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Deborah Baafi April 10, 2023

The Effects of T Follicular Helper (TFH) Cells on Anti-FVIII Antibody Formation

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Abstract

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The development of anti-factor VIII (FVIII) antibodies or inhibitors represents a significant barrier to FVIII replacement therapy in patients with hemophilia A. Despite this, the immune factors that regulate anti-FVIII antibody formation remain incompletely understood. We have previously shown that marginal zone (MZ) B cells are an important initiating factor in the immune response to FVIII. MZ B cell responses can be CD4+ T cell independent or dependent. Additionally, we have previously shown that anti-FVIII antibody formation is a CD4+ T cell dependent process, and it is known that T follicular helper (TFH) cells interact with follicular B cells to produce antibodies in the germinal center (GC) of the spleen. Therefore, we hypothesize that TFH cells play a crucial role in anti-FVIII inhibitor formation. To investigate this hypothesis, we challenged B6 mice with 1-4 weekly doses of FVIII and utilized a FVIII MHC class II tetramer to determine the kinetics of FVIII-specific CD4+ T cell expansion as well as characterize the CD4+ T cell response. In addition, we utilized a conditional knock out (KO) mouse model (TFH KO), which lack the ability to generate TFH cells, to analyze the contribution of TFH cells in anti-FVIII antibody formation. The TFH KO mice and wild type control B6 mice received 4 weekly doses of FVIII and anti-FVIII IgM and IgG in plasma were examined by an enzyme-linked immunoassay (ELISA). Lastly, a Bcl-6 inhibitor, an inhibitor that prevents Bcl-6 expression and thereby should prevent TFH formation, was used to investigate the primary stages for a therapeutic intervention against FVIII inhibitors. B6 mice received an infusion of sheep red blood cells (SRBCs), which have been shown to initiate a GC B cell response, and then were administered the inhibitor, twice daily for 7 days. We found that FVIII specific TFH cells expand after 2-3 exposures to FVIII, TFH cells are necessary for anti-FVIII IgG formation, and 79-6 prevented GC B cell expansion when exposed to a blood-borne antigen. In summary, our present findings and future work will likely reveal important pathways to effectively understand and target anti-FVIII antibody production.

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Introduction

Hemophilia A is a serious bleeding disorder characterized by the absence or deficiency of the coagulation protein, factor VIII (FVIII). FVIII is an essential component of the coagulation cascade and is a large plasma glycoprotein of 2,332 amino acids that is organized into 6 domains: A1-A2-B-ap-A3-C1-C2[1]. At baseline, this 280 kDa protein is present in its inactive form and circulates in the plasma tightly bound to its carrier protein, von Willebrand Factor; it requires activation by thrombin[2]. Upon activation, the B domain and ap (activation peptide) portion are released from the entire sequence and thrombin cleaves the A1 and A2 domains to produce a heterotrimer [2]. In addition to thrombin, factor Xa (FXa) has the ability to directly activate FVIII; either process leads to the production of activated FVIII, which acts as a co-factor for factor IXa (FIXa) and allows for the downstream production of factor X (FX) and eventual fibrin clot formation through the intrinsic coagulation pathway[2].

Hemophilia A is an X-linked recessive disease affecting around 1:5,000 male births[3]. The absence of FVIII can result in severe clinical consequences and decreased quality of life, stemming from the inability to successfully form a cross-linked fibrin clot, the end-product of the blood coagulation cascade, which aids in the cessation of bleeding. The absence of functioning FVIII results in excessive bleeding episodes, including mucosal, gastrointestinal, joint, and muscle bleeds[3]. Till this day, administration of FVIII replacement products has been used as the mainstay of treatment in hemophilia A patients for bleeding episodes as well as prophylaxis to prevent bleeding[3]. Although FVIII replacement therapy has been highly successful in reducing morbidity and mortality for many patients, 20-40% of patients with severe hemophilia A develop FVIII

alloantibodies, known as inhibitors, to the FVIII replacement therapy[2,4-10]. These inhibitors are neutralizing IgG antibodies against FVIII, which render FVIII replacement products ineffective. Thus, inhibitors lead to uncontrollable or difficulty in preventing bleeds and can lead to increased morbidity and mortality, especially in hemophilia A patients who must undergo surgery or in patients who have more severe symptoms[9,10]. This also raises costs for hospitals and negatively burdens the medical system[9,10].

Bypassing agents, including activated factor VIIa and FEIBA (an anti-inhibitor coagulant complex that contains factors II, IX, X, and activated FVII to target clot formation) have been used for bleeding treatment and prophylaxis in patients with inhibitors[11]. However, these treatments are costly, have inconvenient dosing regimens, and do not provide optimal bleeding control or prevention when compared to FVIII replacement products[2,4-5,12-13]. Another prophylaxis treatment for hemophilia A includes emicizumab, a bispecific monoclonal antibody that mimics the function of activated FVIII by cleverly bridging together FIXa and FX to produce FXa[14]. Although emicizumab has been highly effective in reducing bleeding episodes in hemophilia A patients with and without inhibitors, unfortunately it is not an optimal treatment for patients with active bleeding; the administration of FVIII still remains the best therapy for bleeding management[15,16]. Furthermore, in recent studies, antibodies to emicizumab have been documented, which adds another layer of complexity to the treatment of hemophilia A patients[14]. Observing that alloimmunization is at the forefront of complications in hemophilia A patients with inhibitors, it is imperative to analyze the immune system and its response to FVIII to better understand FVIII inhibitor formation as the exact immune mechanisms underlying these

processes remains unknown. Once this is understood, treatment can be developed to predict or prevent inhibitor formation and confer an improved quality of life for hemophilia A patients.

In previous studies, it has been seen that infused FVIII localizes in the spleen of mice with hemophilia A, specifically in the marginal zone (MZ)[17]. Cells located within the marginal sinus of the spleen are ideally positioned to detect and fight pathogens that have entered the blood[17]. The MZ is an area between the white pulp and red pulp of the mouse spleen that contains various immune populations, including MZ macrophages, dendritic cells (DCs), and MZ B cells that directly interact with blood-borne antigens and rapidly produce antibodies[17,18]. Our lab has confirmed that FVIII indeed localizes in the splenic MZ and has also shown that FVIII colocalizes with splenic MZ B cells, which are CD21+ and CD23- (Figure 1). MZ B cells reside in the outer region of the spleen follicle and are a unique immune cell because of their innate and adaptive immune modalities. They are specialized in their direct interaction with blood-borne pathogens, while on the other hand, have the ability to speedily produce antibodies against these pathogens independent of T cell help[19].

The observation that FVIII colocalizes with MZ B cells in the spleen, suggests that MZ B cells play an important role in the pathway of anti-FVIII antibody formation. To test the hypothesis that MZ B cells are required for anti-FVIII antibody formation, MZ B cells with were depleted in a preclinical hemophilia A mouse model using anti-CD11a and anti-CD49d antibodies, followed by weekly doses of recombinant FVIII (rFVIII)[3,20]. Flow cytometry was used to evaluate successful MZ B cell depletion. Plasma was collected one week following rFVIII administration and evaluated for anti-FVIII antibody titers via an ELISA. Compared to saline and isotype controls,

the hemophilia A mice that received MZ B cell depleting antibodies had a complete lack of anti-FVIII IgG titers[3]. This evidence highly suggests that MZ B cells are key players in the initial immune response to FVIII.

With this newfound understanding of the impact of MZ B cells on inhibitor formation, our lab sought to better comprehend the full scope of the immunological pathway underlying alloimmunization in hemophilia A. The schema in Figure 2 illustrates our hypothesized understanding of inhibitor formation. Within the MZ resides MZ B cells, which possess antigen-specific B cell receptors. As an innate-like population, they exhibit the ability to detect and then respond to blood-borne antigens, similar to other innate immune populations, including DCs and macrophages, that act as first responders to antigens and invading pathogens. Our finding that MZ B cells co-localize with FVIII shortly after injection and that they are required for FVIII inhibitor formation, coupled with the known ability of MZ B cells to produce antibodies, leads us to propose that MZ B cells, with their FVIII-specific receptors, interact with FVIII directly and subsequently produce an initial amount of anti-FVIII IgM & IgG[21] .

