnet Consulting Inc (NJ).

2.10. RNA extraction and gene expression analysis

Samples containing PBMCs stimulated with either CD3/CD28 or FV621 for 6 days and total RNA were prepared according to the manufacturer's protocol (RNeasy Mini kit, Qiagen # 74104). RNA samples were labeled using the Affymetrix WTA Plus kit according to manufacturer's guidelines, and probed using the Clariom S Human Array. Raw data generated from Clariom S Arrays were processed using Affymetrix Expression Console Software. CEL files containing feature intensity values were converted into summarized expression values using by Robust Multi-array Average (RMA) which consists of background adjustment, quantile normalization and summarization across all chips. All samples passed QC thresholds for hybridization, labeling and the expression of housekeeping gene controls.

2.11. Statistical analysis

Statistical analysis was performed using Prism software (GraphPad version 8.3). The Student's *t*-test (unless otherwise indicated, unpaired, two-tailed) was used to compare the significance of differences between TT stimulated cells to Tregitope treated cells or the indicated experimental groups. Differences were considered significant when $p < 0.05$ (*), very significant when $p < 0.01$ (**), highly significant when $p < 0.0002$ (***), and extremely significant when $p < 0.0001$ (****). The ordinal test (one-way repeated) ANOVA was used for

gene expression analysis. This gene array featured 1,458 genes that showed contrasting gene expression in cells incubated with FV621 or CD3+CD28 stimulation.

3. Results

3.1. FV621 binds with high affinity to multiple HLA class II HLA DR alleles

The major histocompatibility complex proteins (MHC, also known as HLA in humans) play a critical role in the development of an effective immune response by activating regulatory T cells to prevent or diminish immune responses as they bind to and present processed peptide antigens on the surface of antigen presenting cells to diverse T cell populations. In investigating potential tolerogenic peptides in FV, a high abundance and highly conserved human protein, we used EpiMatrix to identify peptides likely to bind to multiple human class II HLA DRB1 molecules and evaluated them in an *in vitro* binding assay. We found that six FV peptides which had been selected for promiscuous binding affinity to HLA DR showed moderate to strong binding to the panel of HLA alleles evaluated in this assay, although FV548, FV582 and FV1737 were somewhat more restricted in the breadth of binding to the full range of HLA DR alleles (**Fig. 2A**). Both FV621 and FV432 demonstrated binding to nearly all of the HLA DRB1 alleles tested (HLA DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501) with strong affinity as reflected by assay results showing very low half-maximal inhibitory concentration (IC₅₀) values, calculated from the dose-response curve (**Fig. 2A**).

The *in vitro* HLA binding assay was repeated so as to compare, in the same assay, the binding affinity of FV621 to IgG-derived Tregitopes 289, 167 and 84. As shown in **Fig. 2B**, human Tregitope 289 binds with strong affinity across multiple HLA alleles [1,23], while Tregitope 167 binds with moderate affinity to HLA DRB1*0301, *0401, *0701 and with strong affinity to HLA

DRB1*0101 and *1501. Tregitope 84 (a Tregitope found in the Fab domain of IgG [7]) binds with strong affinity to HLA DRB1*0101, *0401, *0701 and DRB1*1501, and with moderate affinity to HLA-DRB1*1101. Thus, as predicted, the six FV peptides including FV621 showed moderate to high and promiscuous binding affinity that is very similar to established IgG Tregitopes. High-binding Tregitope 289 was identified as the most appropriate “positive control” comparator for FV621 for subsequent *in vitro* evaluations using human PBMC. FV621 peptides featuring single amino acid mutations at the HLA-binding P1 and TCR-facing P5 were also synthesized and tested in binding assays for correlation with *in vitro* results in the TTBSA.

3.2. Immunomodulatory effect of Factor V-derived peptides on the CD4+ Tetanus Toxoid recall response

Stimulating PBMCs from healthy donors with TT generates a robust memory CD4+ T cell recall response to TT, as demonstrated by the upregulation of several activation markers on the cell surface by flow cytometry. In Supplementary Fig. 1A we show the gating strategy of CD4 T cell proliferation and in addition, we show that TT consistently stimulated CD4+ T cells by 5-60% in different donors (**Supplementary Fig. 1B**). The variation of TT response in healthy donors is likely due to the TT vaccination history of the donors as well as to the distribution of TT-specific precursor memory CD4+ T cells in the PBMC sample thawed for each of the TTBSA assays.

Using the Tetanus Toxoid Bystander assay (TTBSA), we evaluated the six selected FV peptides for their capacity to inhibit the T effector memory response to TT in PBMCs derived from a panel of donors (**Fig. 3**). FV peptides which demonstrated moderate to strong binding affinity to two or more HLA DRB1 alleles were selected for additional testing *in vitro* and

compared with three previously identified Tregitope peptides (Tregitope 289, 167 and 84) in the TTBSA (**Fig. 4**).

As shown for one representative donor in **Fig. 3A (left)**, Tetanus Toxoid stimulation increased CD4⁺ T cell proliferation by roughly 60-fold over control in a CFSE dilution assay. The histogram in Figure 3A, right side, shows the degree of inhibition of CD4⁺ T cell proliferation by FV621. The addition of FV621 significantly suppressed proliferation of CD4⁺ T cells to TT in a dose-dependent manner by as much as 80% at the highest dose (200 µg/ml) of FV621. This observation was repeated for multiple donors (**Fig. 3**).

