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Title. Prevention of the anti-factor VIII memory B-cell response by inhibition of the Bruton's tyrosine kinase in experimental hemophilia A

Short title. BTK inhibition prevents anti-FVIII memory response

Sandrine Delignat^{1,2,3}, Jules Russick^{1,2,3}, Bagirath Gangadharan^{1,2,3}, Julie Rayes^{1,2,3}, Mathieu Ing^{1,2,3}, Jan Voorberg⁴, Srinivas V Kaveri^{1,2,3}, Sébastien Lacroix-Desmazes^{1,2,3}

¹INSERM, UMR S 1138, Centre de Recherche des Cordeliers, Paris, F-75006 France;

²Université Pierre et Marie Curie-Paris 6, UMR S 1138, Centre de Recherche des Cordeliers,

Paris, F-75006 France; ³Université Paris Descartes, UMR S 1138, Centre de Recherche des

Cordeliers, Paris, F-75006 France, ; ⁴Department of Plasma Proteins, Sanquin Research and

Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam,

The Netherlands.

Corresponding author: Sébastien Lacroix-Desmazes, INSERM UMR S 1138 Equipe 16, Centre de Recherche des Cordeliers, Paris, F-75006 France - Tel: 01 44 27 82 02 - Fax: 01 44 27 81 93. Sebastien.Lacroix-Desmazes@crc.jussieu.fr

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Abstract

Hemophilia A is a rare hemorrhagic disorder due to the lack of functional pro-coagulant factor VIII. Factor VIII replacement therapy in patients with severe hemophilia A results in the development of inhibitory anti-factor VIII IgG in up to 30% of the cases. To date, immune tolerance induction upon daily injection of large amounts of factor VIII is the only strategy to eradicate factor VIII inhibitors. It is however efficient in only 60-80% of the patients. Here, we investigated whether blocking B-cell receptor signaling upon inhibition of the Bruton's tyrosine kinase prevents anti-factor VIII immune responses in a mouse model of severe hemophilia A. Naïve and factor VIII-sensitized factor VIII-deficient mice were fed with the selective inhibitor of the Bruton's tyrosine kinase, (R)-5-amino-1-(1-cyanopiperidin-3-yl)-3-(4-[2,4-difluorophenoxy] phenyl)-1H pyrazole-4-carboxamide (PF-06250112), to inhibit B-cell receptor signaling prior to challenge with exogenous factor VIII. The consequences on the anti-factor VIII immune response were studied. Inhibition of the Bruton's tyrosine kinase during the primary anti-factor VIII immune response in naïve mice did not prevent the development of inhibitory anti-factor VIII IgG. In contrast, the anti-factor VIII memory B-cell response was consistently reduced upon treatment of factor VIII-challenged mice with the Bruton's tyrosine kinase inhibitor. The Bruton's tyrosine kinase inhibitor reduced the differentiation of memory B cells *ex vivo* and *in vivo* following adoptive transfer to naïve animals. Taken together, our data identify inhibition of the Bruton's tyrosine kinase using PF-06250112 as a strategy to limit the reactivation of factor VIII-specific memory B cells upon re-challenge with therapeutic factor VIII.

Introduction

Hemophilia A is a rare X-linked hemorrhagic disorder that results from suboptimal levels of pro-coagulant factor VIII (FVIII). Treatment or prevention of bleeding is managed by replacement therapy using therapeutic FVIII, which restores coagulation. However, in up to 30% of severe hemophilia A patients administration of exogenous FVIII is complicated by the development of anti-FVIII antibodies that neutralize FVIII pro-coagulant activity and are referred to as 'FVIII inhibitors'.^{1,2} To date, the most efficient strategy to eradicate inhibitors in inhibitor-positive patients with the severe form of the disease, consists in repeated injections of high-doses of FVIII and is referred to as 'Immune tolerance induction' (ITI). Proposed mechanisms of action of ITI include the induction of protective anti-idiotypic antibodies that neutralize FVIII inhibitors as observed in hemophilia A patients,^{3,4} and the inhibition of FVIII-specific memory B cells as suggested in experiments in FVIII-deficient mice.⁵ ITI is however prohibitively costly, requires extreme compliance from the patients and their families, and is successful in only 60-80% of the cases.⁶⁻⁸ The direct depletion of B cells with the anti-CD20 antibody Rituximab (Mabthera®) is also used, however with limited success and unpredictable consequences in the long-term in populations of pediatric patients.⁹ The development of FVIII inhibitors results from the engagement of a classical T-cell-dependent immune response¹⁰ as evidenced by the presence of class-switched, high affinity anti-FVIII antibodies. B cells play key roles in primary T-cell dependent immune responses, by forming and sustaining germinal centers (GCs), by differentiating into antibody-secreting plasma cells (ASCs) and possibly, as recently suggested, as antigen-presenting marginal zone (MZ) B cells involved in the initially stages of activation of immune effectors.¹¹ During recall responses, memory B cells can be reactivated upon antigen encounter and differentiate into plasma cells, replenish the memory B cell pool or participate as key professional antigen-presenting cells (APCs) owing to a higher prevalence of the cells and to the expression of a

higher affinity antigen-specific B cell receptor (BCR).^{12,13} Antigen-specific B cells are thus potential targets to prevent primary or recall antigen-specific immune responses.