Together, our data suggests that MZ B cells are key players in inhibitor formation, but that is only one part of the larger story. There are CD4+ T cell-independent and CD4+ T cell-dependent antigens, where the latter process requires T cell help for B cell differentiation to occur, whereas B cells are activated in the absence of T cell help in the first process. In fact, there are two types of T cells: CD8+ and CD4+ T cells. CD8+ T cells, also referred to as cytotoxic T cells, function to directly kill intracellular pathogens and cancers[22]. On the contrary, it is understood that CD4+ T cells, also referred to as helper T cells, support the adaptive immune system by interacting and

regulating various immune cell populations like macrophages, B cells, and DCs that later on activate CD8 T cells, etc[23]. Furthermore, there are multiple subtypes of CD4+ T cells that are associated with disease and involved in shaping an immune response, but we focused on these 5 helper T cell populations: TFH, Th1, Th2, Th17, and T regulatory (Treg) cells. TFH cells express the transcription factor, Bcl-6, and are known to activate B cells so they can produce antibodies to defend against extracellular pathogens[24]. Th1 cells express the transcription factor, T-bet, and are known to activate macrophages, defend against intracellular pathogens, and tumors; Th2 cells express GATA-3 and can activate eosinophils and mast cells[24]. Th17 cells express RORγt and help to activate and recruit neutrophils to the peripheral tissues; Treg cells express FoxP3 and are known to fine tune and suppress the immune system alongside resolving inflammation after an infection[24]. These cells are complex and have other various functions.

We next sought to better understand whether FVIII inhibitor formation is a T-cell independent or dependent process. Preliminary research in our lab and previously published research utilizing a preclinical hemophilia A mouse model, revealed that CD4 T cells are required for antibody production to FVIII[25]. After specifically depleting CD4 T cells, with the depleting antibody (GK1.5) and administering 4 weekly exposures of rFVIII to mice with hemophilia A, there was a complete absence of anti-FVIII IgG, suggesting that CD4 T cells are necessary for antibody formation.

TFH cells help activate the humoral immune response by interacting with B cells in a GC reaction so they can produce antibodies that neutralize pathogens, tag the pathogens for clearance, and fix complement for tagging for clearance and/or lysis of the pathogen[24].

Furthermore, TFH cells are defined by the presence of the nuclear transcription repressor, B-cell lymphoma-6 (Bcl-6). Also, cytokines like IL-6 and IL-21 produced by DCs help differentiate naïve T cells into the TFH cell subset[24]. The Bcl-6 transcriptional repressor effectively inhibits differentiation of other T cell lineages by repressing proteins like Blimp-1 and their respective transcription factors, and thereby driving commitment to the TFH cell lineage.

The red question mark in Figure 2 represents our current hypothesis surrounding CD4 T cell activation. It is possible that MZ B cells may directly drive CD4 T cell activation, which could trigger the GC reaction where FVIII-specific follicular (FO) B cells cycle through the light and dark zone of the B cell follicle to produce high affinity anti-FVIII antibodies. But the specific mechanism of how CD4 T cells and MZ B cells interact and how this contributes to inhibitor formation remains unclear. While our preliminary studies support that CD4 T cells, in their entirety, are needed for inhibitor formation, we next desire to identify the subpopulations of CD4 T cells involved in this process, as well as the kinetics of the development of their response. Since we have seen that anti-FVIII antibody formation is CD4 T cell-dependent, and it is known that TFH cells interact with FO B cells to produce antibodies through a GC reaction in the spleen, we hypothesize that TFH cells help anti-FVIII inhibitor formation in hemophilia A mice[24,26]. We seek to better understand the mechanism of inhibitor formation with the goal of identifying a therapeutic approach to prevent inhibitor formation and improve the quality of life for patients with hemophilia A.

Experimental Aims/Goals

Aim 1: Determine the kinetics of FVIII-specific CD4 T cell expansion and characterize the immunophenotype of the cells involved.

Testable Question: When do FVIII specific CD4+ T cells increase in quantity after varying exposures to FVIII and what is the timing of CD4 T cell subset expansion with each exposure?

Hypothesis & Rationale: Clinical data combined with data from our lab suggest that it requires more than 1 exposure to FVIII before inhibitors form; therefore, we hypothesize that CD4+ T cells will undergo activation and proliferation following the 3rd exposure to FVIII, with subsequent differentiation into the TFH subset.

Aim 2: Analyze the contribution of TFH cells in anti-FVIII antibody formation.

Testable Question: Will mice devoid of TFH cells produce anti-FVIII inhibitors upon exposure to rFVIII? How many exposures to FVIII are required to see inhibitor formation?

Hypothesis & Rationale: As TFH cells are essential for the development of T cell-dependent B cell responses and our preliminary data demonstrates a critical role for CD4 T cells in anti-FVIII antibody formation, we hypothesize that TFH cells are required for inhibitor formation and that it will require 3 exposures of FVIII to detect anti-FVIII IgG titers[27-29]. We hypothesize that without the TFH cell subset, mice will not produce anti-FVIII inhibitors. Also, we hypothesize that it will take 3 exposures of FVIII to see inhibitors.

Aim 3: Investigate the primary stages for a therapeutic intervention against FVIII inhibitors.

Testable Question: Will the Bcl-6 inhibitor, 79-6, inhibit the formation of GCs and TFH cells?

Hypothesis & Rationale: We hypothesize that by inhibiting the Bcl-6 repressor, there will be an absence of TFH cells and thus no GC.

Materials & Methods

Mice

C57BL/6J (B6) mice were purchased from Jackson Laboratories to evaluate aim 1. CD4-Cre and Bcl-6 lox-P mice were purchased from Jackson Laboratories. Additionally, CD4-Cre x Bcl-6 fl/fl (TFH KO) and WT/WT x Cre+ and/or Bcl-6 fl/fl x Cre-mice were bred and genotyped in Emory University's facilities to be used in evaluation of aim 2[20]. TFH KO & B6 mice were utilized to evaluate aim 3. FVIII knockout mice (hemophilia A mice, TKO) on a C57BL/6 background were used for confocal microscopy experiments to examine splenic localization of FVIII[30]. TKO mice possess a deletion of the entire *F8* coding sequence; no *F8* messenger RNA is detectable in these mice. TKO mice exhibit a similar bleeding phenotype as measured by factor activity and a tail-snip assay and develop similar anti-FVIII antibody titers following rFVIII exposure. The Cre-LoxP system was utilized to generate this transgenic mouse strain: CD4-Cre + x Bcl-6 fl/fl. Specifically, Cre mice on a B6 background were crossed with Bcl-6-LoxP mice on a B6 background to generate the genotype: CD4-Cre x Bcl-6 fl/fl. This mouse strain contains all normal immune populations and only lacks CD4+ TFH cells. Eight- to 12-week-old male and female mice were used for all experiments. All animals were housed in cages at the Emory University Department of Animal Resources facilities, and all experiments were performed under animal protocols approved by the Institutional Animal Care and Use Committee of Emory University.

Materials

Full length recombinant human FVIII was generously donated by Hemophilia of Georgia. The FVIII MHC Class II tetramer was provided by the National Institute of Allergy and Infectious

Diseases Tetramer Core Facility at Emory University and consists of the TASSYFTNMFATWSPSKARL FVIII peptide presented on the MHC Class II haplotype H-2b.

Confocal Microscopy

Hemophilia A mice (TKO) received either 4 or 10 μg of full length recombinant human FVIII (rFVIII). Group one consisted of the TKO mice that received either 4 μg of rFVIII or saline, three mice per group. Fifteen minutes after rFVIII administration, the spleens were harvested. Group two consisted of 3 TKO mice that received 10 μg of rFVIII or saline. Fifteen minutes after FVIII administration of the second week, spleens were harvested. All spleens were frozen in isopentane using TissueTek freezing medium (VWR Scientific, Randor, PA), and sectioned, followed by fixation. The 7-μm-thick sections were incubated in 0.5% blocking buffer phosphate-buffered saline (PBS) 10.5% heat inactivated fetal bovine serum (FBS) for 2 hours at room temperature. Sections were then stained for 1 hour at room temperature with polyclonal sheep anti-human FVIII (HTI, Essex Junction, VT) diluted 1/100 in blocking buffer. After washing in PBS, fluorescein isothiocyanate (FITC) anti-sheep IgG secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1/100 in blocking buffer was applied for 1 hour at room temperature. Sections were washed and then subsequently stained with phycoerythrin (PE)-CF594 rat anti-mouse B220, and PE rat anti-mouse CD1d (BD Biosciences, San Jose, CA) diluted 1/100 in blocking buffer for 1 hour at room temperature. Sections were again washed in PBS and mounted using Prolong Gold antifade mounting media (Thermo Fisher Scientific, Waltham, MA). Images using 10X and 40X objectives were captured using the Leica SP8 multiphoton confocal microscope and analyzed using Leica application suite (LAS) Advance Fluorescence lite software.

