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Immune Modulatory Cell Therapy for Hemophilia B Based on CD20-Targeted Lentiviral Gene Transfer to Primary B Cells

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Gene-modified B cells expressing immunoglobulin G (IgG) fusion proteins have been shown to induce tolerance in several autoimmune and other disease models. However, lack of a vector suitable for gene transfer to human B cells has been an obstacle for translation of this approach. To overcome this hurdle, we developed an IgG-human factor IX (hFIX) lentiviral fusion construct that was targeted to specifically transduce cells expressing human CD20 (hCD20). Receptor-specific retargeting by mutating envelope glycoproteins of measles virus (MV)-lentiviral vector (LV) and addition of a single-chain variable fragment specific for hCD20 resulted in gene delivery into primary human and transgenic hCD20 mouse B cells with high specificity. Notably, this protocol neither required nor induced activation of the B cells, as confirmed by minimal activation of inflammatory cytokines. Using this strategy, we were able to demonstrate induction of humoral tolerance, resulting in suppression of antibody formation against hFIX in a mouse model of hemophilia B (HB). In conclusion, transduction of receptor-specific retargeted LV into resting B cells is a promising method to develop B cell therapies for antigen-specific tolerance induction in human disease.

INTRODUCTION

Hemophilia B (HB) is an X-linked disorder occurring in 1:25,000 male births, caused by a deficiency in coagulating factor IX (FIX). In its severe form (<1% FIX activity), it can result in frequent bleeding, reduced quality of life, and early death if left untreated.[1] Currently, treatment is based on intravenous (IV) administration of plasma-derived or recombinant FIX concentrate. Complications in the form of neutralizing antibodies (inhibitors) to the infused protein can occur in 1.5%–3% of HB patients.[2,3] Although the frequency of inhibitor occurrence is low, life-threatening complications, such as allergic/anaphylactic reactions, are frequently associated with inhibitor development.[4,5] Current options for FIX inhibitor management are limited, which places focus on alternative approaches for the prevention or treatment of inhibitors.[6]

Lentiviral vectors (LVs), derived from HIV-1, are potent gene therapy vehicles for robust transduction and long-term stable expression into a wide variety of dividing and non-dividing cells, both in vitro and in vivo. As with other gene-therapy vectors, major hurdles faced by lentiviruses in disease correction have been safety, specificity, and efficacy. In part due to setbacks caused by γ-retroviral vector integration into the target cell genome, third-generation lentiviral packaging systems have undergone rigorous construct optimization and development of self-inactivating long terminal repeats (SIN-LTRs) and heterologous promoters, which minimize risks of replication competence, insertional mutagenesis, or aberrant expression of adjacent cellular coding regions, thus addressing the need for safety and efficacy.[7–9] Hematopoietic stem cell therapies that incorporate these new vectors have been tested in several human diseases (Wiskott-Aldrich syndrome, X-linked severe combined immunodeficiency, β-thalassaemia, adrenoleukodystrophy, metachromatic leukodystrophy, etc).[8,10]

Most synthetic LVs are pseudotyped with an alternative envelope glycoprotein such as VSV-G (VSV-LV) for increased stability and broad cellular tropism. For most in vivo applications, however, cell selectivity is required. Although pseudotyping by specific envelope glycoproteins may alter tropism toward particular cell types such as macrophages or neurons,[11] there is a risk for off-target gene delivery that may result in aberrant expression or immune responses to the transgene or vector. Strategies such as the use of tissue-specific promoter elements or microRNA (miRNA)-based detargeting of irrelevant cell types can restrict gene expression to the cell type relevant for a particular therapeutic application, but are limited to a few targets.[12,13]

B cells are an important target for immunotherapy approaches.[14,15] However, primary resting B cells, like other G_0-arrested quiescent cells of the hematopoietic system, require cytokine- or CD40-mediated stimulation to the G_1 phase to become efficiently transduced