Engagement of the surface exposed BCR by the cognate antigen triggers the formation of an intracellular signaling complex which enhances downstream signaling through the phosphorylation and ubiquitination of proteins. Bruton's tyrosine kinase (BTK) is a key proximal and rate-limiting component of the signaling cascade critical for B-cell activation, proliferation and survival.¹⁴ This cytosolic Tec kinase is activated only when BCR signaling promotes its recruitment at the inner cell membrane. Activated BTK in turn phosphorylates the phospholipase C γ 2, which leads to the downstream production of inositol triphosphate and diacylglycerol, resulting in calcium flux and finally to the activation of the NF- κ B and NFAT-dependent pathways.¹⁶ BTK is a strategic therapeutic target for B-cell malignancies that require BTK signaling for cell survival, and for autoimmune diseases associated with the presence of pathogenic autoantibodies such as rheumatoid arthritis¹⁵ or lupus.¹⁶ Several small-molecule inhibitors of BTK have been developed.¹⁷ (R)-5-amino-1-(1-cyanopiperidin-3-yl)-3-(4-[2,4-difluorophenoxy] phenyl)-1H pyrazole-4-carboxamide, or PF-06250112, is a selective potent, orally bioavailable, small-molecule inhibitor of BTK. PF-06250112 forms a covalent but reversible adduct with BTK upon binding to the Cys481 residue that is proximal to the ATP-binding pocket.¹⁸ Using the mouse model of severe hemophilia A, we evaluated the therapeutic potential of BTK inhibition by PF-06250112 on the development of a primary anti-FVIII immune response and on the FVIII-specific memory B-cell recall response.

Methods

PF-06250112 formulation

For *in vitro* studies, PF-06250112 (Pfizer, New York, NY, USA) was solubilized at 1 mg/ml in dimethylsulfoxide (Sigma-Aldrich, St Louis, MO, USA). For *per os* administration, PF-

06250112 was prepared in 0.5% methylcellulose, 0.5% hydroxypropylmethylcellulose acetate succinate H grade and 20 mM Tris at pH 7.4.

Flow cytometry

BTK inhibition with PF-06250112 was evaluated on splenocytes labelled with anti-CD86-FITC (BD Pharmingen, San Jose, CA, USA), and anti-B220-PE (BioLegend, San Diego, CA, USA). Phenotypic analyses were performed using anti-CD4-Alexa 700, anti-CD19-Pacific Blue (Biolegend), anti-CD45-APC (eBiosciences, San Diego, CA, USA); anti-CD3e-FITC, anti-CD11b-PE (BD Pharmingen), anti-CD21-APC Cy7, anti-CD23-PE Cy7 and anti-GL7 Alexa 488 (Biolegend). FACS analysis was done on live cells using BD LSRII and FACSDiva software.

Treatment of FVIII-deficient mice

Seven to 11 week-old exon 16 FVIII-deficient mice on the C57Bl/6 background (gift from Prof H.H. Kazazian, Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia) were handled in agreement with ethical authority guidelines (experimentation on mice was approved by Animal Ethics Committee, authorization #02058.04 granted by the “Direction générale de la recherche et de l’innovation”). PF-06250112 and analogs have half-lives of about 7 hours.^{18,19} For preventive treatment, mice were fed for 5 consecutive days per week, during 4 weeks with PF-06250112 (15 mg/kg) or Vehicle in order to cover about 10 FVIII half-lives.²⁰ Mice were injected with human recombinant B domain-deleted FVIII (0.5 µg BDD-FVIII, Refacto, Pfizer) once a week, 2 hours after the second feeding of the week. Mice were bled 5 days after the fourth FVIII injection. To investigate the effect of PF-06250112 on recall immune responses to FVIII, mice were injected intravenously with 0.5 µg of BDD-FVIII or PBS once a week for 4 weeks. Ninety days after the last FVIII injection, FVIII-sensitized mice with anti-FVIII circulating antibodies were fed for 5 days a week, during 2 weeks with PF-06250112 (15 mg/kg) or Vehicle. Only one challenge with FVIII was

performed with 1 µg of BDD-FVIII, 2 hours after the second day of the first week of feeding. Mice were bled before, 7 and 14 days after FVIII challenge.

Evaluation of anti-FVIII immune responses

Anti-FVIII IgG in mouse serum were measured by ELISA and FVIII inhibitory titers were measured with a chromogenic assay (Siemens, Marburg, Germany).²¹ Proliferation of splenocytes was assessed in 96-well plates ($0.25 \cdot 10^6$ cells/well) with concanavalin A (Sigma) or BDD-FVIII for 72 hrs.²² Cell proliferation was measured by incorporation of [³H]-thymidine (0.5 µCi/well) for an additional 18 hours, using a beta counter (Microbeta 1450, Perkin Elmer).

Stimulation of adoptively transferred memory B cells

Spleens from FVIII-sensitized mice that had developed detectable anti-FVIII IgG (typically 80% of the mice) were collected 7 days after the fourth injection with 1 µg of BDD-FVIII. After lysis of erythrocytes, splenocytes were pooled and plasma cells were depleted using a goat anti-mouse CD138 polyclonal antibody (BD Pharmingen), conjugated to anti-goat IgG coated Dynabeads (Thermo Fisher, Waltham, MA, USA).²³ Naïve FVIII-deficient mice were then injected intravenously with 10^7 CD138-depleted splenocytes. Feeding of mice with PF-06250112 or Vehicle was initiated 24 hours after adoptive transfer for 5 consecutive days a week during 2 weeks, 1 µg of BDD-FVIII was injected intravenously only once 2 hours after the first feeding. Mice were bled 14 days after FVIII injection.

***Ex vivo* memory B cell differentiation assay**

Pooled splenocytes from 5 FVIII-sensitized mice having developed anti-FVIII IgG were cultured at $1.5 \cdot 10^6$ cells/ml in RPMI-1640, with 10% fetal calf serum (Thermo Fisher), 100 U/mL penicillin, 100 mg/mL streptomycin, and 50 µM 2-mercaptoethanol without CD138 depletion for 6 days. At day 0, PF-06250112 or Vehicle was added at different concentrations. FVIII (1µg/ml Advate®, Shire, Dublin, Ireland) was added to the culture 2 hours later.

Advate was used instead of Refacto to follow as closely as possible the seminal protocol established by Hausl et al.²³ Of note, Refacto and Advate present with the same immunogenicity in FVIII-deficient mice.^{21,24} After 6 days, the formation of FVIII-specific ASCs was assessed by enzyme-linked immunospot (ELISPOT) assay with FVIII-coated plates (0.5µg Advate/well). After 2 hours of blocking with 10% FCS in RPMI medium, cells were incubated on the membrane and cultured overnight at 37°C in 5% CO₂. After washing with PBS, 0.1% Tween 20, antibodies were detected using a goat anti-mouse IgG Alkaline-Phosphatase conjugated antibody (Southern Biotech, Birmingham, Alabama, USA), and ASCs were revealed with Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma). Plates were scanned on an ImmunoSpot Analyzer (CTL, Shaker Heights, OH, USA). Spots were automatically counted by ImmunoSpot Software using the SmartCount™ and Autogate™ functions.