FVIII Specific CD4+ T Cell Isolation

B6 mice received 1, 2, 3, or 4 weekly injections of 2 μg rFVIII or saline by retro-orbital (RO) injection. Additionally, 2 hours prior to the FVIII infusion, for the positive control: B6 mice received 100 μg Polyinosine: polycytidylic acid (Poly I:C) by intraperitoneal (IP) injection. One week following the last injection, spleens were harvested in IMDM media (Gibco IMDM: 15mM HEPES + L-Glutamine). Cells were centrifuged at 1500 rpm for 10 minutes, and subsequently resuspended in IMDM media containing 2.4G2 Fc block (BD Bioscience) + 18 μg/mL FVIII MHC Class II tetramer (I-Ab: FVIII2210-2229) and incubated for 1 hour at room temperature. Cells were then washed with cold IMDM media and resuspended in IMDM media containing 50 μL anti-PE microbeads (Miltenyi Biotech). Samples were incubated on ice for 30 minutes and subsequently washed with cold IMDM media. Tetramer+ cells were then enriched by resuspending samples in 5 mL cold FACS buffer (1x DPBS + 2% bovine serum albumin) and passing the cells over a magnetized LS column (Miltenyi Biotech). Columns were washed two times with 3 mL cold sorting buffer (1x DPBS + 2% BSA + 1% HEPES) and then removed from the magnet. A plunger was used to push 5 mL sorting buffer through the column for elution of bound tetramer+ cells. Following centrifugation, bound and unbound fractions were resuspended to 100 μL and 2 mL cold FACS buffer, respectively. To determine absolute counts, 5 μL from each sample were added to 200 μL AccuCheck counting beads (Invitrogen). The remaining cell suspensions were incubated for 30 minutes on ice with BV40 anti-mouse CD44, BV711 anti-mouse PD-1, PerCP anti-mouse CD4, APC Fire 810 anti- mouse CD3, Live/Dead Zombie NIR, Spark NIR 685 anti-mouse CD11b/Cd11c, PE-Cy7 anti-mouse CXCR5, BV785 anti-mouse CD40L, APC-R700 anti-mouse FAS(CD95), PerCP-

eflour710 anti-mouse GL-7, BV570 anti-mouse B220, BV510 anti-mouse CD21, and Pacific Blue anti-mouse CD23 (Thermofisher Scientific). Polarization of the CD4 T cell response was investigated by fixing and permeabilizing cells post surface stain for 30 minutes on ice using eBioscience Foxp3/Transcription Factor Fixation/ Permeabilization buffer (Thermofisher Scientific). Fixed and permeabilized cells were washed in 1x Permeabilization Wash Buffer (Thermofisher Scientific), and subsequently stained overnight at 4°C with BV421 anti-mouse Bcl-6, PE-CF594 anti-mouse GATA3, BV605 anti-mouse Tbet, BV650 anti-mouse RORγt, and APC anti-mouse FoxP3. All samples were run on a 3 laser Cytek Northern Lights flow cytometer and analyzed using FlowJo software.

Bcl-6 Inhibitor

Small molecule inhibitor 79-6-Calibochem (197345, Sigma-Aldrich) is a transcriptional repressor that selectively inhibits the Bcl6 gene activity[31]. In efforts to eliminate the production of GCs in a mouse, this inhibitor was utilized. B6 mice received intraperitoneal (IP) injections of 200 μL of 50 mg/kg of 79-6 in DMSO and the control mice (B6 and TFH KO) with 10% DMSO (vehicle). All groups were transfused with 50 μL of packed sheep red blood cells (SRBCs) reconstituted in PBS on day 0 and then the mice were administered twice daily injections of 79-6 or the vehicle for 6 days. The experimental group of mice received one dosage of the 79-6 one hour prior to the SRBC infusion to initiate immunological response[20]. 2 hours after the last injection, the spleens were harvested in R10 media (RPMI + 10% heat inactivated FBS). Cells were centrifuged at 1200 rpm for 15 minutes and subsequently incubated in 2 mL of Sigma red cell lysing buffer at room temperature for 5 minutes. After 5 minutes, 18 mL of PBS was added to

each sample and cells were centrifuged for 10 minutes at 1200 rpm. Cells were resuspended in 600 μl of PBS for counting. To count, a 1:20 dilution of trypan blue and cell sample was made. Cells were counted by using an automated cell counter (Nexcelom, Lawrence, MA). 300 μL of each sample was added to a U-bottom 96 well plate and thereafter centrifuged at 1200 rpm for 2 minutes. Cells were washed in 200 μl of PBS buffer and centrifuged as mentioned previously. Cells were incubated in Fc block and FACS buffer at 1:100 dilution for 10 minutes in the dark at 4°C. The plate was washed with PBS and then incubated in 50 μl (1:200 dilution) of Zombie NIR dye at room temperature for 20 minutes in the dark. 150 μl of FACS buffer was added to the plate and briefly agitated to wash. Cells were centrifuged for 2 minutes at 1200 rpm. To view the TFH cells, MZ B cells, and GCs, all mice were and stained for 60 minutes in the dark, on ice, with the following surface markers: APC Fire 810 anti-mouse CD3, PerCP anti-mouse CD4, PE-Cy7 anti-mouse CXCR5, BV711 anti-mouse PD-1, BV785 anti-mouse CD40L, BV40 anti-mouse CD44 (T cell markers); BV570 anti-mouse B220, BV510 anti-mouse CD21, Pacific Blue anti-mouse CD23 (B cell markers); APC-R700 anti-mouse Fas (CD95) and PerCP-eflour710 anti-mouse GL-7 (GC markers). Cells were wash and centrifuged with FACS buffer at 1200 rpm for 2 minutes for a total of 3 washes. Intracellular markers were investigated by fixing and permeabilizing cells post surface stain for 30-60 minutes on ice using eBioscience Foxp3/Transcription Factor Fixation/ Permeabilization buffer (Thermofisher Scientific). Fixed and permeabilized cells were washed in 1x Permeabilization Wash Buffer (Thermofisher Scientific), and subsequently stained overnight at 4°C with APC anti-mouse FoxP3 & PE anti-mouse Bcl-6. All samples were run on a Cytek Norther Lights flow cytometer and analyzed using FlowJo software.

Plasma Analysis for Anti-FVIII Antibodies

To examine anti-FVIII antibody formation in the TFH KO mice, one week following each FVIII administration, blood was collected from the orbital venous plexus with heparinized capillary tubes. Plasma was isolated and an ELISA used for measuring anti-FVIII IgG and FVIII antibody titers, as done previously[30, 32, 33].

Statistics

Statistical analysis was performed using a non-parametric Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparison post-test. Prism (GraphPad Software, La Jolla, CA) was used to perform all statistical analyses. P values <0.05 were considered statistically significant.

Results

FVIII co-localizes with MZ B cells in the mouse spleen.

As previous studies revealed that FVIII localizes in the mouse spleen, we sought to confirm these results and to further investigate the other type of immune populations in the MZ that may possibly be interacting with FVIII[17]. This was accomplished by administrating 4 and 10 μg of recombinant FVIII to hemophilia A mice. After 15 minutes, the spleens were harvested, sectioned, stained, and analyzed via confocal microscopy. 4 and 10 μg of FVIII were sufficient dosages to detect FVIII in the spleen of the mice.