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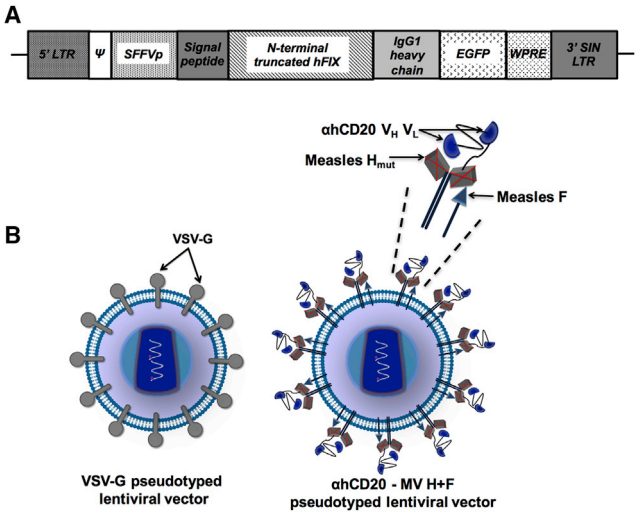

Figure 1. Retargeting LVs with Specificity for Human CD20 Expressing B Cells

(A) Schematic representation of the lentiviral construct expressing the IgG-hFIX fusion protein. LTR, long terminal repeat; ψ, packaging element; SFFVp, spleen focus forming virus promoter; eGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; 3′ SIN LTR, self-inactivating at the U3 region of the 3′ LTR. (B) Representative cartoon depicting the principle of CD20-LV. As compared to VSV-LV, CD20-LVs are pseudotyped with the MV H and F envelope glycoproteins. Retargeting is achieved by mutating surface receptor binding sites on the H protein (H_{mut}) to abolish binding to the natural receptors SLAM and CD46. Addition of scFv, consisting of the heavy and light elements of the variable region of an Ig molecule specific for hCD20 connected by a linker, redirects receptor specificity to hCD20-expressing cells.

by conventional lentiviral or retroviral vectors, which is not desirable for in vivo applications or for tolerogenic studies.[16–18] Measles virus (MV) hemagglutinin (H) and fusion (F) envelope protein pseudotyped LVs (MV-LV) have recently been shown to efficiently enter and integrate into non-activated B and T lymphocytes.[19] Importantly, MV-LV can also be used to generate targeted vectors that restrict gene delivery to cells of choice at the level of cell entry.[20,21] This strategy involves mutating the receptor-binding sites in the H protein and fusion of a single chain variable fragment (scFv) specific for the target receptor of choice. Among others, LVs delivering genes to lymphocytes with unprecedented selectivity have been generated.[22] When targeted to CD19 and CD20, selective gene delivery into resting human B lymphocytes was achieved.[21,23–25]

In this study, we expanded upon previous studies that used human CD20 (hCD20) as a target antigen for scFv binding to B cells in an MV-LV vector system. We were able to confirm that MV-LV with receptor specificity for CD20 (CD20-LV^{GFP}) efficiently transduced resting primary human B lymphocytes or transgenic (tg) murine B cells expressing hCD20. Wild-type (WT) mouse B cells were not targeted by this vector, even upon lipopolysaccharide (LPS) activation, demonstrating the remarkable specificity of this approach. CD20-LV^{GFP} was used to package an immunoglobulin G (IgG) heavy chain conjugated human FIX cassette (CD20-$LV^{hFIX\text{-}IgG}$), and autologous gene-modified hCD20-tg B cells were adoptively transferred to mediate immune tolerance to FIX in a mouse model of HB. The use of resting B cells as tolerogenic antigen-presenting cells (APCs), in combination with an IgG fusion cassette to establish tolerance,[26,27] resulted in significant prevention of inhibitory antibody development to FIX. Further, we were able to show that ex vivo lentiviral transduction of human B cells did not result in a detectable elevation of inflammatory cytokines, suggesting minimal immune responses to this tolerance approach.