For comparison, five other FV peptides (FV432, FV548, FV582, FV1737 and FV1802) were also tested for their ability to inhibit TT-specific immune-response in the TT-bystander suppression assay using PBMC from three HLA-diverse donors. Despite binding nearly as well as FV621, as shown in **Fig. 3B**, the five other FV peptides did not inhibit the CD4⁺ T memory response in the TTBSA as much as was observed for FV621 for the same donors. In particular, despite having similar binding affinity across multiple HLA DR alleles as FV621 (**Fig. 2**), FV432 failed to significantly inhibit CD4⁺ T cell proliferation in the TTBSA to the same extent as FV621. We also examined the effect of FV621 and other FV peptides (FV432, FV548, FV582, FV1737 and FV1802) on CD4⁺ T cell proliferation or the CD4⁺ T cell activation markers CD69 and HLA DR on CD4⁺ T cells. Of the six FV peptides, only FV621 inhibited T effector proliferation and modified CD69 and HLA DR expression (data not shown).

3.3. Effect of FV621 delivery in HSA-bioconjugate in TTBSA

Translation of Tregitope peptides such as FV621 to potential clinical use will necessitate establishing optimal pharmacokinetics, prolonging the half-life of the peptide and enhancing its

stability under physiological conditions. Albumin prolongs the half-life of conjugated payloads by binding to the neonatal Fc receptor (FcRn) and recirculating. Albumin is also avidly taken up by antigen-presenting cells, potentially decreasing the need for more frequent or repeat dosing of Tregitopes in human therapy. We recently demonstrated that a human serum albumin (HSA)- Tregitope (IgG Tregitopes 084+167) fusion protein administered with Pre-pro-insulin (PPI) peptides promoted antigen-specific tolerance in Non Obese Diabetic (NOD) mice [7]. The HSA-Tregitope fusion, when administered with the target PPI peptide in vivo, significantly diminished blood glucose levels, as compared to HSA-Tregitope fusion without PPI, demonstrating improved glucose control in overtly diabetic mice, over 49 days after initiating treatment [7]. However, Tregitope peptides fused to albumin were subsequently shown to be cleaved in yeast expression systems, making large scale production of this fusion product unlikely.

We therefore evaluated the maleimide linkage method [31],[32] of attaching the FV621 Tregitope to albumin, and evaluated whether a FV621-linked HSA bioconjugate would perform well in the TTBSA, as compared to free FV621 peptide. As expected, FV621 peptide alone (no conjugation) inhibited CD4+ T cell proliferation in a dose-dependent manner, while FV621 conjugated with HSA also significantly inhibited CD4+ T cell proliferation in vitro but at tenfold lower doses than that of total Tregitope FV621 in the peptide-only treatment wells. The amount of FV621 delivered by the FV621-HSA-conjugate was 0.25, 1.0, 2.5 $\mu\text{g/ml}$ (for 10, 40 and 100 $\mu\text{g/ml}$ of HSA-conjugate, respectively), which is 10-30 fold lower than that represented in the free peptide treatment wells (8, 16, 24 $\mu\text{g/ml}$). In comparison, HSA alone (not conjugated

to FV621) did not have any impact on the TT mediated CD4+ T cell proliferation in the TTBSA (Fig. 3C).

The greater efficacy of the FV621-HSA bioconjugate on suppression of T effector memory cells *in vitro* may be related to the increased half-life of the conjugates or to increased efficiency of Tregitope presentation by antigen presenting cells. These data further suggest that HSA-bioconjugation using the maleimide linkage is worth exploring as a flexible delivery vehicle for FV621 alone, or in combination with target antigens, for inducing tolerance *in vivo*.

3.4. Effect of FV621 on immune response to OVA *in vivo*

We have previously reported that Tregitopes derived from IgG were effective in a wide range of *in vivo* autoimmune disease models. Prior to performing more intensive autoimmune model studies, we decided to evaluate the effect of FV621 treatment in a simple *in vivo* model involving OVA immunization of C57Bl/6 mice. C57Bl/6 mice were immunized with OVA emulsified in Complete and Incomplete Freund's Adjuvant (CFA/IFA) on days zero and 14. In the treatment arm, FV621 was added to the OVA in CFA at day zero. In the control arm, saline was added to the OVA/CFA. Titers of anti-OVA antibodies were measured in serum of treated mice and compared to untreated mice. OVA-induced IFN γ production by ELISpot was also measured in T cells obtained from mouse spleens at day 17. As compared to mice given a sham (saline) treatment with OVA, co-administration of FV621 peptide administered with OVA antigen inhibited anti-OVA antibody titers in serum and also suppressed IFN γ production in spleen cells (Fig. 3D).