Statistical analysis

Statistical analysis was performed using the non-parametric two-tailed Mann-Whitney U test with a confidence interval 95%. P values ≤0.05 were considered significant (ns: not significant). Analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

PF-06250112 inhibits BCR signalization

BCR engagement initiates an intracellular signaling cascade that induces the BTK-dependent activation of B cells.¹⁴ PF-06250112 has recently been described to inhibit BCR-mediated B-cell signaling, activation and proliferation.¹⁸ We confirmed the effect of PF-06250112 on splenic B cells by monitoring the upregulation of CD86 in response to B-cell triggering with anti-IgM or anti-IgD for *in vitro* and *in vivo* assays, respectively. Pre-treatment of purified splenic B cells by PF-06250112 prevented in a dose-dependent manner the induction of CD86

expression on B cells upon stimulation with anti-IgM F(ab')₂ fragments (Figure 1A). It however marginally impacted CD86 expression upon stimulation of the cells with an anti-CD40 antibody. The calculated IC₅₀ was 1.1±0.6 nM (Figure 1A, inset), which is in agreement with previous observations.¹⁸ The *in vivo* validation of PF-06250112 was performed using an anti-IgD antisera instead of the anti-IgM antibody in order to avoid quenching of the triggering antibody by endogenous circulating IgM. The treatment of FVIII-deficient mice with PF-06250112 two hours prior to the injection of the anti-IgD antisera prevented anti-IgD-mediated CD86 induction, as measured *ex vivo* on splenic B cells (Figure 1B), and only marginally affected anti-CD40-mediated CD86 induction. The dose of 15 mg/kg of PF-06250112 was sufficient to prevent induction of CD86 expression in the mice and was used for further experiments.

BTK inhibition does not prevent primary anti-FVIII immune responses

We then investigated the potential of PF-06250112 to prevent the development of a primary anti-FVIII immune response in FVIII-deficient mice (Figure 2A). The effect of four weeks of treatment with PF-06250112 was first evaluated on the splenic B and T-cell compartments of FVIII-injected mice. Analysis by flow cytometry revealed that chronic inhibition of BTK with PF-06250112 results in an increase in CD11b-positive cells (P=0.040, Figure 2B), which include monocytes, macrophages and natural killer cells, and in a significant decrease in CD4⁺ T cells (P=0.031) and in different B-cell subsets, including follicular (Fo) and marginal zone (MZ) B cells (P=0.038, Figure 2C). Although statistically significant, changes in percent of the cell populations were biologically marginal. The anti-FVIII IgG titers measured after the fourth injection of FVIII in PF-06250112-treated mice (565±170 AU, mean±SEM, Figure 2D) were not statistically different from that in Vehicle-treated mice (1133±376 AU, P=0.488). Similarly, inhibitory titers did not statistically differ between PF-06250112-treated mice (123±88, Figure 2E) and control mice (457±219, P=0.572). The capacity of splenic T

cells to proliferate in the presence of FVIII or concanavalin A was similar in the two groups of mice (Figures 2F and 2G). Thus, inhibition of BTK at the time of FVIII injection does not prevent the onset of a primary anti-FVIII immune response.

BTK inhibition alters memory FVIII-specific B-cell responses

We investigated the effect of BTK inhibition in the context of a memory anti-FVIII B-cell response, where the FVIII-specific BCRs in principle have a higher affinity for FVIII than BCRs of naïve B cells. FVIII-deficient mice were injected with FVIII once a week for four weeks, and then left untouched for 90 days to allow the spontaneous elimination of FVIII-specific short-lived plasmocytes.²⁵ FVIII-sensitized mice were then fed with Vehicle or PF-06250112 and treated with FVIII as described in Figure 3A. Anti-FVIII IgG titers measured prior to FVIII re-challenge were heterogeneous among mice (Figure 3B), as previously described.^{21,26,27} The levels of anti-FVIII IgG depicted in Figure 3C were therefore normalized with respect to the initial levels of anti-FVIII IgG measured for each individual mouse. Feeding mice with PF-06250112 significantly reduced the increase of anti-FVIII IgG response, as compared to control mice (Figure 3C).

We then exploited an alternative model in order to study the effect of PF-06250112 in the absence of a potential bias provided by the presence of plasma cells and circulating anti-FVIII IgG. Splenocytes from FVIII-treated mice or naïve mice were depleted of CD138-positive plasmocytes and adoptively transferred to naïve FVIII-deficient mice as previously described.²³ Feeding of the recipient mice with PF-06250112 or Vehicle was initiated on the next day, and one injection of FVIII was performed 2 hours after the first feeding (Figure 4A). The treatment of mice with PF-06250112 prevented the production of anti-FVIII IgG as compared to control mice (0.9 ± 0.3 versus 124.0 ± 115.7 , $P=0.011$, Figure 4B). Anti-FVIII IgG titers in PF-06250112-treated mice injected with FVIII were similar to that of control mice adoptively transferred with splenocytes from FVIII-sensitized mice injected with PBS and to

that of control mice adoptively transferred with splenocytes from naïve and FVIII-injected mice. We further performed re-stimulation of FVIII-specific memory B cells *ex vivo*. Splenocytes from FVIII-sensitized FVIII-deficient mice having developed a humoral response to FVIII were isolated and pre-treated with PF-06250112 or Vehicle before stimulation with FVIII. After 6 days of culture, FVIII-specific ASCs were measured by ELISPOT. A statistically significant dose-dependent reduction of ASCs formation was observed when splenocytes were pre-incubated with PF-06250112 as compared to splenocytes pre-incubated with Vehicle, prior to stimulation with FVIII ($P \leq 0.003$, Figure 5). Thus, inhibition of BTK prevents the activation of FVIII-specific memory B cell response.