RESULTS

Development of a Lentiviral Tolerance Induction Approach Targeting hCD20

Previously, we had applied an established concept for tolerance induction to HB by ex vivo retroviral transduction of LPS-stimulated mouse B cells with a truncated human FIX protein lacking the pre-pro peptide (rendering it non-functional), engineered in frame with an autologous IgG1 heavy chain lacking the signal sequence for secretion.[27] It has earlier been shown that endogenous processing of the protein by B cells could tolerize T cells directly.[28,29] The use of IgG as a carrier, combined with the capability of B cells to function as tolerogenic APC, has been applied to various animal models of autoimmune and other diseases.[26,27,30–33] We were able to demonstrate the prevention of inhibitor formation to FIX and suppression of pre-existing inhibitors in FIX-primed HB mice by this approach. However, because retroviral vectors can only transduce dividing cells, we sought to translate this strategy into LV using mice transgenic for hCD20 as a model.

It has been shown that CD20-targeted, MV glycoprotein pseudotyped LV can effectively transduce resting primary human B cells with minimal activation, characterized by a small change in cell cycle progression from the resting G_0 to the G_{1b} phase.[25] This makes these vectors a very attractive candidate in comparison to VSV-LV, especially for in vivo cell targeting.

We therefore engineered a truncated version of the human FIX coding sequence (retaining all known T cell epitopes in mice) at the N terminus of the IgG heavy chain scaffold and cloned it into the pSEW lentiviral transfer vector (Figure 1A). This was then packaged into CD20-LV^{GFP} (CD20-$LV^{hFIX\text{-}IgG}$, Figure 1B).

CD20-LV^{GFP} Selectively Transduces hCD20-Expressing Primary B Cells

Although viral titers of CD20-LV^{GFP} were lower (2 to 3 × 10^6 transducing units/mL, titrated on Raji cells) than those obtained by conventional VSV-LV, targeted specificity allowed for 39%–60% transduction of both primary human B cells and hCD20-tg resting B cells, as quantified by GFP expression of 7-AAD-excluded live cells (Figure 2). Both primary human and hCD20-tg mouse cells were transduced at a similar frequency at various MOIs, confirming that transduction depended only on expression of the targeted receptor, in this case, CD20 (Figure 2). Similarly, previous studies confirmed

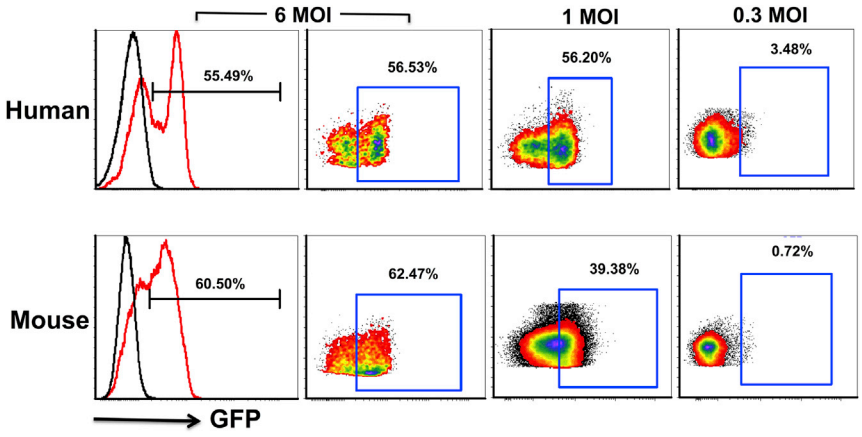

Figure 2. Representative Histogram and Intensity Dot Plots Indicating Transduction Efficacies Achieved with CD20-LV^{GFP} at Different MOIs

Primary human and hCD20 transgenic mouse B cells were transduced at 6, 1, and 0.3 MOI of LV, and percent GFP$^+$ cells were quantified after 7-AAD exclusion of dead cells. Histogram overlay at 6 MOI (black histogram: untransduced cells, red histogram: LV-transduced cells) indicate the intensity of GFP expression. Data are representative of at least two experiments.

high-target versus non-target cell discrimination upon transduction of mixed lymphocyte populations of human B and T cells.[21] Notably, the high transduction frequencies observed in this study occurred in the absence of B cell stimulation by cytokines or other factors, with implications for tolerogenic studies and in vivo gene therapy. In fact, LPS stimulation had no effect on the frequency of transduced primary human or hCD20-tg mouse cells (data not shown).

