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ORIGINAL ARTICLE

Factor IX administration in the skin primes inhibitor formation and sensitizes hemophilia B mice to systemic factor IX administration

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Abstract

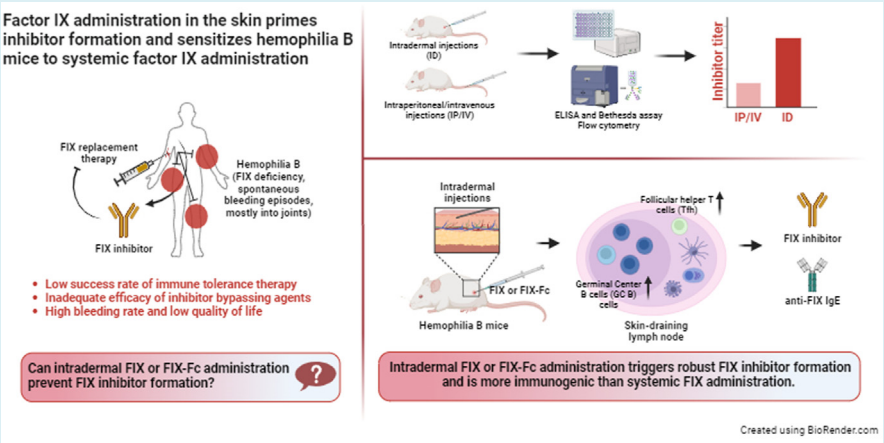
Background: Factor IX inhibitor formation is the most serious complication of replacement therapy for the bleeding disorder hemophilia B, exacerbated by severe allergic reactions occurring in up to 60% of patients with inhibitors. Low success rates of immune tolerance induction therapy in hemophilia B necessitate the search for novel immune tolerance therapies. Skin-associated lymphoid tissues have been successfully targeted in allergen-specific immunotherapy.

Objectives: We aimed to develop a prophylactic immune tolerance protocol based on intradermal administration of FIX that would prevent inhibitor formation and/or anaphylaxis in response to replacement therapy.

Methods: We measured FIX inhibitor, anti-FIX immunoglobulin G1, and immunoglobulin E titers using the Bethesda assay and enzyme-linked immunosorbent assay after 4 weeks of twice-weekly intradermal FIX or FIX-Fc administration followed by 5 to 6 weeks of weekly systemic FIX injections in C3H/HeJ hemophilia B mice. We also measured skin antigen-presenting, follicular helper T, and germinal center B cell frequencies in skin-draining lymph nodes after a single or repeat intradermal FIX administration.

Results: Intradermal administration enhanced FIX inhibitor formation in response to systemic administration. We further found that intradermal administration alone triggers inhibitor formation, even at a low dose of 0.4 IU/kg, which is 100-fold lower than the intravenous dose of 40 IU/kg typically required to induce inhibitor development in hemophilia B mice. Also, intradermal administration triggered germinal center formation in skin-draining lymph nodes and sensitized mice to systemic administration. Factor IX–Fc fusion protein did not modulate inhibitor formation.

Conclusion: Intradermal FIX administration is highly immunogenic, suggesting that the skin compartment is not amenable to immune tolerance induction or therapeutic delivery of clotting factors.



KEYWORDS
anaphylaxis, factor IX, hemophilia B, immune tolerance, inhibitor

Essentials

- People with factor IX inhibitors urgently need better immune tolerance therapy.
- We attempted tolerance induction by administering FIX or FIX-Fc in the skin of hemophilia B mice.
- Administration of FIX or FIX-Fc in the skin is more immunogenic than systemic injections.
- The skin is not amenable to tolerance induction toward or therapeutic delivery of FIX.