The confocal microscopy revealed that FVIII resides in the mouse spleen and co-localizes with MZ B cells in the marginal sinus (Figure 1). The color change of the antibodies from red, which represents FVIII to blue, MZ B cells, provides evidence that MZ B cells interact with FVIII shortly after it enters the blood and spleen. This suggests that MZ B cells may play a role in the early immune response to FVIII.

FVIII specific CD4+ T cells begin to proliferate and expand after 2 weekly administrations of FVIII.

Prior to tetramer technology, T cell activity could only be assessed via indirect mechanisms that included restimulating cells from immunized mice in vitro and assessing the cytokines and/or proliferation from them[34]. Scientists have been able to harness the power of the interaction between a T cell's receptor and an MHC class II molecule to study antigen-specific T cell interactions via the fluorescent tetramer tool. A tetramer is composed of 4 MHC class II

molecules bound to a peptide specific to the antigen of interest and these 4 molecules are then attached to a streptavidin molecule that is bound to a fluorochrome (PE) so that these cell interactions can be detected via flow cytometry. As illustrated by previous lab data, depletion of CD4 T cells led to the complete absence of anti-FVIII IgG antibody formation in hemophilia A mice. Therefore, to further define the contribution of CD4 T cells in FVIII inhibitor formation, FVIII-specific T cell activation and proliferation was examined. To do this, FVIII specific CD4 T cells from B6 mice were enriched one week after 1 to 4 weekly doses of saline, FVIII, or Poly I:C & FVIII utilizing the FVIII MHC Class II tetramer, (I-$A^{(b)}$: $FVIII_{2210-2229}$) loaded with the peptide: TASSYFTNMFATWSPSKARL, obtained by the NIH tetramer core (Figure 3A). Poly I:C was utilized for the positive control group to detect tetramer positive cells after weeks 1 and 2. Poly I:C is a double stranded (ds)RNA that is recognized by the innate immune system via toll-like receptor 3, which leads to the activation of the type-1 interferon pathway that produces an inflammatory response[35]. The bound fractions of these samples were stained with CD3, CD4, CD44, T-bet, GATA-3, RORγt, Bcl-6, and FoxP3 to identify the CD4+ T cells and the 5 most common CD4+ helper T cells that are associated with disease: Th1, Th2, Th17, TFH, and Treg cells (Figure 3B).

B6 mice produce FVIII specific CD4+ T cells that begin to proliferate and expand after 2 weekly injections of FVIII (Figure 3C). Though there is no statistical difference between the saline group and B6 mice that received 2 weekly injections of FVIII, there are clearly FVIII tetramer+ CD4+ T cells after 2 weekly injections (Figure 3C). Thus, cells start to expand after 2 weekly injections but do not reach a statistically significant amount of expansion until after 3 injections. There was a significant expansion of FVIII specific CD4+ T cells that were also CD44+ in comparison to the saline group after 3 and 4 weekly exposures to FVIII. This data suggests that

CD4+ T cells require multiple exposures to FVIII prior to activation and expansion in the immunological response.

TFH cells increase and proliferate after 2 exposures to FVIII.

It is currently accepted and known that TFH cells interact with FO B cells in the GC to provide proliferating and survival signals to the B cells so that the B cell with the highest affinity B cell receptor, BCR, to the antigen that is being presented by the FO dendritic cells, will be able to undergo further maturation and expansion to produce high-affinity antibodies against the pathogen[24]. Accordingly, it was imperative to examine the effects and kinetics of TFH cell expansion in the presence of FVIII. Next, the FVIII-specific CD4 T cell tetramer bound samples received intracellular staining with T-bet, GATA-3, RORγt, Bcl-6, and FoxP3, and subsequently cells that were tetramer+ and Bcl-6+ were analyzed (Figure 3B). Although it was expected for the TFH cells to proliferate and expand after 2-3 weekly exposures to FVIII, there was a significant expansion of TFH cells after the 4th week (Figure 4). This data illustrates a trend that TFH cells are increasing after the 2nd weekly dosage of FVIII, suggesting that TFH cells begin to expand after the 2nd exposure to FVIII. Additionally, there was a significant increase in Th1 and Th2 cells after the 2nd exposure to FVIII (Figure 4). Surprisingly, instead of steadily increasing after the 2nd week, the Th1 and Th2 cell values decreased after the 3rd weekly exposure (Figure 4). Unexpectedly, the B6 mice that received saline, had high levels of Treg cells possibly indicating that they had an unrelated immunological response occurring due to their environment (Figure 4). This data suggests that TFH cells may interact with FVIII from an early timepoint and that other helper T cells may be involved in the complex immune response.

Mice require 3 administrations of FVIII for anti-FVIII antibody production and completely lack anti-FVIII IgG titers in the absence of TFH cells.

To test the hypothesis that without the TFH cell subset, mice do not produce anti-FVIII antibodies and that 3 doses of FVIII are required for inhibitor formation, a transgenic, conditional knock out (KO) mouse model was made. Bcl-6 is the key transcription factor in CD4 T cells that drives commitment to the TFH cell differentiation[24]. Consequently, a TFH KO mouse was created using the Cre-recombinase system. After genotyping the mice, a CD4-Cre+ mouse was crossed with a Bcl-6-LoxP mouse to create a Bcl-6 fl/fl x CD4 Cre+ mouse (Figure 5A, B). The TFH KO mice in addition to B6 mice were then administered rFVIII for 4 weeks. After each weekly timepoint, plasma was collected from the mice to evaluate the anti-FVIII IgM and IgG titers by an ELISA (Figure 5C).

TFH KO mice did not produce anti-FVIII IgG after 2, 3, and 4 weekly exposures to FVIII (Figure 5D). Additionally, there was a slight decrease in the anti-FVIII IgM over time. This data suggests that TFH cells are required for anti-FVIII IgG production.

The Bcl-6 inhibitor, 79-6, did not significantly reduce the percent of T and B cell lymphocyte populations in B6 mice.

The development of inhibitors for hemophilia A patients has further complexified patients' treatment and care[3,9-10]. In attempts to better the quality of life for patients, we investigated the primary stages of a therapeutic intervention against anti-FVIII antibodies, with a Bcl-6 inhibitor, 79-6[31]. Understanding that TFH cells express Bcl-6 and that they interact with FO B cells to form a GC response to produce high-affinity antibodies, led us to ask the question of

whether this inhibitor would prevent the formation of GC B cells and TFH cells. To test the hypothesis that by inhibiting Bcl-6, there will be an absence of TFH cells and thus no GC B cells, we utilized SRBCs as they have been shown to be a powerful immunogen that produces GC reactions in mice[20]. On Day 0, control B6 and TFH KO mice were challenged with SRBCs. The B6 experimental group was administered the Bcl-6 inhibitor (79-6), followed by SRBCs 2 hours later (Figure 6A, B). Thereafter, twice daily administration of 79-6 over the course of 7 days was given to the experimental group (Figure 6B)[20].

To examine if 79-6 had any off-target effects to other immune populations, splenocytes were harvested from experimental and control mice followed by analysis of B and T cell populations by flow cytometry. The total percentage of B cells, alongside MZ B cells and FO B cells were not impacted nor were they significantly decreased by the inhibitor across all groups of mice (Figure 6C). 79-6 did not significantly reduce the percent of total CD4+ T cell population in B6 mice that were infused by it. In fact, they had comparable percentages of total CD4+ T cells to the positive and negative control and the naïve mice that did not receive any infusions (Figure 6D). Unexpectedly, the TFH cell population did not significantly decrease in the experimental group (Figure 6D). There were comparable percentages of the TFH cell subset in all the groups. By examining TFH cells via their surface markers (CXCR5+ and PD1+) and their intracellular makers, Bcl-6, the data reveals that there is no significant impact of 79-6 on these cells (Figure 6D). This data suggests that the Bcl-6 inhibitor does not have any off-target effects for the other T and B cell lymphocytes in the spleen.

The Bcl-6 inhibitor, 79-6, decreases germinal center formation in mice that are transfused with SRBCs.

After examining the potential off-target effects of the Bcl-6 inhibitor, 79-6, we tested the hypothesis that 79-6 would decrease GC B cell formation in response to SRBCs. The same protocol as described above was utilized to investigate this hypothesis. After the 7th day, spleens were harvested and stained with B220, CD21, CD23, FAS, and GL-7 (Figure 7A).