In contrast, the transduction efficacy in primary B cells from WT BALB/c mice was comparable to that of untransduced cells (0.7% GFP$^+$, Figure 3A). Transduction in WT mice was significantly lower as compared to hCD20-tg mouse B cells (Figure 3B). Thus, we were able to ensure minimal off-target gene delivery by this approach.

CD20-LV-based ex vivo transduction was also found to elicit minimal inflammatory cytokine response by real-time RT-PCR quantification. mRNA levels of interferon (IFN)-α, IFN-β, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 remained unchanged in primary human B cells at 24 hr post transduction with CD20-LV^{GFP} particles (data not shown). Incubation of cells with the TLR9 agonist CpG (5 μM ODN 2066) as a positive control resulted in 31.56 ± 2.7-fold upregulation in IL-6 as compared to control untreated cells. We were unable to consistently quantify increased mRNA expression of other inflammatory cytokines like IFN-α, IFN-β, and TNF-α by the TLR9 agonist, which has also been documented by others.[34]

B Cell-Based Immune Modulatory Therapy Prevents Inhibitor Formation in HB Mice

hCD20-tg mouse B cells transduced with CD20-LV$^{hFIX\text{-}IgG}$ were adoptively transferred into BALB/c-HB mice at 5–10 × 10^6 cells/mouse. The truncated FIX protein was earlier shown to be just as effective in generating inhibitory antibodies as the full-length protein, while consistently yielding higher-titer virus.[27] Because the frequency of transduced cells was 39%–60%, we did not flow sort for GFP$^+$ cells before adoptive transfer. HB mice on the BALB/c background do not respond as robustly to FIX protein administration, resulting in a lower incidence of inhibitor formation compared to, for example, C3H/HeJ mice.[35] In order to elicit a stronger immune response to FIX administration, BALB/c-HB mice were challenged 1 day following adoptive transfer of lentivirus-modified B cells with 1 IU FIX in adjuvant delivered by the subcutaneous (SC) route. Functional inhibitor and anti-FIX IgG1 titers were quantified 2 and 3 weeks later (Figure 4A). B cell gene transfer recipient mice developed significantly lower anti-hFIX IgG1 (4,428.01 ng/mL, Figure 4B) and Bethesda titers (0.58 BU/mL, Figure 4C) as compared to control BALB/c-HB mice that received nothing (anti-hFIX IgG1: 10,524.2 ng/mL, BU/mL: 2.33) at 2 weeks. This difference was sustained at 3 weeks (3,566.3 ng/mL versus 17,902.6 anti hFIX IgG1; 0.06 versus 2.79 BU/mL, Figures 4B and 4C).

Next, prevention of inhibitor formation to hFIX administered via the more clinically relevant IV route was tested. BALB/c-HB mice received CD20-LV$^{hFIX\text{-}IgG}$ transduced hCD20-tg mouse B cells at 5–10 × 10^6 cells/mouse as before. The following day, mice were administered with 3 IU hFIX, 1x/week for 8 weeks before quantification of functional inhibitor and anti-FIX IgG1 titers (Figure 5A). Three of four mice receiving B cell gene transfer did not develop inhibitors against FIX (0 BU), whereas one-fourth of the animals (25%) formed an inhibitor (Figure 5C). This is in contrast to the 81% inhibitor response rate seen in control animals that received protein therapy only (Figure 5C). A similar difference was seen for anti-hFIX IgG1-binding antibodies, with a 4.6-fold reduction in the average titer (Figure 5B). Because of the one mouse that failed to respond to the tolerogenic therapy, the differences in average antibody formation (856 versus 3,931 ng IgG1/mL and 2 versus 3.4 BU/mL) failed to reach statistical significance. Statistical analysis defined this animal as an outlier. On outlier removal, the treated group and control group were found to be significantly different for anti-FIX IgG1 titers (Figure 5B, $p = 0.0014$).