Discussion

The occurrence of FVIII inhibitors following FVIII replacement therapy remains a major clinical and societal challenge in hemophilia A. B cells are a key effector of the anti-FVIII immune response. Depending on their subtype and localization and depending on the stage of the immune response, B cells play different roles as i) APCs either in the initiation phase of immune response (i.e., MZ B cells involved in FVIII capture and trafficking in the spleen),¹¹ or in recall responses (higher number of FVIII-specific memory B cells with high affinity FVIII-specific BCRs), and ii) precursors of ASCs (that produce FVIII inhibitors) localized in the spleen or in the bone marrow. Therefore, B cells represent an ideal therapeutic target either to prevent the initiation of anti-FVIII immune responses or to eradicate ongoing responses in inhibitor-positive patients. Incidentally, the only strategies known to eradicate FVIII inhibitors in patients target B cells. ITI was shown to favor the development of anti-idiotypic antibodies that neutralize FVIII inhibitors^{3,4} and, at least in mice, to eliminate FVIII-specific memory B cells.⁵ Likewise, the use of the therapeutic anti-CD20 antibody Mabthera (Rituximab) specifically targets CD20-expressing naïve and memory B cells, thus preventing

the replenishment of the pool of plasma cells upon re-challenge with therapeutic FVIII. Both strategies fail to target long-lived plasma cells that reside in niches in the bone marrow and lack expression of CD20 and of the BCR.²⁸ Attempts to target plasma cells in FVIII-deficient mice using either inhibitors of the proteasome²⁹ or cocktails of immunosuppressive agents³⁰ have met with limited success or lack of antigen specificity. Here, we investigated a new approach to prevent the activation of antigen-specific B cells by inhibiting BTK, a kinase involved in upstream BCR signalization. Inhibition of BTK targets only those B cells that are activated at the time of administration of therapeutic FVIII, thus providing some degree of specificity for FVIII.

We first validated that, upon BCR triggering, PF-06250112 blocks BCR signaling *in vitro* and *in vivo* by measuring the induction of CD86 expression, a co-stimulation molecule involved in anti-FVIII immune response.³¹ PF-06250112 inhibited BCR signalization *in vitro* with an IC₅₀ of about 1 nM, a value close to that reported previously.¹⁸ PF-06250112 used at 15 mg/kg also inhibited the induction of CD86 *in vivo* following stimulation of splenic B cells with an anti-IgD serum. A marginal effect of PF-06250112 was observed on B cells stimulated by CD40 ligation, a BCR-independent pathway. This moderate alteration of CD40 signaling had also been reported in the case of PCI-32765, another BTK inhibitor; a possible interference of BTK inhibitors on CD40-induced the NFκB-signaling was evoked.³²

As previously reported in an experimental model of SLE¹⁸ or using immunization of mice with the T-dependent model antigen SRBC,¹⁹ the regular *per os* administration of PF-06250112 of FVIII-deficient mice led to a statistically significant decrease in the percentages of MZ and Fo B cells. In contrast to the latter reports however, there was no change in percentages of splenic GC B cells, possibly owing to the use of a different antigen or of a different disease model. In our hands, the treatment of FVIII-deficient mice with PF-06250112 failed to prevent the onset of a primary anti-FVIII immune response. Exogenously

administered FVIII was previously shown to closely associate with MZ macrophages and MZ B cells.^{11,33,34} The depletion of MZ macrophages or MZ B cells was independently shown to delay the onset of anti-FVIII immune response.^{11,33} However, it is not clear whether reduced immune responses to FVIII following depletion of MZ macrophages or B cells result from the direct implication of these cells in FVIII internalization and presentation to T cells, or trafficking to the GC, or from a mere disruption of the spleen architecture.³⁵ The lack of effect of chronic BTK inhibition on the development of primary anti-FVIII humoral responses, despite an alteration of peripheral B-cell populations including MZ B cells, suggests that MZ macrophages are the principal APCs at play in the onset of primary anti-FVIII immune responses, at least in mice. PF-06250112 feeding was also associated with a modest decrease in percentages of CD4+ T cells. PF-06250112 has been shown to be 10,000-fold more specific for BTK than for interleukin-2-inducible T-cell kinase (ITK)¹⁸. In addition, there is no modulation of splenic T-cell populations in BTK-deficient mice.¹⁴ Hence, the decrease in the percentage of CD4+ T cells does probably not reflect a direct effect of PF-06250112 on T cells in FVIII-sensitized mice, but rather a modulation of T-cell homeostasis consecutive to a decrease in splenic Fo and MZ B cells. This illustrates a possible moderate off-target effect of PF-06250112 in naïve mice. Signaling through CD40 and CD40L is essential for the onset of primary immune responses, including in the case of therapeutic FVIII.^{36,37} The lack of effect of PF-06250112 on the primary anti-FVIII immune response presumably reflects the lack of effect of BTK inhibition on CD40 signaling.

The inhibition of BTK using PF-06250112 was able to limit the memory B-cell response towards therapeutic FVIII. We first demonstrated that the inhibition of BTK in primed mice limits the increase in anti-FVIII IgG after a single re-challenge with FVIII. Plasma cells do not express BCR at their membrane and are therefore insensitive to BTK inhibition.^{19,38} The continuous endogenous production of anti-FVIII IgG by resident plasma cells may have