3.5. FV621 peptide inhibits CD4⁺ T cell proliferation in TT responsive donors to a degree that is similar or greater than IgG Tregitope

We then asked whether there was a dose response effect of FV621 in suppression in the TTBSA and whether FV621 could inhibit very high level responders to TT. We therefore performed a dose-response assessment of FV621 and IgG Tregitopes and compared their inhibition of TT induced memory CD4⁺ T effector responses (Fig. 4). Tregitope 289 (Fig. 4A), Tregitope 84 (Fig. 4B) and Tregitope 167 (Fig. 4C) significantly inhibited the TT induced memory response to CD4 T cell proliferation. Comparing the effect of Tregitopes 289, 167, 84 with FV621 (Fig. 4D) in donors that respond to TT at the 5-20% level of proliferation, we found that

the inhibition by FV621 Tregitope is similar to the inhibition by Tregitopes 289, 167 and 84. For this comparison across donors, data representing the maximum (%) of CD4+ T cell proliferation in response to TT was normalized to 100% of the total; all other conditions resulted in a reduction of the TT response by as much as 80%.

To better elucidate the inhibitory capacity of FV621 as compared to Tregitope 289 we evaluated their suppressive effect in robust TT responsive donors. (30-40% CD4+ T cell proliferation in response to TT), in the TTBSA. We found that the FV621 Tregitope is better able to inhibit CD4+ T cell proliferation compared to Tregitopes 289 in high TT responsive donors with significant reduction in proliferation at the highest dose tested (20.0µg/ml) compared to Tregitope 289 (**Fig. 4E**). Thus, while exhibiting similar HLA binding affinity, FV621 more potently suppresses robust TT-induced CD4+ T memory cell proliferation as compared to Tregitope 289. Variation in Tregitope-specific Treg precursor frequencies and/or alternative modes of action by which FV621 Tregitopes suppress T effector cells may explain the observed differences in inhibitory capacity by the recruited Tregs.

The strong suppressive effect of FV621 was confirmed in TTBSA performed using PBMCs from 20 healthy donors at *in vitro* Tregitope dosing levels of 15.0µg/ml and 20.0µg/ml (**Supplementary Fig. 1B**). FV621 also inhibited TT induced CD4+ T memory responses across a broad spectrum of donor HLA types (**Supplementary Table 1**) suggesting that FV621 has the potential to suppress effector T cell responses across population groups.

Next, we evaluated whether a modification of the TCR facing amino acid residue within the predicted HLA-binding domain of FV621 would impact the suppressive effect of FV621. A point mutation at position G628H (glycine changed to histidine) significantly diminished the

suppressive capacity of FV621 on TT-induced memory CD4 T cell proliferation in the TTBSA (Supplementary Fig. 2). The HLA binding capacity of this peptide (G628H) was identical to FV621, suggesting that the impact of the modification is limited to TCR recognition by CD4 T cells. In contrast, a peptide containing a single modification to the HLA-facing amino acids (I624A) bound with slightly lower affinity to HLA DR as expected, also shown in Supplementary Fig 2C., but this lower binding affinity did not diminish the suppressive effect of FV621.

3.6. FV621 peptide inhibits CD4+ T effector cell activation and induces CD4+ T regulatory cell proliferation

To further characterize the inhibitory capacity of FV621 on the CD4+ T effector cell populations and investigate its impact on T regulatory cells, we evaluated the effect of FV621 in culture with PBMC from donors with diverse HLA DR haplotypes. CFSE labeled PBMCs from healthy donors were stimulated with TT in the presence or absence of FV621 for 6 days and the proliferation of T effector and regulatory cells was assessed. T regulatory cells were identified by the expression of CD127^{low}, CD25^{hi} and FoxP3^{hi} (FoxP3 is a transcription factor and major regulator of Treg development but is also transiently expressed in activated T effector cells) [33] while CD4+ T effector cells were identified as CD25^{hi}FoxP3^{int} in the CD4+ gated population. As shown for a single representative donor **Fig. 5A** (lower panel), proliferation of CD4+CD25^{hi}FoxP3^{int} T effector cells was detected in the presence of TT alone, while co-treatment of the cultures with increasing concentrations of FV621 significantly inhibited the percentage of activated CD4+ T effector cells. Reciprocally, we observed a dose-dependent increase in percentage of Tregs in cultures treated with FV621 plus TT (**Fig. 5B**).

The observed increase in the absolute number of Tregs with increasing dose levels of FV621 may be due to (1) conversion of TT-specific T effectors to adaptive or iTregs (likely); (2) elimination of TT-specific T effectors from the mixed population of cells (possible); or (3) proliferation of natural Tregs (unlikely). We hypothesize that FV621 treatment may be increasing the proportion of antigen-specific T regulatory cells by decreasing the TT-specific T effector population as indicated in the same TTBSA assay (Fig. 5); this is supported by our granzyme B studies (see below). Alternatively, the shift in Treg to T effector ratios may be due to conversion of T effectors to adaptive or iTregs. This hypothesis is supported by previous studies that showed that Tregitope treatment of human PLMCs *in vitro* converted tetramer-stained Birch Pollen specific T effector cells to adaptive or iTregs [1,22]. Conversion of antigen-specific T effector cells to antigen-specific Tregs has also been documented in D011.10 mice treated with Tregitopes 167 and 289 [21].

Regardless of the mechanism, we note that the ratio of activated T regulatory cells to T effector cells has been described as a determining factor in the maintenance of tolerance and in the potential for treatment of allergic and autoimmune diseases [34,35]. FV621 treatment in the presence of TT shifts the balance of T effector cells and T regulatory cells by increasing the overall ratio of Treg to Teff cells (**Supplementary Fig. 3**).