79-6 decreases GC B cells in B6 mice to a similar degree as demonstrated in TFH KO mice (Figure 7B). Since the TFH KO mice do not have TFH cells nor do they produce anti-FVIII IgG, we would expect them to not produce GCs (Figure 5B, D). The B6 mice that received 79-6 displayed a similar phenotype to these B6 mice, which suggests that the inhibitor decreased GC formation in these mice.

Discussion

Throughout the years, FVIII inhibitors to FVIII replacement therapy have made it difficult to treat hemophilia A patients. This phenomenon has warranted deeper study into the immune mechanisms underlying anti-FVIII antibody formation, which remain incompletely understood. It is imperative to identify and understand the distinct cell populations involved in the process of inhibitor formation. Data has shown that FVIII localizes in the mouse spleen and co-localizes with MZ B cells shortly after administration, and this points to the potential importance of MZ B cells in initiating the immune response to FVIII (Figure 1). MZ B cells seem to be the first cells that FVIII encounters and engages with after entering the blood, but this only illustrates part of the larger immunological response. We hypothesized that CD4+ T cells play an important role in anti-FVIII antibody formation due to the potential interactions they may have with MZ B cells and FO B cells (Figure 2). But even more specifically, we speculated that TFH cells were the unique subset of CD4+ T cells that mediate anti-FVIII inhibitor formation.

Classically, TFH cells upregulate CXCR5 and downregulate CCR7 (homes them to the T cell zone), which drives them to the interfollicular region where they interact with FO B cells[27-29]. The FO B cells do the opposite (upregulate CCR7 and downregulate CXCR5) so that both immune populations meet at the interfollicular region[33]. At this point is where the full differentiation of the T cell into the TFH cell that expresses Bcl-6 occurs. TFH cells help to activate FO B cells. Also, TFH cells migrate into the follicle and the B cells migrate deeper into the center of the follicle where they cycle in and out of the light zone (LZ) and dark zone (DZ). In the DZ, the B cells (CXCR5+

and CXCR4+) are densely packed and are rapidly proliferating and are undergoing processes of somatic hypermutation and class-switching, which has an end-goal purpose for the B cells to create the highest affinity BCR for the antigen that is being presented by the follicular dendritic cells (fDCs)[33]. The B cells will then lose expression of CXCR4 and move to the LZ, where the GC B cells compete to bind to the antigen displayed by the fDCs with their varied BCR affinities. The GC B cell with the highest affinity will present its peptide:MHC class II complex to the TFH cell where the GC B cell receives "help" in the form of proliferative and survival signals from the TFH cells[25-27]. This process will occur several times and the result will be the formation of long-lived plasma cells that reside in the bone marrow to provide life-long immunity and high-affinity antibodies. Memory B cells will be produced in which they can differentiate into plasma cells that can make antibodies that will neutralize the antigen upon the second exposure of the pathogen and/or enter the GC to produce more memory B cells to repeat this cycle[24,36].

Nevertheless, we examined that FVIII specific CD4+ T cells begin to proliferate and expand after 2 weekly administrations of FVIII (Figure 3). This highly suggests that FVIII engages antigen presenting cells (APCs) that in turn interact with CD4+ T cells to produce an immune response. Specifically, it was also shown that TFH cells increase after 2 exposures to FVIII, suggesting that CD4+ T cells differentiate into this key cell population to interact with FVIII specific B cells (Figure 4). While this data did not provide information into whether TFH cells were involved in the production of anti-FVIII antibody formation, it provides insightful details on the kinetics of the involvement of CD4+ T cells in the immune response to FVIII. Understanding the kinetics of FVIII-specific TFH cell expansion, will provide information and/or measures that would aid in predicting inhibitor development in patients.

To further understand the contribution of TFH cells to anti-FVIII antibody formation, we created a mouse model that lacks the ability to generate TFH cells. As aforementioned, Bcl-6 is the key transcriptional repressor, amongst other factors, that causes naïve T cells to differentiate into TFH cells. Thus, without this transcription factor, the CD4+ T cells will lack the ability to differentiate into TFH cells. This led us to evaluate the necessity of TFH cells in anti-FVIII antibody formation. The data suggested that anti-FVIII IgG antibody formation is dependent upon TFH cells (Figure 5). Henceforth, we speculate that when FVIII enters the bloodstream it engages with APCs that then eventually activates TFH cells, and the subsequent GC reaction occurs producing anti-FVIII antibodies that have the potential to neutralize FVIII. It has been shown that not all anti-FVIII IgG have inhibitory function therefore, in the future, it would be imperative to test the plasma for inhibitory antibody levels utilizing a Bethesda assay[37]. Additionally, we will take this experiment the next step further by creating a CD4-Cre^{ERT2} mouse crossed with loxP-flanked Bcl-6 mouse whose offspring will be CD4-Cre^{ERT2} x Bcl-6 fl/fl mice[38]. This is an inducible Cre-loxP system that will induce the deletion of the Bcl-6 gene upon the application of the drug tamoxifen (TAM)[38]. As we know that multiple exposures of FVIII are required to induce anti-FVIII antibodies, this will allow us to analyze the timeline in which the TFH cells are necessary for inhibitor formation. This is clinically relevant because we will be able to understand when we need to potentially stop inhibitor formation when administering FVIII to hemophilia A patients.

Since TFH cells are known to interact with FO B cells in the GC of a spleen and it's highly suggestive that TFH cells are required for anti-FVIII IgG formation, we sought to investigate a therapeutic intervention that could possibly inhibit TFH cells and reduce GC B cells[24,31]. It was imperative that we first understand the impact of the inhibitor with an immunogen that is known

to produce GCs. Desiring to target GC formation, SRBCs were the proper first immunogen to test the inhibitor because previous studies have revealed that SRBCs produce significant GC B cells in mice[20]. Although the Bcl-6 inhibitor, 79-6, did not deplete or significantly decrease the TFH cell population, this does not mean that the inhibitor had no effect on the antigen specific TFH cells (TFH cells that are specific to SRBCs). (Figure 6). On the other hand, 79-6 was able to decrease GC formation to a comparable extent as the TFH KO mice that served as the negative control (Figure 7). This reveals that the inhibitor may be able to decrease GC formation to a sufficient degree for antibody formation to cease. Although promising, this reveals a need for further studies to analyze whether the Bcl-6 inhibitor stopped the GC formation due to direct inhibition of B cells or direct inhibition of antigen specific CD4+ T cells that differentiated into TFH cells. Future studies must be conducted where mice are challenged with FVIII and then administered the Bcl-6 inhibitor. We would then examine their anti-FVIII IgG titers to better understand whether this inhibitor will be able to reduce anti-FVIII IgG production.

In summary, the findings of these studies have revealed the importance of TFH cells in anti-FVIII antibody formation. These results reveal the need for further repeats of each experiment to address the variability within the mice and to identify trends more confidently. Overall, these results will advance our understanding of anti-FVIII antibody formation for further experimentation of possible therapeutic interventions to stop inhibitor prevention and to better the quality of life for patients.