introduced a bias in our experimental setup. Indeed, the formation of immune complexes between circulating anti-FVIII IgG and the administered FVIII neutralizes FVIII and alters its immunogenicity.³⁹ In addition, immune complexes between FVIII and anti-FVIII IgG facilitate FVIII internalization by dendritic cells⁴⁰ and could skew the target APC population from memory B cells to dendritic cells or macrophages. In support of our initial observation however, the inhibition of BTK also prevented the *in vivo* re-activation of FVIII-specific memory B cells following adoptive transfer to naïve mice and administration of FVIII, as well as the *ex vivo* differentiation of memory B cells into ASCs. Immune responses to FVIII in FVIII-deficient mice are notoriously heterogenous.^{21,26,27} This is illustrated by the fact that 80% of the mice developed primary immune responses to FVIII, and that memory responses were heterogenous in the 3 experimental models used in the present study. Of note, although the effect of the inhibition of BTK on the anti-FVIII memory response was heterogenous among mice, results from all three experimental models lead to a significant reduction in the intensity of the anti-FVIII memory response. Our finding that BTK inhibition was efficient in blocking anti-FVIII recall but not primary immune responses is reminiscent of recent observations. The chronic inhibition of BTK with an analog of PF-06250112 was shown to prevent immune responses to T-independent antigen or to T-dependent antigens only provided that BCR has a strong affinity for the antigen, and provided that antigen ligation induces a strong signaling of the BCR.^{14,19} Memory B cells generally express BCRs of high affinity for their cognate antigen owing to affinity maturation in the GCs. In the case of the anti-FVIII B cell response, the rare human monoclonal anti-FVIII IgG studied to date were obtained following immortalization of memory B cells from inhibitor-positive patients. The affinity of the anti-C2 domain IgG4k BO2C11 for FVIII⁴¹ is 10^{-11} M^{-1} and that of the anti-C1 domain IgG4k LE2E9 is greater than the affinity of von Willebrand factor for FVIII (i.e., $K_D < 0.1 \text{ nM}$).⁴² Likewise, the affinity of polyclonal anti-FVIII IgG from inhibitor-positive

patients was described to be in the low nanomolar range.⁴³ Indirectly, the efficacy of PF-06250112 to block the recall anti-FVIII immune response confirms that FVIII-specific memory B cells express high affinity BCR.

A strong predictor of ITI failure is the intensity of the inhibitory titer peak that is reached within the two weeks that follow ITI initiation.⁴⁵ Yet, the molecular and cellular mechanisms that underlie the reduced efficiency of ITI when the inhibitor peak is high after initiation of ITI are unknown. In particular, it is not understood whether a high level of circulating FVIII inhibitor reflects the quantitative or qualitative properties of the circulating anti-FVIII IgG, the affinity for FVIII of the BCR of memory B cells or the number of FVIII-specific memory B cells. Although the experimental protocols employed in the present work do not reflect the clinical situation, and although no animal model of ITI has been validated yet, we propose here that the inhibition of BTK-dependent BCR signaling as an adjunct therapy in patients starting ITI could increase the chances for ITI success by preventing the reactivation of FVIII-specific memory B cells. Further, reduction of the potential toxicity of PF-06250112 could be achieved by targeting it to FVIII-specific B cells using CD22L/FVIII-coated nanoparticles⁴⁴, thus paving the way towards their use in hemophilia A.

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Authors' contributions

Designed research: SD, BG, JV, SLD

Performed research: SD, JRu, BG, JRa, MI

Analyzed data: SD, JRu, SVK, SLD

Wrote the paper: SD, SLD

Conflict of interest

The authors declare no conflict of interest.

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Legends to figures

Figure 1. Inhibition of splenic B-cell activation by PF-06250112. Panel A. Splenic B cells from C57Bl/6 mice, purified by negative selection were plated in RPMI, 10% FCS and treated with PF-06250112 or Vehicle (DMSO) for 2 hours. F(ab')₂ fragments of goat anti-mouse IgM (10 µg/ml) or a monoclonal hamster anti-mouse CD40 antibody (5 µg/ml) were added. After 24 hours, the expression of CD86 by live B220-positive cells was analyzed by flow cytometry. Results are representative of two independent experiments performed in triplicates (mean±SD). Treatment of B cells *in vitro* with PF-06250112 suppressed induction of CD86 expression with an IC₅₀ of 1.1±0.6 nM (Inset). Panel B. C57Bl/6 mice were fed with PF-06250112 or Vehicle. After 2 hours, goat anti-IgD antisera (400 µl, eBiosciences), or rat anti-mouse CD40 IgG (100 µg, Biolegend), were injected intraperitoneally. Eighteen hours later, mice were sacrificed and the expression of CD86 by live splenic B220-positive B cells was analyzed by flow cytometry. Representative of two independent experiments performed with 3 mice per group (mean±SD).

Figure 2. Treatment with PF-06250112 does not prevent the onset of a primary anti-FVIII immune response. Panel A. Experimental scheme for the preventive treatment of FVIII-deficient mice. Mice were fed with PF-06250112 (15 mg/kg) or Vehicle for 5 days a week, and injected with FVIII once a week, 2 hours after the second feeding of the week. Mice were sacrificed 5 days after the 4th injection with FVIII (day 27). Spleens and serum were recovered. Panels B and C. At sacrifice, the isolated splenocytes were labeled with anti-CD45, anti-CD11b, anti-CD19, anti-CD3 and anti-CD4 antibodies (% of live CD45-positive cells, Panel B). Follicular B cells (Fo) were identified as CD19⁺CD23^{high}CD21^{low}, marginal zone B cells (MZ) as CD19⁺CD21^{high}CD23^{low}, germinal center B cells (GC) as CD19⁺GL7⁺ (% of live cells, Panel C). Panels D, E. Anti-FVIII IgG titers (panel D) and inhibitory titers (panel

E) were measured in the serum of the mice by ELISA with the mouse monoclonal anti-FVIII IgG mAb6 as a standard (expressed in arbitrary units, AU), and chromogenic assay, respectively. Panels F and G. Splenocytes were incubated for 72 hours with FVIII (panel F) or with concanavalin A (ConA, panel G). The incorporation of tritiated thymidine is depicted as count per minute (CPM) after an additional 18 hours of incubation (mean \pm SEM). Data are representative of 2 independent experiments with 9 mice per group.

Figure 3. Treatment with PF-06250112 controls the recall response to FVIII. Panel A. FVIII-deficient mice were injected with FVIII once a week for 4 weeks. After 90 days, mice were fed for 5 days a week, during 2 weeks with PF-06250112 or Vehicle, and injected with FVIII only once, 2 hours after the second day feeding. Panel B. Levels of anti-FVIII IgG were assessed by ELISA using the mouse monoclonal anti-FVIII antibody mAb6 as a standard. Mice were then randomly attributed to either the PF-06250112- or the Vehicle-fed groups. Panel C. Levels of anti-FVIII IgG were measured 7 and 14 days after the last injection of FVIII. IgG levels were normalized with respect to the initial levels of anti-FVIII IgG of each respective mouse measured at Bleed 0. Graphs depict means \pm SEM of three independent experiments with a total of 17 mice/group.