DISCUSSION

Tolerance induction using gene-modified primary B cells is highly promising in various mouse models of genetic disease, such as hemophilia and autoimmune diseases.[26,27,31,33,36] However, inability to efficiently transduce human B cells has kept this approach from moving forward. Here, we addressed this problem by developing a lentiviral delivery system that specifically targets hCD20. Not only were we

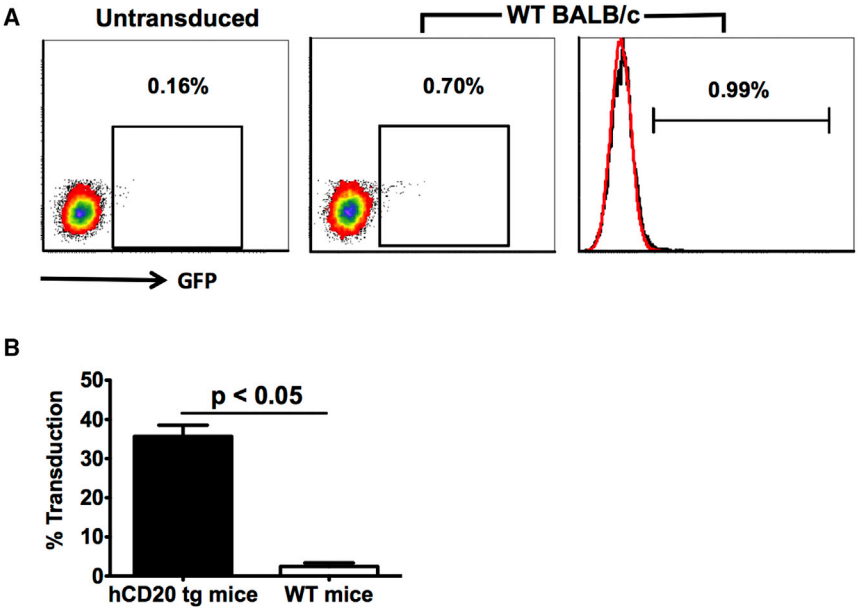

Figure 3. CD20-LV^{GFP} Does Not Transduce WT Mouse B Cells

(A) Representative histogram and intensity dot plots showing negligible transduction (% GFP$^+$) of WT BALB/c B cells by CD20-LV^{GFP} at an MOI of 6. (B) Comparison of transduction efficacies of hCD20-tg mouse B cells and WT BALB/c B cells (% GFP$^+$) by CD20-LV^{GFP} at an MOI of 1. Data are representative of at least two experiments.

able to demonstrate remarkable cell-specific targeting by this method, but the availability of a hCD20 transgenic mouse strain also allowed us to test for the effectiveness of this approach for tolerance induction in vivo. Taken together, we report a highly specific and effective protocol for tolerance induction, which indicates the feasibility of this approach for translational studies.

Prevention and management of inhibitors in HB is not addressed as commonly as that of hemophilia A because inhibitor development is less frequent. However, 9%–23% of severely affected patients develop inhibitors, of which approximately 80% are high responding ($\geq$5 BU/mL).[2,37] In addition, the frequent and simultaneous occurrence of allergic/anaphylactic reactions with the development of inhibitors is unique to HB.[4,5] Tolerance approaches to prevent or reverse FIX inhibitors are, therefore, highly desirable.[3]

We were able to show in an earlier study that B cell-based therapy for HB effectively prevented inhibitor formation to FIX and desensitized mice with a pre-existing response. This, however, involved retroviral transduction of LPS-activated murine B cells.[27] In addition, it is known that LVs transduce B cells poorly and cannot enter truly quiescent B cells, making gene transfer studies difficult for B cells. Our approach to retarget the vector to CD20-expressing B cells allowed for transduction of resting B cells without the risks of an immune response caused by activation signals provided to the B cells during gene transfer. We demonstrated receptor-specific targeting and improved tolerance to FIX by gene-modified hCD20-tg mouse B cells, resulting in a significant prevention in the development of inhibitory antibodies to FIX in a mouse model of HB. Importantly, incidence in inhibitor formation was lower in mice receiving gene-modified B cells, suggesting that this protocol may be advantageous in preventing humoral responses to coagulation factor therapy. In our previous studies in C3H HB mice, using a murine retroviral vector, we found that transplant of B cells expressing the hFIX-IgG fusion was also able to reverse pre-existing inhibitors and eliminate anaphylactic reactions.[27] Because the LV expresses the same hFIX-IgG fusion, it is likely that this vector will also be effective in inhibitor reversal. To prove this point, it will be necessary to backcross hCD20-tg mice onto the C3H background to subsequently test for inhibitor reversal in C3H/HeJ FIX$^{-/-}$ mice (without intervention, these animals have a more robust and lasting inhibitor response to hFIX, which is needed to distinguish active tolerance induction from spontaneous decline in titers).