Figures

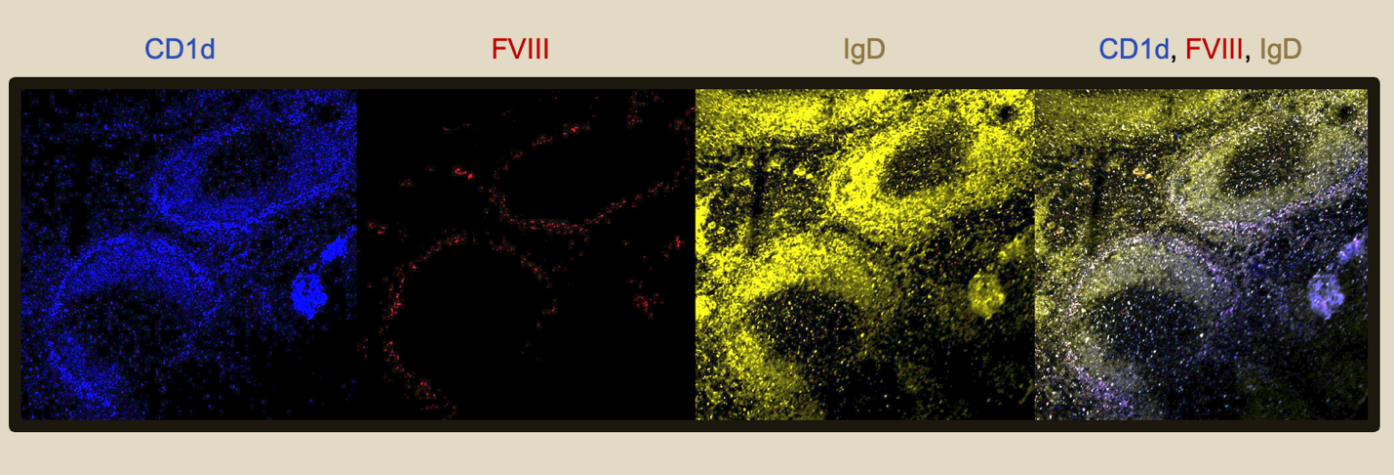


Figure 1. FVIII colocalizes with MZ B cells in the mouse spleen. Mice with hemophilia A were injected with 10 μg of rFVIII, followed by splenic harvest 15 min post injection. Frozen spleens were sectioned and stained with polyclonal sheep anti-FVIII (red), IgD (yellow) and CD1d (blue). Images were obtained using the Leica Sp8 multiphoton confocal microscope and are shown at 10x magnification.

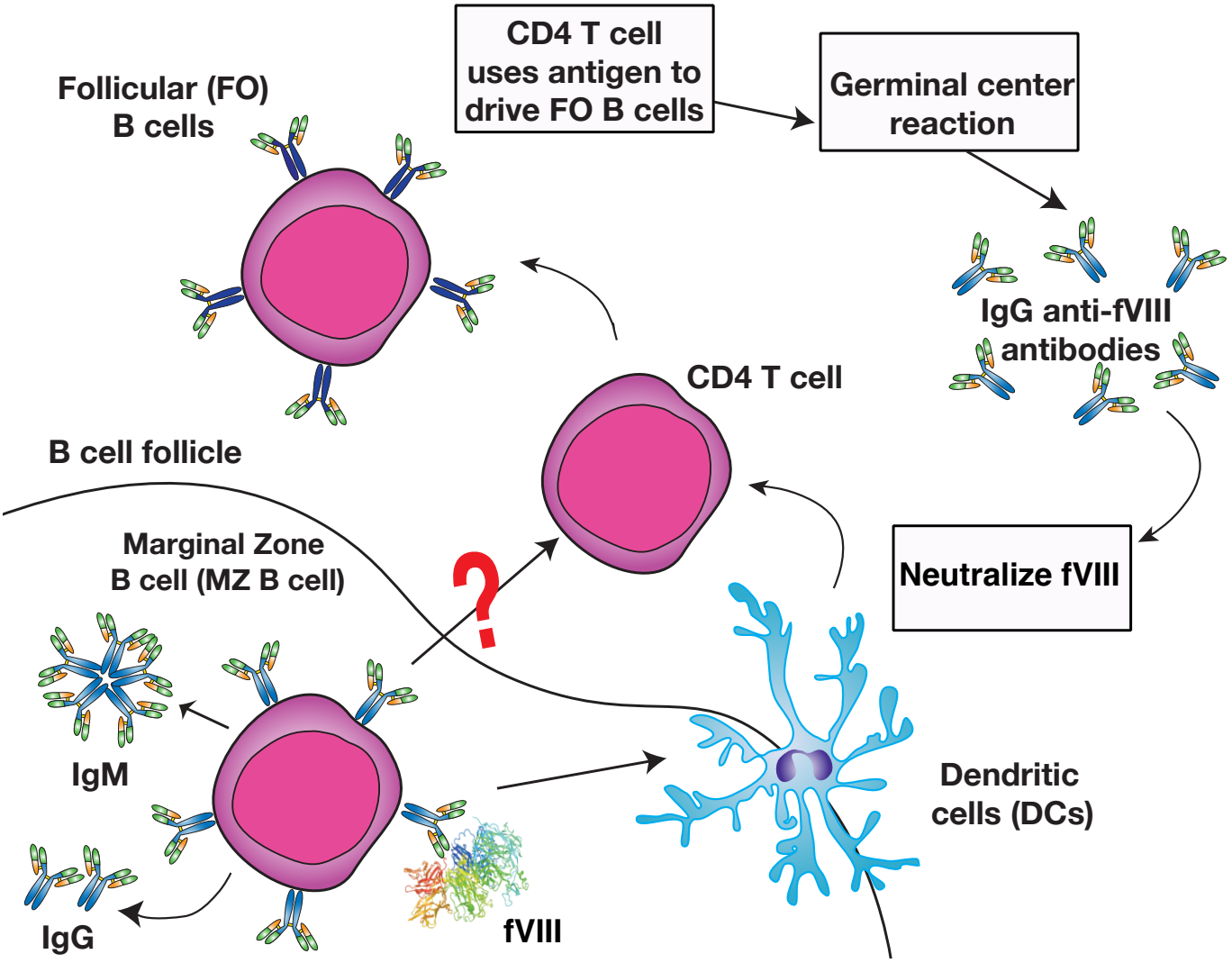
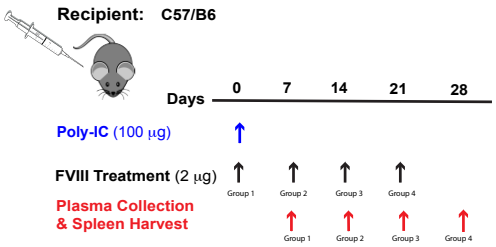


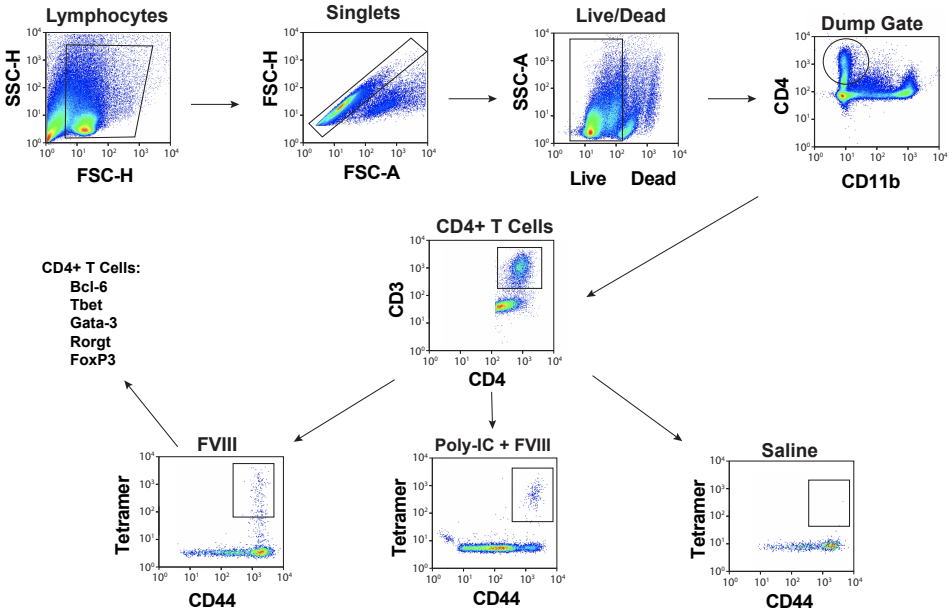
Figure 2. Schema illustrating the aims of the research, supporting the hypothesis that *TFH cells are a key component in anti-FVIII inhibitor formation in Hemophilia A mice.* **Aim 1**: Examine kinetics of FVIII-specific CD4 T cell expansion. **Aim 2**: Investigate contribution of TFH cells in FVIII inhibitor formation. **Aim 3**: Investigate Bcl6 inhibitor.