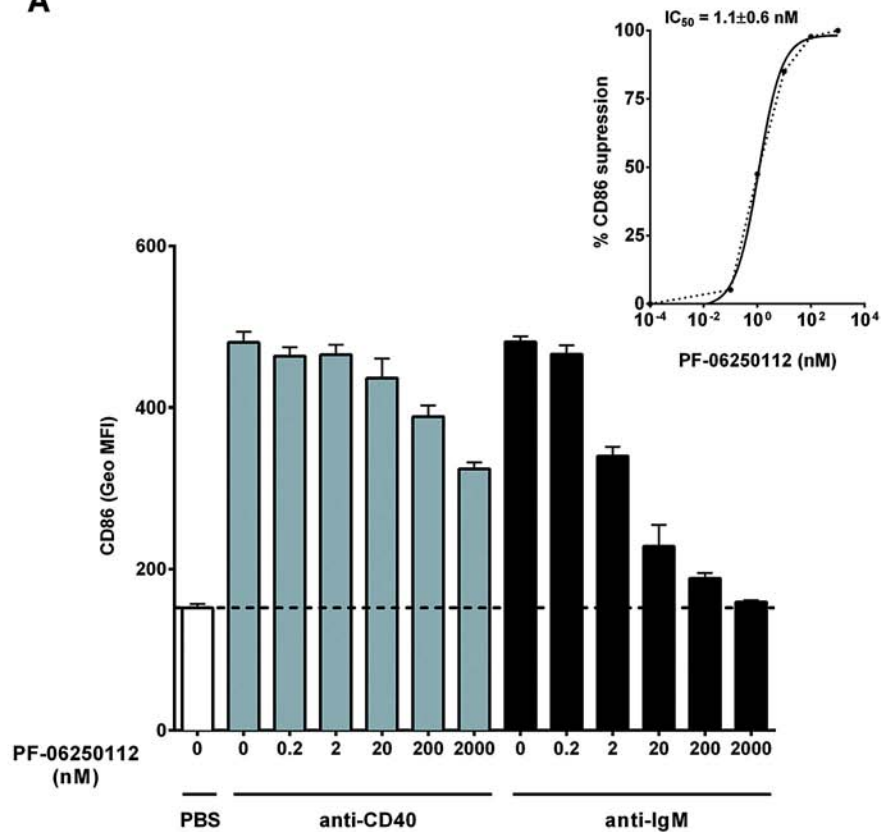
Figure 4. Treatment with PF-06250112 inhibits the anti-FVIII memory B-cell response. Panel A. FVIII-deficient mice were injected with PBS or FVIII once a week for 4 weeks. Seven days after the last injection, mice were sacrificed and spleens were collected and pooled. CD138-depleted splenocytes from FVIII-treated or naïve (PBS-treated) FVIII-deficient mice were adoptively transferred to naïve FVIII-deficient mice. From the next day onwards, host FVIII-deficient mice were fed during 2 weeks with 15 mg/kg of PF-061250112 (18 mice) or Vehicle (20 mice), daily for 5 days. Two control groups were injected with PBS

or were adoptively transferred with splenocytes from naïve mice (7 mice per group). Mice were injected with FVIII or PBS only once 2 hours after the first feeding. Panel B. Levels of anti-FVIII IgG were measured by ELISA 14 days after FVIII challenge. Means \pm SEM are depicted on the graph.

Figure 5. PF-06250112 inhibits the *ex vivo* differentiation of FVIII-specific memory B cells into ASCs. Splenocytes from FVIII-sensitized FVIII-deficient mice having developed a humoral response to FVIII were isolated 14 days after the last injection of FVIII. Pooled splenocytes were cultured for 6 days in the presence of 1 μ g/ml FVIII. Splenocytes were incubated with Vehicle or PF-06250112 two hours before adding FVIII. At day 6, newly differentiated anti-FVIII ASCs were detected by ELISPOT. The graph depicts means \pm SEM.

Figure 1

A



B

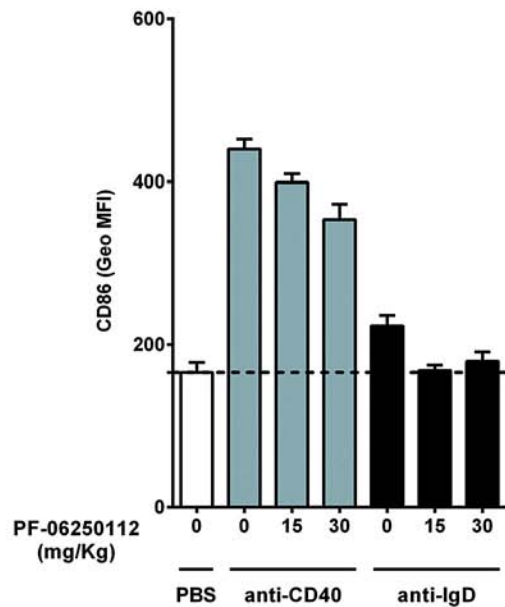
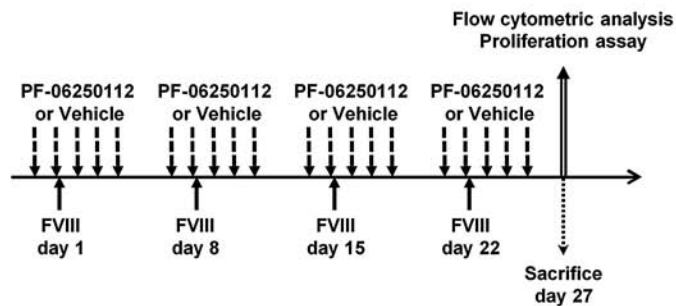
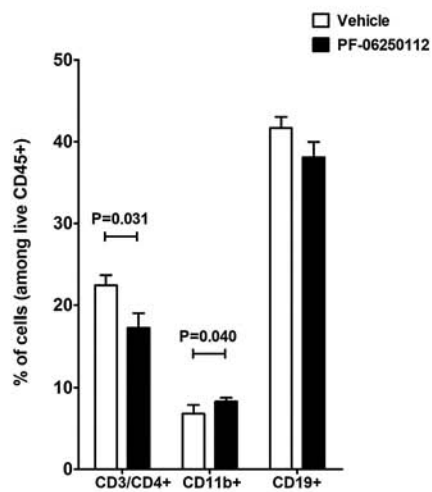