3.7. Inhibition of CD8+ T cell response by FV621

Another potential mechanism by which FV621 activated Tregs could mediate an inhibitory effect on T eff cells is by secretion of suppressor cytokines and/or modulation of cell surface inhibitory receptors on dendritic cells, and which could explain the effect on both activated CD4+ and CD8+ T cells via inhibition arising from APCs [36]. CD8+ T effector memory cells can

be induced to proliferate in response to engagement with peptide-loaded MHC class I molecules wherein the peptides are derived from antigens to which most of the population have been previously exposed, either through vaccination or by natural infection. As shown in **Fig. 6A**, where FV621 was incubated with class I restricted (CEF) T cell epitopes, FV621 strongly inhibited memory CD8⁺ T cell activation (CD25^{hi}) and proliferation (CFSE^{low}) in response to CEF stimulation of PBMCs in a dose-dependent manner (panels **A** and **B**) but CD4⁺ T cells were not significantly stimulated by CEF peptides (**Fig. 6C**). This suggests that the inhibitory effect of FV621 on T cells is not due to competition for HLA binding on the APC (binding of Tregitope FV621 to MHC class II cannot interfere with CEF peptide binding to MHC Class I, and that Tregitopes can modulate both CD4⁺ and CD8⁺ effector T cell responses. The independent effect of IgG Tregitopes on Class I-restricted T cell responses was also previously observed using individual MHC Class I-restricted, (AAV, EBV and CMV) virus-derived class I epitopes [11]. This effect appeared to be due to direct contact between Tregs and CD8⁺ T cells, as suggested by the observation that IgG Tregitope-stimulated PBMCs were not suppressive of CD8 T cell responses when separated from the CD8⁺ T cells in transwell plates [22].

3.8. FV621 modulation of T cell response following non-specific T cell activation by CD3/CD28

To further evaluate whether FV621 is broadly suppressive and corroborate the previous observation, we also performed an *in vitro* polyclonal T cell activation assay using healthy donor PBMCs. Cells were labelled with CFSE, cultured in complete media in a 96 well round bottom plate overnight and stimulated with anti-human CD3/CD28 dynabeads at a cell to bead ratio of 5:1 for 6 days alone or in combination with FV621. After six days of incubation, cells were

stained with surface and intracellular markers and analyzed by flow cytometry. FV621 inhibited CD4⁺ T cell proliferation similar to its effects in the TTBSA (**Supplementary Fig. 4**) following polyclonal CD4⁺ T cell stimulation by the CD3/CD28 dynabeads, supporting our previous observation, that the effect of FV621 is not due to binding competition with antigen at the MHC-T cell interface.

We have also tested the relative effect of Tregitope FV621 on T cell proliferation to multiple immune stimulatory CD4 epitopes (CEFT, a pool of 23 peptides used as MHC Class II-restricted controls in T cell assays) using an *in vitro* bystander suppression assay. We demonstrated that CEFT stimulates CD4 T cells and co-incubation with FV621 significantly inhibit proliferation of CD4 T cells in a dose dependent manner (**Supplementary Fig. 5**), confirming that FV621 is immunomodulatory, whether added to peptide or to whole antigen, *in vitro*.

3.9. FV621 stimulation downregulates antigen presenting cell surface markers

We then investigated whether FV621 and IgG Tregitopes may modulate T cell responses (both CD8 and CD4) by modulating antigen presenting cells (APCs) from an immunostimulatory to a tolerogenic phenotype (also known as ToIDC) [37]. The expression of HLA-DR, CD80 and CD86 by APCs critically contributes to the activation of antigen specific Treg and T effector cells. The dendritic cell surface markers CD11c, HLA DR, CD80 and CD86 have been shown to be down-modulated by known immune suppressive agents including IVIG [38] and other Tregitope-like peptides [5].

We therefore compared the effect of incubating FV621 and Tregitope 167 with PBMCs on the expression of CD11c and HLA-DR (**Fig. 7**) *in vitro*. Healthy donor PBMCs were incubated with the immunostimulatory Influenza hemagglutinin HA₃₀₆₋₃₁₈ (negative control), Tregitope 167

(positive control) and FV-derived peptides, and cells were stained for the surface expression of HLA-DR on day 4. Compared to stimulation with HA₃₀₆₋₃₁₈ alone, both Treg167 and FV621 stimulation induced significant downregulation of HLA-DR (**Fig. 7A**). We also compared the mean fluorescence intensity (MFI) of HLA-DR in CD11c⁺ cells (**Fig. 7B**) and percent changes in MFI over media alone stimulation (**Fig. 7C**). Comparing both MFI and percent changes to the expression of HLA DR demonstrated that FV621 was more efficient than Tregitope 167 and the other FV peptides at reducing HLA DR expression on antigen presenting cells. FV621 stimulation also downmodulated CD86 expression in CD11c⁺ cells compared to HA stimulation (data not shown). A recent observation that Tregs “steal” HLA DR from dendritic cells [39] suggests that this may be one mechanism by which CD11c dendritic cells lose their HLA DR when co-cultured with FV621-activated Treg cells.