A)

Group	Infusion
Positive Control	Poly-IC & FVIII
Negative Control	Saline
Experimental	FVIII



B)



C)

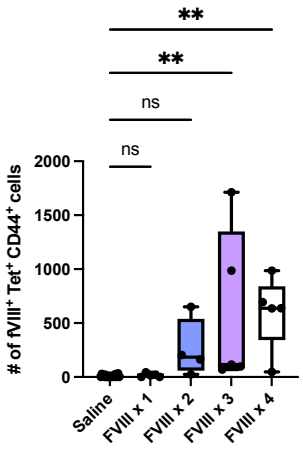
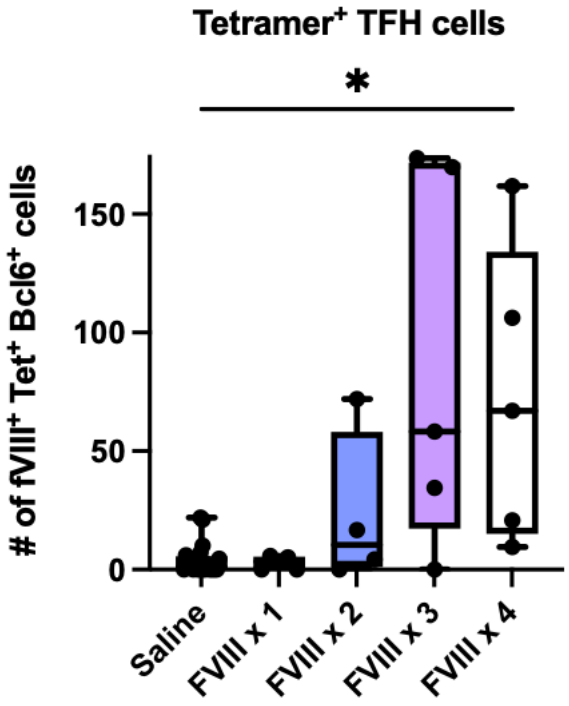
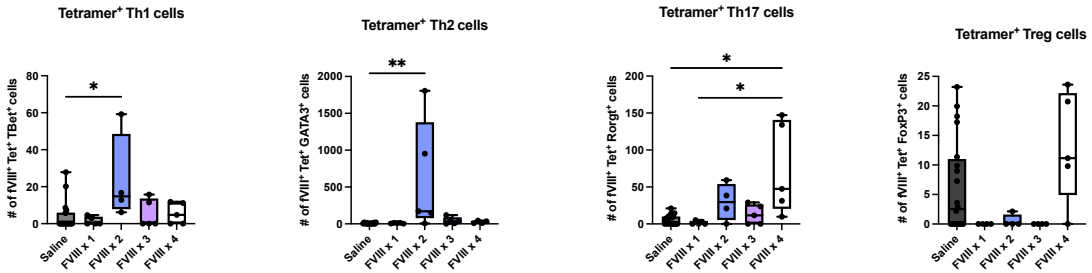


Figure 3. FVIII specific CD4+ T cells begin to proliferate and differentiate after 2 weekly administrations of rFVIII. (A) Recipient B6 mice received 1 (Group 1), 2 (Group 2), 3 (Group 3), or 4 (Group 4) injections of either saline or FVIII with or without Poly I:C. Spleens were

harvested at the indicated timepoints and incubated with a FVIII-specific MHC Class II tetramer. Tetramer-positive cells were isolated by running samples through a sorting column after incubation with magnetic beads. Finally, cells were stained for surface and intracellular markers and run on flow cytometry. (B) Gating strategy is shown for analysis of FVIII-specific CD4 T cells (tetramer positive). (C) Absolute numbers of FVIII tetramer positive cells are shown for each group. **P < 0.01, ns = not significant.



A)



B)

Figure 4. TFH cells trend to increase and proliferate after 2 exposures to FVIII. Tetramer positive cells were analyzed for expression of intracellular markers to identify CD4 T cell subsets including (A) T follicular helper (TFH) cells, (B) Th1 cells, Th2 cells, Th17 cells and regulatory T cells (Tregs). Absolute numbers are shown for each population. ** P<0.01, *P < 0.05, ns = not significant.

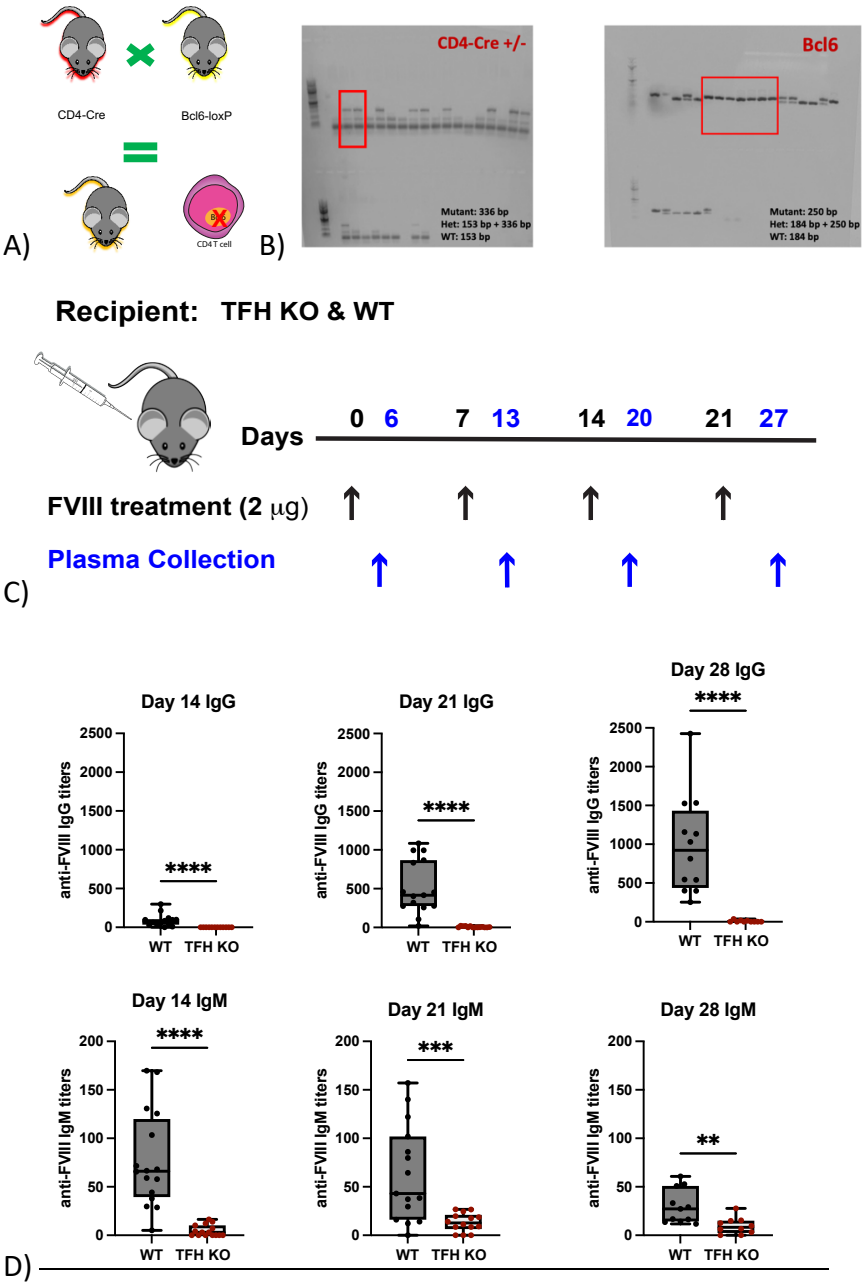
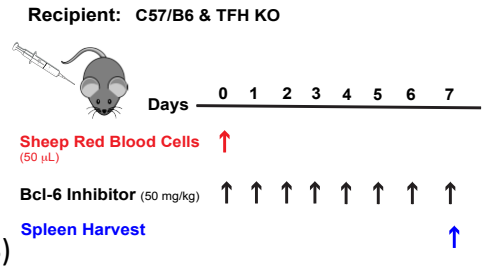


Figure 5. Anti-FVIII IgG production is a TFH cell dependent process that requires 3 administrations of FVIII. TFH KO mice received 4 weekly injections of 2 μg rFVIII. Plasma was obtained one week following each injection and was analyzed by ELISA for anti-FVIII IgM and IgG. ****P <0.0001, ***P< 0.001, **P < 0.01, ns = not significant.