Figure 2

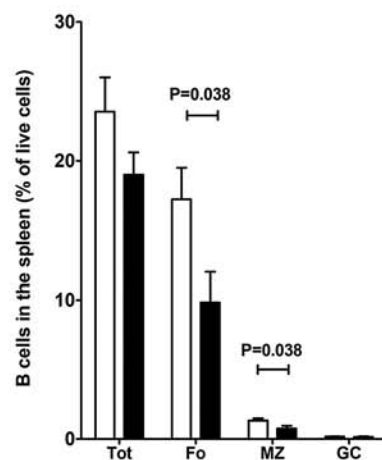
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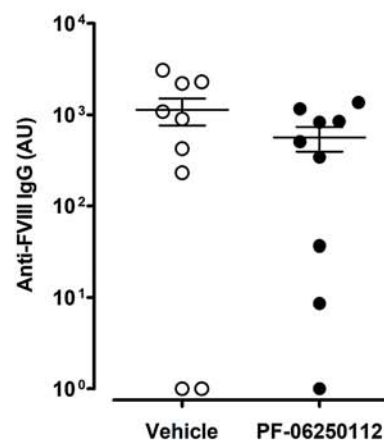
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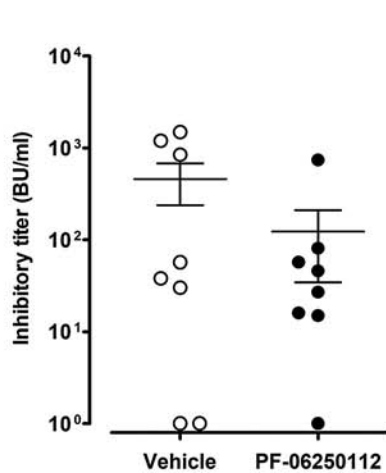
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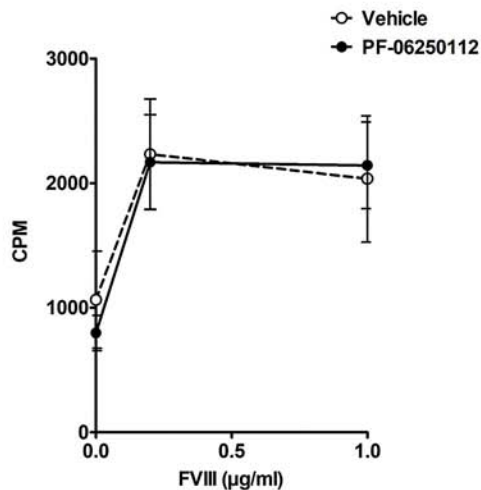
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E



F



G

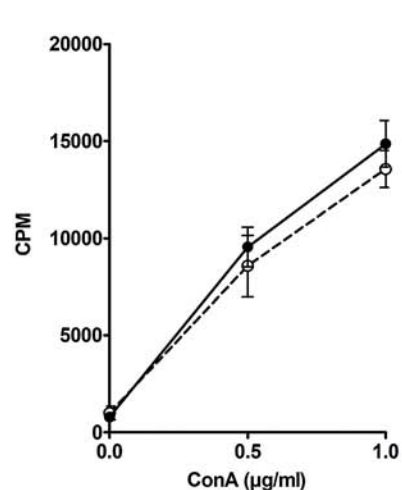
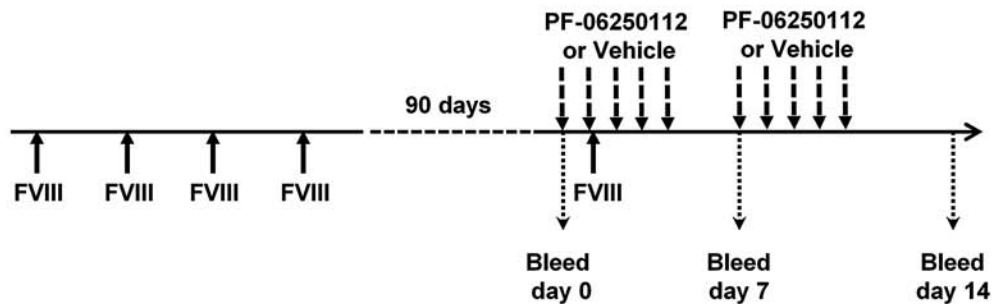
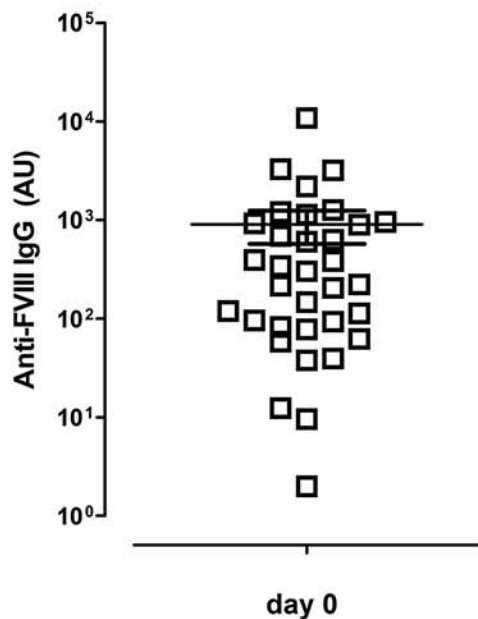