3.10. *Effect of FV621 peptide on granzyme B expression in regulatory T cells upon activation*

T regulatory cells have been shown to upregulate the expression of granzyme-B and perforin, which can lead to cytotoxicity of T effector cells and induce immune suppression by this means [40,41]. We thus evaluated the expression of granzyme-B in T regulatory cells after stimulating with TT in the presence or absence of FV621. We gated on highly activated Tregs (CD25^{hi}FoxP3^{hi}) in the granzyme-B expressing cell population (**Fig. 8A**). FV621 treatment increased the (%) frequency of granzyme-B⁺ activated T regulatory cells and decreased activated T effector cells in a dose-dependent manner (**Fig. 8B**). Thus, granzyme B release [42] may be one mechanism by which FV621-responding Tregs regulate CD4⁺T effector function.

3.11. FV621 stimulation shows differential gene expression profile as compared to conventional T cell stimulation

To understand the mechanism of action of FV621 treatment in suppressing T effector memory T cell response, we analyzed PBMC gene expression analysis by Affymetrix Clariom S assays. The heatmap in Supplementary Fig. 6 shows that the effect of FV621 on cultured PBMC has a differential effect on expressed genes, as compared to conventional stimulation with CD3/CD28. Comparing with selected gene clusters between inhibitory FV621 and activating CD3/CD28 stimulation, we found that FV621 stimulation differentially upregulates cluster 1 genes and down-regulates cluster 2 genes.

3.12. Effect of FV621 on cell viability *in vitro*

To rule out the possibility that the effect of FV621 was related to toxic effects of the peptide, which might simulate a suppressive effect *in vitro*, we evaluated the viability of cells in culture with FV621 using two different methods. Cells were cultured in the presence of FV621 in a 96 well plate for 2, 3, 5 and 7 days after which cells were collected and stained with live-dead viability dye and analyzed by flow cytometry (**Supplementary Fig. 7**) and by trypan blue exclusion and counting with a cellometer (Nexcelom). The absolute number of live cells that was counted at days 2, 3, 5 and 7 after stimulation with different doses of FV621 Tregitope was similar by flow cytometry. Although we observed a slight (10-15 %) decreased viability at the highest dose of FV621 peptides by trypan blue exclusion, this may have been due to assay variability in the measurement of live cells or active killing of bystanders by activated Tregs (**Supplementary Fig. 7**).

4. Discussion

T regulatory cells constitute a critically important population of cells that limit the immune response against self and non-self-peptide antigens and are necessary for the induction and maintenance of peripheral tolerance. Here we define a new Factor V-derived regulatory T cell epitope that activates epitope-specific regulatory T cells as measured by *in vitro* and *in vivo* assays.

We suggest that T reg cells responding to FV621 are epitope specific. Antigen-specific regulatory T cells have been described in the literature (see for example, Shevach EM, et al., reference [4]). Moreover, natural Tregs that are activated by antigen-specific epitopes may be critical to the development of tolerance to enzyme replacement therapeutics in patients with partial enzyme deficiency disease [43]. Tregitopes, otherwise known as the cognate epitopes of circulating IgG-specific Tregs, were initially discovered in 2008 [1] and shown to suppress inflammatory responses to co-administered antigens *in vitro* and *in vivo* [7,8].

FV 621 Tregitope may act on Tregs in conjunction with target antigens to drive antigen-specific tolerance [22]. This was exemplified by *in vivo* studies of IgG Tregitopes in NOD mice, in which we have shown that suppression of diabetes was more effective when Tregitope treatment was combined with pre-pro-insulin (PPI) peptides [7]. In a prior publication, with David W. Scott, we demonstrated that Tregitope treatment converts Teffector cells to antigen-specific induced Treg cells (iTreg) in D011.10 mice [7]. More recently, collaborators Bruce Mazer and Marieme Dembele have shown that treatment with IgG Tregitopes and allergens resulted in the development of allergen-specific tolerance (as demonstrated by transfer of Tregs to allergic recipients) [12]. Thus we hypothesize that the role of natural regulatory T cell epitopes

such as FV621, described here, and IgG Tregitopes, is to activate natural Tregs that induce tolerance by converting activated T effectors to iTreg indirectly (through antigen presenting cells) or directly, as suggested by AAV studies carried out by Federico Mingozi and Danny Hui [22].

The purpose of the present work was to explore the hypothesis that Tregitopes are present in prevalent human serum proteins other than IgG and can contribute to tolerance induction by processing of an endogenous protein. Here we demonstrate that some, but not all, promiscuous HLA DR-binding T cell epitopes identified in the Factor V protein with properties similar to known Tregitopes, were able to down-modulate the memory T effector response. Only one human FV-derived peptide (FV621) was found to consistently inhibit *in vitro* CD4+ and CD8+ T cell effector responses in donors across the span of nine major HLA DRB1 supertypes representative of more than 95% of the general population, whereas others were not found to have the same inhibitory properties. FV621 was a very strong binder to multiple class II HLA DR alleles, different from the other FV peptides with the notable exception of FV432, which was used as a control in the assays, due to its promiscuous binding. It is intriguing to note that both FV621 and FV432 bound across nine HLA DR supertype alleles and that the TCR facing residues of these peptides share homology with sequences in Factor VIII, and/or with hephaestin and ceruloplasmin, prevalent serum proteins that are involved in copper and iron transport [44]. FV621 has also been eluted from HLA class II molecules [20,21] thus processing and presentation of the relevant peptide may be an additional determinant of efficacy to explore when searching for Tregitopes.