A)

Group	Infusion
Positive Control - B6	sRBC
Negative Control - TFH KO	sRBC
Experimental - B6	sRBC & Bcl-6 Inhibitor
B6	Naive

B)



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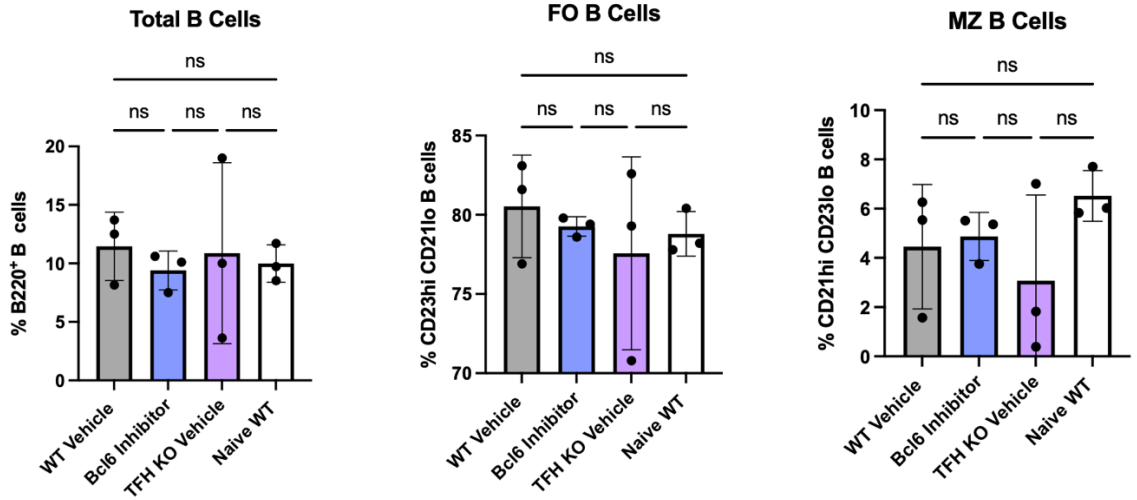

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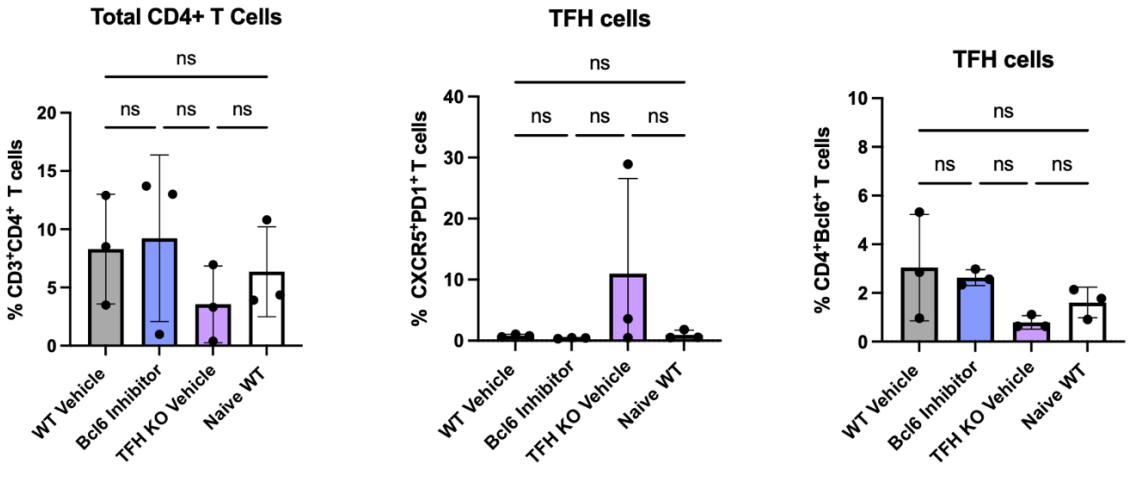
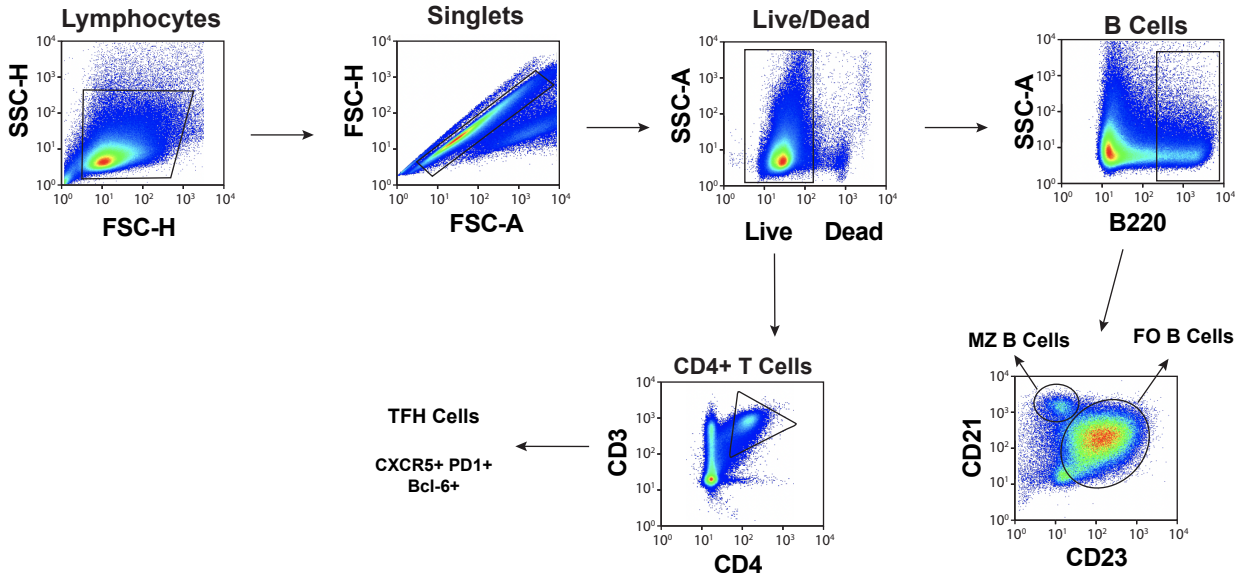


Figure 6. The Bcl-6 inhibitor, 79-6, did not significantly alter T and B cell populations. (A, B) WT (B6) or TFH KO mice received one tail vein infusion of sheep red blood cells (SRBCs) followed by

IP injections every 12 hours of either vehicle or Bcl6 inhibitor for one week. Splenocytes were then harvested, stained for surface and intracellular markers, and analyzed by flow cytometry. (C) Percentages are shown of total B cells, follicular (FO) B cells and marginal zone (MZ) B cells. ns = not significant.

A)



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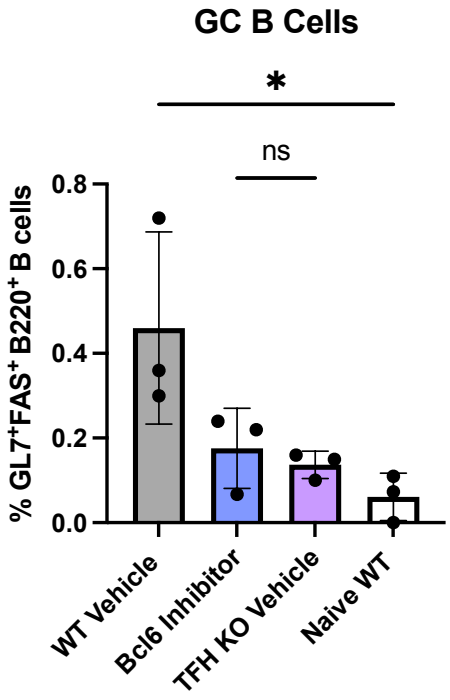

Figure 7. The Bcl-6 inhibitor, 79-6, decreases germinal center formation in mice that are transfused with sheep red blood cells. (A) Germinal center (GC) B cells were gated on, and percentages of each experimental group are shown (B). *P < 0.05, ns = not significant.

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