We had previously shown that nucleofection of plasmid DNA, although achieving high gene transfer efficacy, yielded strong immune responses to FIX, which was due to TLR9-dependent activation of innate immune mediators and inflammatory cytokine expression.[27] Ex vivo lentiviral transduction did not result in a detectable immune response, as confirmed by a lack of inflammatory cytokine elevation. Consistent with results initially described by Kneissl et al., we also found that transduction by CD20-LV caused minimal activation in resting B cells, eliminating the need for potential activators of innate immunity.[25]

Toward clinical translation of this approach, upscaling and production of CD20-LV in a GMP-like process will be an important issue. Large-scale production of vector may be difficult to reach with the engineered MV glycoproteins used in this study. High transduction efficiencies on B cells allowing active hFIX secretion have recently been described for lentiviral vectors pseudotyped with the baboon envelope glycoprotein.[38] Although this could be very efficient for ex vivo B cell transduction, this vector lacks selectivity for B cells, making direct in vivo application difficult. A recent paper demonstrated that the Nipah virus (NiV) glycoproteins can be engineered for receptor targeting of LV, resulting in substantially improved vector production yields, while retaining the high selectivity of the MV-based system.[39] In particular, when the same CD20-specific scFv that was used here on the MV glycoproteins was used on the NiV glycoproteins, vector production yields increased by more than two orders of magnitude, making a scale-up for the production of clinical vector lots conceivable.

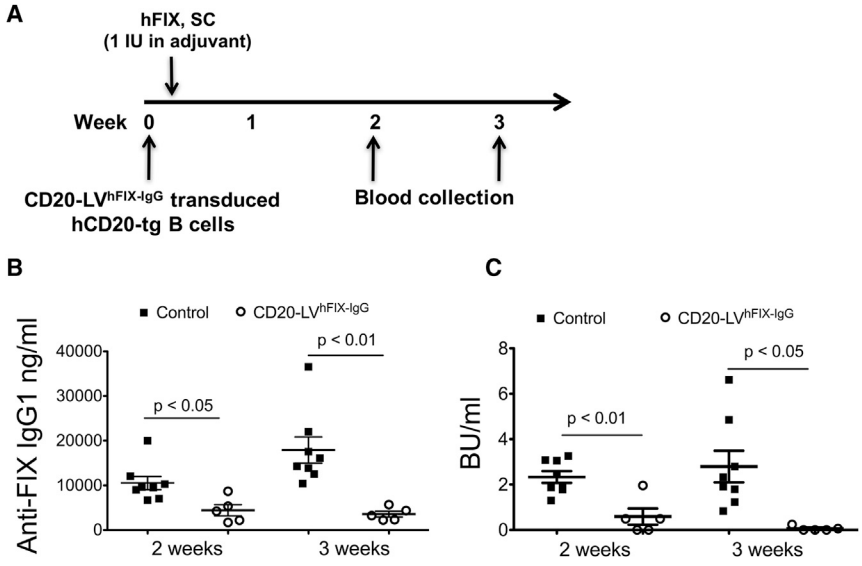

Figure 4. Adoptive Transfer of CD20-LVh$^{FIX\text{-}IgG}$ Transduced B Cells Prevents Inhibitor Formation in Response to Adjuvant Challenge with hFIX

(A) Experimental outline and timeline. BALB/c HB mice (n = 6) received CD20-LVh$^{FIX\text{-}IgG}$ transduced hCD20 transgenic mouse B cells at 5–10 × 10^6 cells/mouse. This was followed 24 hr later by SC injection of 1 IU of hFIX in adjuvant. Blood was collected on weeks 2 and 3. (B) Comparison of anti-FIX IgG1 titers (ng/mL) from control and cell transfer recipient mice. (C) Inhibitor titers (BU/mL) from control mice and mice that received transduced B cells. Data are average ± SD. Statistical differences are indicated for each time point.