In comparative studies investigating the inhibitory effect of previously identified and validated Tregitopes, FV-mediated inhibition of tetanus toxoid specific effector T cell responses is similar to, or stronger than, that of IgG-derived Tregitopes 289, 167 and 84.

The mechanisms by which Tregitopes, such as the FV621 Tregitope described here, cause Tregs to exert their immune suppressive activity have been described in several publications: IgG-derived Tregitopes 167 and 289 induced the expression of IL-10 in FoxP3+ T regulatory cells *in vitro* following culture of PBMCs after stimulation with Tregitopes [45]. Down-modulation of HLA DR expression on antigen presenting cells may lead to indirect effects on activation of CD4 and CD8 T cells, as previously suggested [45].

Other mechanisms by which Tregitopes such as FV621 may exert immunosuppressive activity could include, but are not limited to the following: i) production of immunosuppressive cytokines, e.g., TGF β , IL-10 and IL-35, ii) upregulation of effector T cell specific transcription factors important for the expression of CXCR3 and survival of Tregs [46](e.g. T-bet), iii) competing with effector T cells for the growth factor IL-2 by sustained expression of the IL-2Ra subunit, CD25, iv) inducing cytotoxicity of T effector cells by producing perforin and/or granzymes such as granzyme B, and v) modulating dendritic cell maturation and function [47].

We explored several alternative mechanisms for the effect of FV621. Competition for HLA binding was excluded by the demonstration that FV621 was also able to suppress CD8+ T cell responses to class I-restricted T cell epitopes; theoretically the peptide cannot 'compete' for binding on a separate (MHC class I) molecule. Stronger proof that the suppression is not due to competition was further demonstrated by suppression of T effector cell activation by non-antigen specific T cell stimulation in an assay using anti-CD3/CD28 antibody dynabeads. Moreover, FV621

was also shown to modify DC expression of cell surface markers of immunogenic DCs including HLA DR expression, an effect that is not due to MHC-competition.

We also demonstrate that FV621 increases the proportion Tregs under culture conditions. This may be due to activation of Tregs, which alter the phenotype of nearby T effectors to induce them to become iTregs, through the direct expression of cytokines, or reduction of the number of T effector cells through cytotoxicity via the release of granzyme B from T regulatory cells. We note that additional slight decreases of Treg frequency at the highest concentration of FV621 treatment *in vitro* may be due to Granzyme B-mediated killing of Treg cells [48]. Further studies are needed to investigate other potential mechanisms which may be of importance *in vitro* and *in vivo*.

Even though the original hypothesis leading to the discovery of FV621 was that FV might modulate immune response to FVIII replacement therapy, we have not yet explored the potential for FV621 to induce tolerance to FVIII. While we plan to explore the potential role of FV621 in tolerance to Hemophilia A, we also suggest that the FV621 peptide may be useful for inducing tolerance in a wide variety of immunogenic antigens and in autoimmune diseases. For example, we have tested whether the delivery of IgG Tregitopes have been shown to modulate allergic responses to allergens in a mouse model of allergy [12] and, IgG Tregitopes fused to albumin and administered with antigenic insulin peptides to NOD mouse significantly reduced the development of diabetes [7]. Thus, given the observation that antigen-specific tolerance can be triggered by HLA specific presentation of FV621 or other Tregitopes in conjunction with multiple antigens, there is no compelling reason to limit therapy with Tregitopes to a specific antigenic protein. Therefore, FV621 Tregitope linked to albumin, or integrated into a gene-

therapy vector or transgene may be an attractive solution to the problem of immunogenicity in gene therapy and transplantation.

Although fusing Tregitopes to albumin represents a simple and attractive means of delivering Tregitope peptides, we have been interested in developing flexible platforms for Tregitope delivery, by which multiple Tregitopes and various target antigens can be delivered *in vivo*, together. In preparation for translation to *in vivo* studies, we prepared a FV621 conjugate using maleimide-linkage technology and tested it in our *in vitro* TTBSA for the inhibitory effect on TT induced memory CD4 T cell proliferation. Delivery of the FV621-HSA-conjugate *in vitro* was more effective compared to FV621 free peptides, in the TTBSA, supporting the hypothesis that HSA-conjugates may be a suitable delivery method for translating the effect of Tregitopes into human clinical trials. HSA is also an FDA-approved excipient and drug delivery method. Future studies will define thresholds (how many Tregitopes will be needed to induce tolerance to single effector T cell epitope) and address the feasibility of FV621-HSA bioconjugate delivery in a mouse model of allergy and autoimmune disease.

These studies extend from our first discovery of IgG Tregitopes in 2008. Beyond their importance in tolerance to self, we note that Tregitopes are also conserved across species, and homologs to promiscuous human Treg epitopes are found in bacterial, viral, and parasitic pathogens. JanusMatrix and a version of the TTBSA have also been used to identify Tregitopes in surface proteins of pathogens [49,50] and engineering these pathogen “Tregitopes” or human-like epitopes out of the sequence of the antigen improves immune responses, specifically B cell response to the target pathogen antigen [51]. This observation has now been

followed by new immune-engineering studies to improve the immune responses to surface proteins of influenza [52], HIV envelope, and SARS-CoV-2.