1 | INTRODUCTION

Congenital hemophilia B is an X-linked bleeding disorder caused by mutations in the *F9* gene, which lead to deficiency of coagulation factor IX. The standard of care is protein replacement therapy using clotting factor concentrates [1]. However, approximately 5% of patients develop inhibitors, which are neutralizing antidrug alloantibodies that make the therapy ineffective [2]. This continues to be the most serious complication of hemophilia B treatment, dramatically increasing morbidity. Management of bleeding events in patients with FIX inhibitors requires by-passing agents, which show lower efficacy than FIX replacement therapy. Immune tolerance induction therapy eradicates inhibitors only in 20% to 30% of patients and necessitates frequent intravenous (i.v.) infusions of FIX concentrates, placing a heavy burden on patients and generating high treatment costs [3]. In addition, up to 60% of individuals with FIX inhibitors develop allergic reactions, further increasing morbidity and discouraging healthcare providers from attempts at tolerization [4,5]. Several clinical trials evaluate new nonfactor replacement therapies that promise to improve outcomes in persons with hemophilia B and FIX inhibitors, but inhibitor eradication and restoration of response to the replacement therapy remain paramount [6]. Therefore, development of a novel, noninvasive tolerance induction protocol is an urgent need for persons with hemophilia B with inhibitors.

We have previously induced tolerance to FIX protein replacement therapy in murine and canine models of hemophilia B via oral delivery of FIX in transgenic plants [7,8]. Twice per week oral gavage of transplastomic lettuce starting 1 month before initiation of FIX treatment suppressed FIX inhibitor formation and fatal anaphylactic reactions in mice and dogs with hemophilia B, and reversed preexisting inhibitors in hemophilia B mice by activation of regulatory T cells [9,10].

Here, we adapted our prophylactic oral tolerance protocol to attempt tolerance induction by administering FIX or FIX-Fc fusion protein (FIX-Fc) in the skin of hemophilia B mice. The skin is an immunologically active organ, harboring high numbers of antigen-presenting cells and regulatory T cells, and this environment is relatively easily accessible [11]. Importantly, the intradermal route has been successfully used for drug delivery and tolerance induction in peanut allergy [12]. We therefore hypothesized that this approach to antigen-specific immunotherapy may be effective in FIX replacement therapy, which is similarly plagued by antibody formation (including immunoglobulin G [IgG] and immunoglobulin E [IgE]) and anaphylactic reactions. Disappointingly, we found that intradermal FIX or FIX-Fc administration triggers strong inhibitor responses even at markedly lower doses than i.v. administration and sensitizes hemophilia B mice to systemic FIX delivery.

2 | METHODS

2.1 | Animals

Hemophilia B mice on the C3H/HeJ genetic background were as published and bred at Indiana University [13,14]. Male mice aged approximately 8 weeks were housed and treated under Institutional