Figure 3

A



B



C

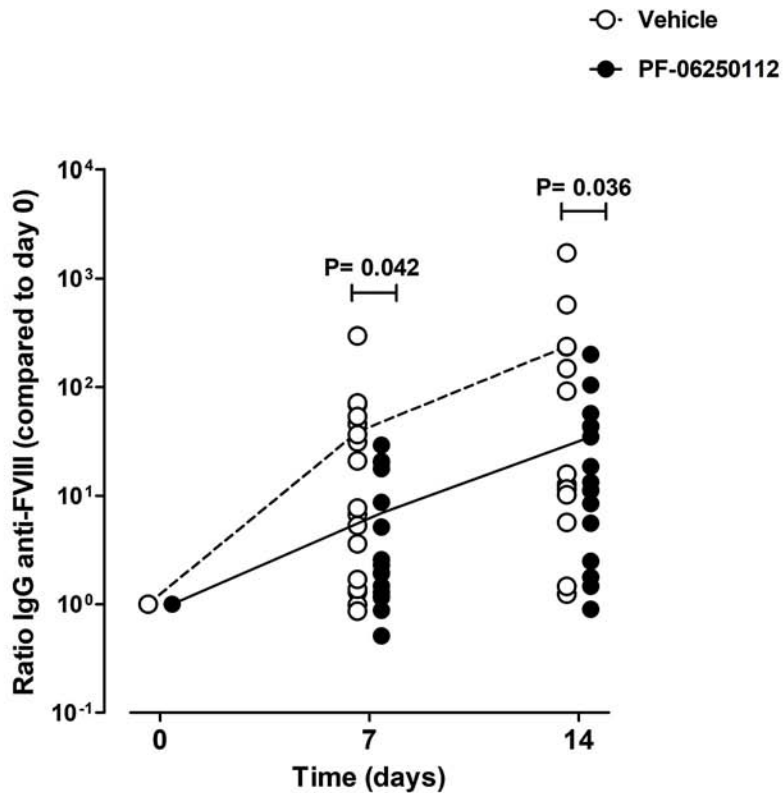


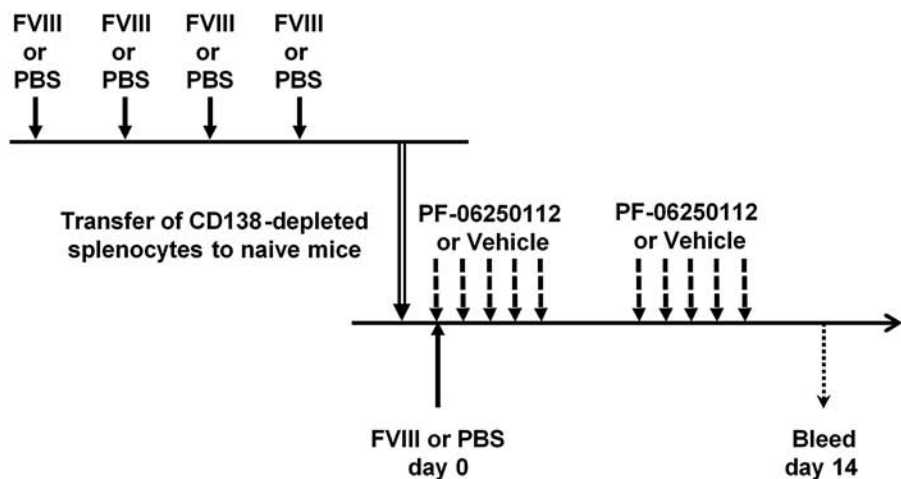
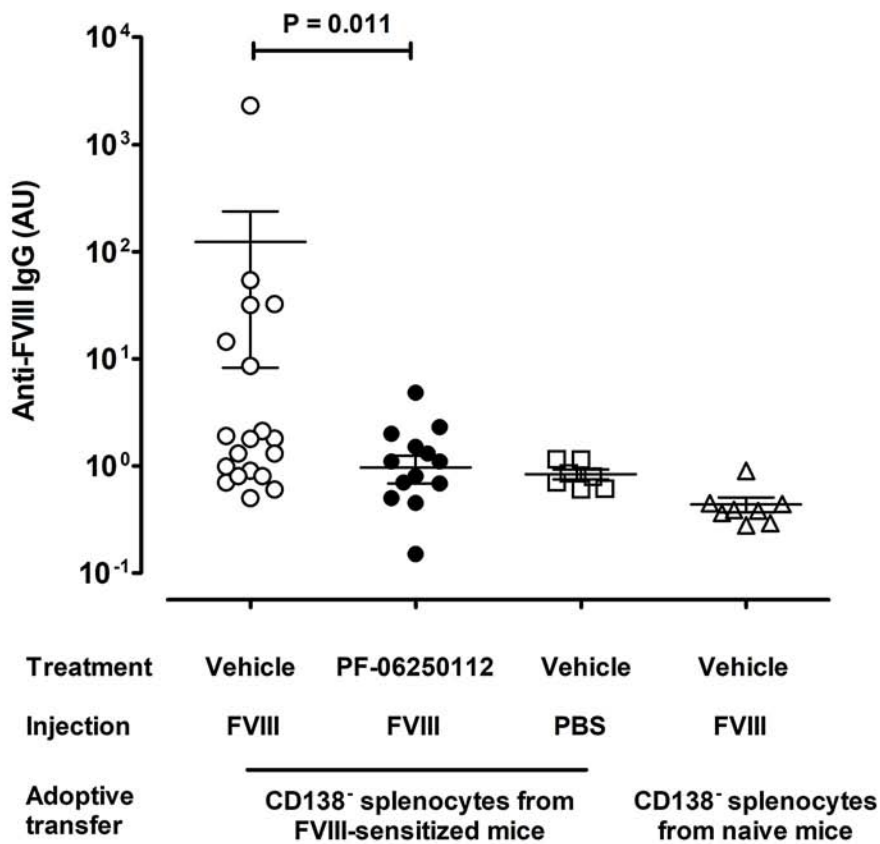
Figure 4**A****B**

Figure 5