We describe one novel Tregitope here, however many more non-IgG Tregitopes remain to be discovered in humans and in human pathogens. We have provided the principal steps used to define Tregitopes and new methods (JanusMatrix, the TTBSA) for finding Tregitopes in this publication. The assays described here and used for validation of the effects of FV621, may assist with their identification. Defining and validating endogenous Tregitopes may be especially critical to understanding immune responses to biologics and to replacement therapies, as well as to the discovery of new biological interventions that will improve human and animal health.

The new regulatory peptide “Tregitope FV621” is derived from the prevalent serum protein, coagulation factor V (FV). We have shown that the novel FV621 peptide binds across multiple HLA-DRB1 and significantly suppresses rhesus Toxoid-induced CD4+ and CD8+ T memory cell responses in an *in vitro* bystander suppression assay. FV621 appears to expand T regulatory cells in some subjects, and upregulates granzyme B expression while downmodulating HLA-DR expression in APCs. Similar to the previously defined IgG Tregitopes, the novel FV Tregitope also has immunomodulatory effects *in vivo*. Delivering FV621 in HSA-bioconjugates enhances its suppressive ability *in vitro*, indicating that the bioconjugation strategy could be suitable delivery method for the translation of a FV621-Tregitope therapeutic into human clinical trials.

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Contributions

ADG and WDM conceived of the original Tregitope hypothesis and proposed that Tregitopes were present in other highly prevalent proteins in the human genome; AR identified FV as a potential source of Tregitopes and WDM and FT selected the new FV Tregitopes by comparing the sequences of FV and FV¹¹¹ using EpiMatrix and JanusMatrix. ADG, WDM and AR contributed to the design, planning, evaluation and interpretation of the assays described here. BR, SM, SL, GS developed the detailed plans for the laboratory assays with assistance from EG and DK (acknowledged above), performed and /or interpreted the results of the assays and collated the results. All of the manuscript authors contributed to the synthesis of the results for publication, participated in the writing of the manuscript and have approved the manuscript for submission.

Conflict of Interest

ADG and WDM are senior officers and shareholders of, SM, SL and BR are employees of, and GS is a consultant for EpiVax, Inc., a privately-owned biotechnology company located in Providence, RI. AR and WDM are co-authors of an intellectual property filings related to FV621, which is jointly held by the FDA and EpiVax. All of these authors acknowledge that there is a potential conflict of interest related to their employment status and/or ownership relationship with EpiVax and attest that the work contained in this research report is free of any bias that might be associated with the commercial goals of the company.

Supplementary data Supplementary material

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Fig. 1. Engagement and activation of nTregs by Tregitope presentation via APCs. Tregitopes were initially found in IgG and may also be present in other prevalent human proteins. We hypothesize that their role is maybe to recruit naturally circulating Tregs that suppress the immune response to nearby T effector epitopes (green) found in the variable CDR region of IgG providing natural adaptive tolerance induction to the CDR. As shown here, conjugating Tregitopes (blue peptide) and their target antigens (green peptide) to carrier proteins such as albumin enables their delivery to antigen-presenting cells while improving half-life *in vivo*.

Fig. 2: HLA DRB1 binding of candidate FV peptides and comparing with IgG Tregitopes.

A) Selected Factor V peptides were evaluated for their ability to bind HLA DRB1 *in vitro* and IC₅₀ values were calculated. FV432, FV621 and FV1802 peptides bind to multiple HLA DRB1 alleles (DRB1*0101, *0301, *0401, *0701, *1101, *1301 and *1501) whereas FV548, FV582 and FV1737 peptides had more limited binding to HLA DR. A seven-point competition assay using a validated control peptide was performed; the color coding reflects the binding affinity, IC₅₀ were determined by interpolation. FV621 binds with strong affinity to binder all HLA DR alleles tested; variation in binding affinity varied less than 10-20 % in repeat assays. **B) Comparison of FV621 to previously published Tregitope peptides (084, 167, and 289) for binding to HLA DRB1 alleles.** Binding assays were performed as described above for FV peptides. Tregitopes 167 and 289 are Fc-derived: 84 is Fab-derived.

Fig. 3: Evaluating the FV peptides and HSA-FV621 for inhibition of CD4⁺ T cell recall response in the Tetanus Toxoid Bystander Suppression Assay (TTBSA). PBMCs from healthy donors were stimulated with 0.5 µg/ml of TT and increasing concentrations of FV peptides (5, 10, 15 and 20µg/ml). Proliferation of CD4⁺ T cells was measured six days post-stimulation by flow cytometry by CFSE dilution. **A)** Representative flow and histogram plots indicate a dose dependent effect on the proliferation of CD4⁺ T cells with increasing concentrations of FV621. **B)** Graphs showing the percent inhibition of CD4⁺ T cell proliferation for each of the FV-derived peptides compared to TT stimulation alone for donors 157, 135 and 143. FV621 (dark blue line) demonstrated consistent suppression of the TT response across donors as compared to the

other FV peptides (light blue lines). **C)** Inhibition of the CD4⁺ T cell recall response by HSA-conjugates in TTBSA. PBMCs from healthy donors were stimulated with 0.5 µg/ml of TT with or without FV621 or HSA-FV621 and analyzed at six days post-stimulation by flow cytometry for inhibition of CD4⁺ T cell proliferation. Data shown represents the average 3 donors. P values *** = 0.0002 and **** = <0.0001 represents the statistical significance between TT and FV621 stimulation using a two-tailed t test. **D)** FV621 Treatment inhibited OVA-induced immune response in vivo. C57BL/6 mice were immunized with OVA in CFA at day 0 and day 14 and treated with FV621 in CFA at day 0. Serum and splenocytes were collected at day 21 and anti-OVA antibody titers in serum were measured by ELISA (left) and IFN γ production by splenocytes was measured in a FluoroSpot assay (right).