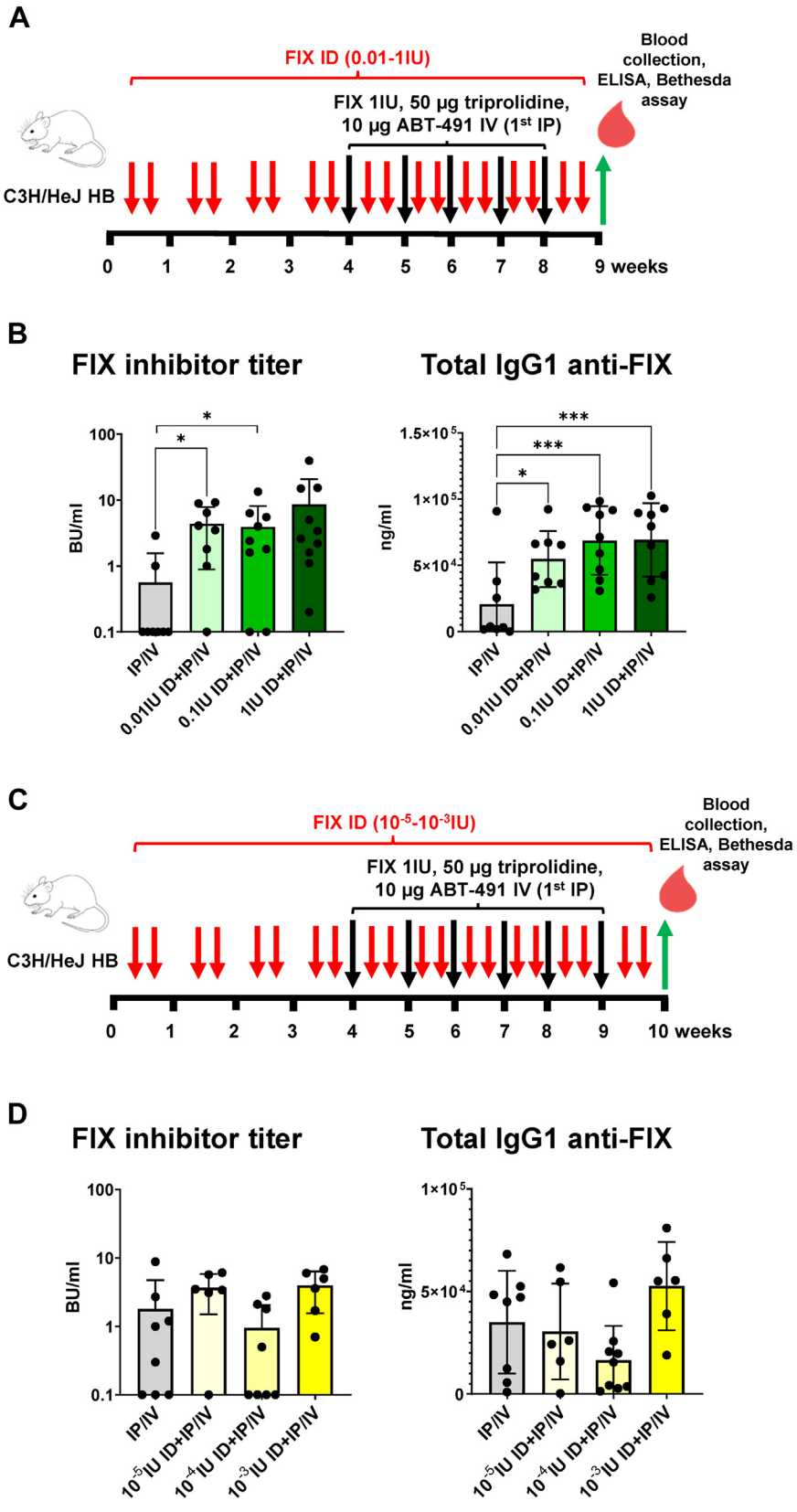


FIGURE 1 Intradermal factor IX administration does not induce immune tolerance toward FIX. (A) Experimental timeline. C3H/HeJ hemophilia B (HB) mice received FIX intradermally (ID) (0.01-1 IU) twice weekly for 4 weeks before initiation of once weekly intraperitoneal (IP)/intravenous (i.v.) FIX (1 IU) + triprolidine + ABT-491 administration for 5 weeks with intradermal injections continued throughout (n = 8-9 per group). At 9 weeks, blood was collected for enzyme-linked immunosorbent assay (ELISA) and Bethesda assay. (B) Bethesda assay and anti-FIX immunoglobulin G (IgG) 1 ELISA results in mice that received FIX IP/i.v. only (IP/i.v.) or ID (0.01-1 IU) + IP/i.v. (C) Experimental timeline. C3H/HeJ HB mice received FIX ID (10^{-5}-10^{-3} IU) twice weekly for 4 weeks before initiation of once weekly IP/i.v. FIX (1 IU) + triprolidine + ABT-491 administration for 6 weeks with intradermal injections continued throughout (n = 6-8 per group). At 10 weeks, blood was collected for ELISA and Bethesda assay. (D) Bethesda assay and anti-FIX IgG1 ELISA results in mice that received FIX IP/i.v. only (IP/i.v.) or ID (10^{-5}-10^{-3} IU) + IP/i.v. Shown are means ± SDs and P values from analysis of variance (*P < .05, **P < .01, ***P < .001, ****P < .0001). BU, Bethesda unit.

TABLE 1 Inhibitor and anti–factor IX immunoglobulin G1 titers in hemophilia B mice that received 10^{-5} to 1 IU of FIX intradermally + intraperitoneally/intravenously.