MATERIALS AND METHODS

Generation of Constructs and LV Production

The plasmid pCG-Hmut-αCD20-ScFv was as previously described.[24] Briefly, the αCD20-ScFv was linked to the ectodomain of the pCG-H_{mut} plasmid via the factor Xa cleavage site. This composed an 18 amino acid (aa) cytoplasmic tail truncation and mutations in the CD46 (Y481A, S548L, and F549S) and signaling lymphocyte activation molecule (SLAM, R533A) interaction regions of the H protein of the MV Edmonston strain.[40] The pCG-Fcd30 plasmid composed a 30 aa cytoplasmic tail truncation. Cytoplasmic tail truncations of MV H and F proteins facilitated efficient incorporation of these proteins into the lentiviral membrane. All plasmids were isolated using the EndoFree Plasmid Maxi Kit (QIAGEN). CD20-LV^{GFP} particles were produced by polyethyleneimine (PEI)-based co-transfection of HEK293T cells with the HIV-1 packaging plasmid pCMVΔR8.9, the GFP transfer plasmid pSEW-GFP, and the two plasmids encoding the modified H and F proteins, respectively (H:F ratio of 1:7), as described.[25] Vector particles were concentrated by ultracentrifugation over a 20% (weight/vol) sucrose cushion (100,000 × *g* for 3 hr at 4°C) and titrated by transduction on Raji cells.

To generate the LV transfer vector plasmid CD20-LV$^{hFIX\text{-}IgG}$ encoding the hFIX-IgG fusion protein, a 0.9-kb truncated version of the human FIX coding sequence (lacking the N terminus) was PCR amplified from the AAV2-EF1α-FIX vector.[27] The truncated version retained all known T cell epitopes in mice. This was subcloned into BSSK-IgG, a murine IgG1 H chain cassette plasmid containing *Not*I and *Xho*I cloning sites at the N terminus of the IgG1 H chain

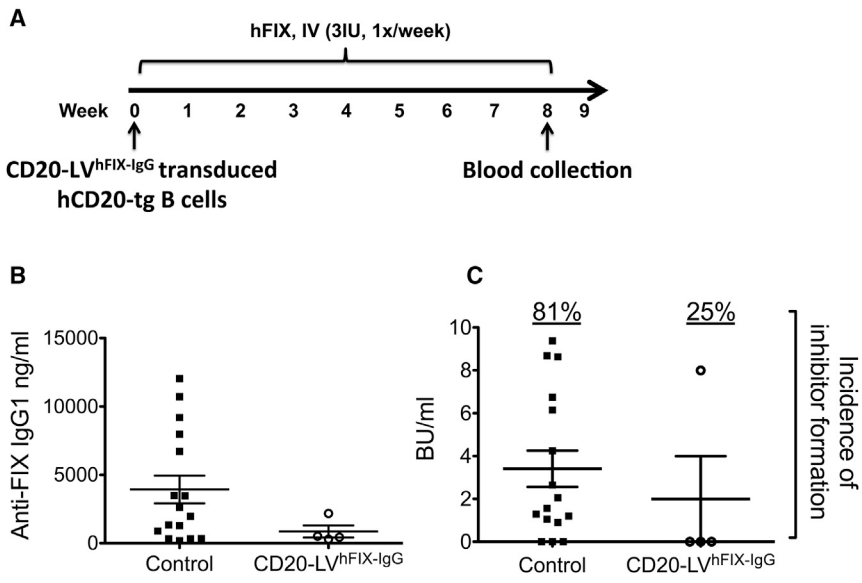

Figure 5. CD20-LV$^{hFIX\text{-}IgG}$ Transduced B Cells Reduce Inhibitor Formation in Response to IV Challenge with hFIX

(A) Experimental outline and timeline. BALB/c HB mice (n = 4) received CD20-LVh$^{FIX\text{-}IgG}$ transduced hCD20-tg mouse B cells at 5–10 × 10^6 cells/mouse. This was followed by eight weekly IV injections of 3 IU of hFIX. Blood was collected on week 8. (B) Comparison of anti-FIX IgG1 titers (ng/mL) from control and cell transfer recipients. (C) Inhibitor titers (BU/mL) from control mice and mice that received transduced B cells. The incidence of inhibitor formation for both groups of mice is indicated. Data are average ± SD. Statistical differences are indicated for each time point.