Fig. 4: Comparison of the inhibition of CD4⁺ T cell recall response by the FV621 peptide with previously validated IgG derived Tregitopes 289, 84 and 167 in the TTBSA. PBMCs from healthy donors were stimulated with 0.5 µg/ml of TT with or without the addition of the indicated concentrations of FV621 peptide or Tregitope 289, 84 or 167 and analyzed at six days post-stimulation by flow cytometry. TT induced memory CD4⁺ T cell proliferation was normalized to 100% and the effect of Tregitope costimulation with TT on the inhibition of CD4⁺ T cell proliferation by Tregitope 289 **A)**, Tregitope 84 **B)**, Tregitope 167 **C)** and FV peptide FV621 **D)** were plotted. Data were compiled from 6-10 donors from 4-5 independent experiments. FV621 exhibited a similar effect on CD4⁺ T cell proliferation as compared to the established IgG derived Tregitopes. **E)** PBMCs were stimulated with 0.5 µg/ml of TT with or without the indicated concentrations of FV621 or 289 and analyzed at six days post-stimulation by flow

cytometry. Data are combined from 3 donors. P values * = <0.05, P values ** = <0.01, P values *** = <0.0002 and **** = <0.0001 represents statistical significance vs. TT using a two-tailed t test.

Fig. 5: FV621 peptide effect on effector and regulatory CD4 T cell response in the bystander assay.

A) The upper left panel shows the gating strategy and the bottom panel summarizes the effect of FV621 on the effector CD4⁺ T (CD25^{hi}FoxP3^{int}) cell population in this example donor. The bar graph on the right shows the inhibition of T effector cell percentage with increasing concentrations of FV621. **B)** To further discriminate T regulatory cells, CD3⁺ cells were gated for CD4⁺/CD127^{low} followed by CD25^{hi}/FoxP3^{hi} as shown. The lower panel shows the effect of FV621 on regulatory CD4⁺ T cells. The bar graph on the right in Fig. B shows that FV621 increased the percentage (%) of T regulatory cells upon stimulation with FV621. PBMCs from healthy donor were stimulated with TT in the presence or absence of FV621 and analyzed six days post stimulation by flow cytometry. These data are representative of 6-8 individual donors.

Fig. 6: Effect of FV621 on CD8⁺ and CD4⁺ T cell response in PBMCs from healthy donors stimulated with CEF peptides.

A) Effect of increasing FV621 concentrations on CD8⁺ T cell proliferation (CFSE low) and activation (CD25 high) CD8⁺ T cells in PBMCs stimulated with 2 µg/ml CEF peptides. Lower panels show the effect of increasing concentration of FV621 peptide on the proliferative response of CD8⁺ T cells **B)** or CD4⁺ cells **C)** in the same assay, to increasing concentrations of CEF peptides.

Fig. 7: Factor V peptides on the downregulation of HLA-DR in antigen presenting cells (APCs).

Healthy donor PBMCs were incubated with Tregitope (Treg167), flu peptide (HA306-318) and different factor V peptides for three days and analyzed by flow cytometry for the expression of CD11c and HLA-DR. **A)** Shows the representative FACS plot for expression of HLA-DR vs CD11c. **B)** Comparing MFI of HLA-DR expression in CD11c⁺ cells and **C)** Graph of (%) change in MFI of HLA-DR expression for the total CD11c⁺ relative to media and peptide stimulation. Data are representative plots for one donor from three individual experiments.

Fig. 8: Effect of FV621 stimulation on the level of granzyme B in T regulatory (blue square) and

T effector (red circle) compartment of T cells. PBMCs were stimulated with TT and sorted into granzyme B positive CD4⁺ T cell populations. **A)** Flow cytometry gating strategy for the activated Treg (GrB⁺CD25^{hi}FoxP3^{hi}) and Teff (GrB⁺CD25^{hi}FoxP3^{int}) cells granzyme B production.

B) Comparing the effect of FV621 on the (%) of Granzyme-B positive Treg and Teff cells. Data are representative of 5 individual donors.

Highlights

- Novel Factor V (FV) 621 Tregitope binds across multiple HLA-DRB1 alleles *in vitro*.
- FV621 is a potent suppressor of CD4⁺T effector responses to Tetanus Toxoid in the TTBSA.
- FV621-albumin conjugates significantly enhance the suppressive effect of FV621.
- FV621 upregulates Granzyme-B in Tregs and downmodulates HLA-DR expression in APCs.
- A point mutation in the TCR facing residue of FV621 abrogates its suppressive capacity.