(a gift of Dr. R. Tisch, University of North Carolina).[31] The signal sequence of the IgG H chain was previously deleted from the BSSK-IgG vector by site-directed mutagenesis. The hFIX-IgG region was then inserted into the transfer plasmid pSEW-GFP, thereby replacing the GFP reading frame, and LV particles were produced as described.[25]

B Cell Transduction and Flow Cytometry

WT mouse B cells and hCD20-tg B cells were purified using the mouse B cell isolation kit (Miltenyi Biotec), which isolates untouched resting B cells. Primary human $CD19^+$ B cells (mostly $CD20^+$), negatively selected from peripheral blood mononuclear cells (PBMCs), were purchased from Precision for Medicine. Resting human and mouse B cells were transduced with CD20-LV^{GFP} or CD20-$LV^{hFIX\text{-}IgG}$, respectively, by spinoculation at 1,200 × *g* for 90 min on retronectin (20 μg/mL, Clontech) coated six-well plates. The percentage of GFP^+ cells was determined by flow cytometric analysis after 72 hr. Dead cells were excluded by 7-AAD staining (eBioscience). Data were collected on an LSR-II flow cytometer (BD Biosciences) and analyzed with FCS Express 5 (De Novo Software).

Mouse Strains

Animals were housed under specific pathogen-free conditions under institutional Animal and Care and Use Committee approved protocols. All animals were male and 6–8 weeks old at the onset of the experiments. HB mice with a targeted deletion (promoter and first three exons) of murine *F9* (null mutation) were bred on a BALB/c background for >10 generations.[41] Human CD20-tg BALB/c mice were a kind gift from Dr. Mark J. Shlomchik (University of Pittsburgh). Mice were generated by injecting a bacterial artificial chromosome carrying the gene for hCD20 into the pronuclei of C57BL/6 x SJL/j embryos.[42] hCD20-tg mice were backcrossed for seven generations into the BALB/c background.[42]

Prevention of Inhibitor Formation

BALB/c-HB mice were adoptively transferred with 5–10 × 10^6 primary murine hCD20-tg B cells transduced with CD20-$LV^{hFIX\text{-}IgG}$. This was followed by either SC immunization with 1 IU hFIX (Benefix, Pfizer) in adjuvant (Sigma Adjuvant System) or 3 IU hFIX replacement therapy by the IV route, once a week, for 8 weeks. Plasma samples were collected by tail bleed into 0.38% sodium citrate, and inhibitory antibodies to FIX were measured by Bethesda assay, as described.[43] Measurements were carried out in a Diagnostica Stago STart Hemostasis Analyzer. ELISA-based measurements of IgG1 antibodies to hFIX were carried out as described.[43]

Statistical Analysis

Statistical significance was determined using two-way ANOVA or Student's t test with GraphPad Prism 5 software. Incidence of inhibitor formation was calculated using Fisher's exact test. Values at $p < 0.05$ were deemed significant. Outlier analysis was performed using the Grubb's test on GraphPad Prism 6.

AUTHOR CONTRIBUTIONS

X.W., S.R.P.K., Q.Z., and M.B. performed the experiments. X.W., R.W.H., B.J.B., C.J.B., and M.B. designed the experiments. X.W., R.W.H., B.J.B., C.J.B., and M.B. analyzed and interpreted the data. X.W., R.W.H., B.J.B., C.J.B., and M.B. wrote the manuscript. R.W.H. supervised the study.

CONFLICTS OF INTEREST

C.J.B. is listed as inventor on patent applications for receptor-targeted lentiviral vectors that have been out-licensed.

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