Animal group	1 IU FIX IP/i.v.	10^{-5} IU FIX ID + IP/i.v.	10^{-4} IU FIX ID + IP/i.v.	10^{-3} IU FIX ID + IP/i.v.	0.01 IU FIX ID + IP/i.v.	0.1 IU FIX ID + IP/i.v.	1 IU FIX ID + IP/i.v.
Inhibitor (BU/mL), mean ± SD	0.6 ± 1.0 (5w) 1.8 ± 3.0 (6w)	3.7 ± 2.2	1.0 ± 1.1	4.0 ± 2.4	4.4 ± 3.5	3.9 ± 4.2	8.6 ± 12.2
IgG1 (μg/mL), mean ± SD	20.6 ± 31.5 (5w) 35.0 ± 25.0 (6w)	30.4 ± 23.4	16.4 ± 16.8	52.6 ± 21.5	54.8 ± 21.2	68.8 ± 25.8	69.3 ± 27.7

BU, Bethesda unit; FIX, factor IX; ID, intradermally; IP, intraperitoneal; i.v., intravenous.

Animal Care and Use Committee–approved protocols at Indiana University.

2.2 | Intradermal and systemic administration of FIX

The animals received 10^{-5} to 1 IU FIX (Benefix, Pfizer) or 0.01 to 1 IU FIX-Fc (Alprolix, Sanofi) intradermally (ID) in the groin area twice per week for 4 weeks and continued throughout at the same frequency after initiation of intraperitoneal (IP) and i.v. administration of 1 IU FIX (Benefix, Pfizer) ±50 μg triprolidine (antihistamine; Sigma) and 10 μg ABT-491 (platelet-activating factor receptor antagonist; Sigma) in the tail vein once per week for 5 to 6 weeks (1 IP followed by 4 or 5 i.v. injections). IP administration before continuing with i.v. injections ensured more consistent inhibitor formation in the control groups with lower nonresponse rates. Triprolidine and ABT-491 were coinjected to prevent anaphylaxis-related mortality.

2.3 | Antibodies

Fluorescent anti-mouse monoclonal antibodies used in flow cytometry were CD4 (RM4-5), CXCR5 (L138D7), CD95 (SA367H8), GL7 (GL7), PD-1 (29F.1A12), CD11b (M1/70), CD8α (53-6.7), CD11c (N418), CD207 (4C7), and EpCAM (G8.8) from BioLegend and CD19 (1D3) and MHC-II (M5/114.15.2) from eBioscience.

2.4 | Bethesda and anti-FIX antibody enzyme-linked immunosorbent assay

For plasma samples, mice were anesthetized with isoflurane, and blood was collected via the retro-orbital plexus. Sodium citrate (3.2%) was added to the samples at one-tenth total volume. Plasma was isolated by centrifugation. Factor IX inhibitor titers were measured by Bethesda assay, and total anti-FIX immunoglobulins in plasma were measured by enzyme-linked immunosorbent assay (ELISA) as published [14–16]. Bethesda assay measurements were performed using a Diagnostica Stago STart Hemostasis Analyzer. Titers greater than 0.6 Bethesda units were considered positive. To measure IgE titers, IgG was removed from plasma samples using protein G Sepharose columns prior to ELISA (GE HealthCare).

2.5 | Flow cytometry

Single-cell suspensions of inguinal lymph nodes were prepared by passing them through a 70-μm cell strainer in cold phosphate-buffered saline (PBS). Upon pretreatment with Fcγ receptor block (anti-mouse CD16/CD32, BD Biosciences), cells were stained with antibodies at 4 °C for 15 minutes in the dark, followed by a viability dye. Live cells were stained using Zombie Aqua (BioLegend) according to the manufacturer's instructions. Data were collected on LSRFortessa (BD Biosciences) and analyzed with FCS Express (De Novo Software).

2.6 | Statistical analysis

Data are reported as means ± SDs. Significant differences were determined using the Mann–Whitney U-test, 1-way analysis of variance, or log-rank test. P values <.05 were considered significant ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$). All analyses were performed using GraphPad Prism.

3 | RESULTS

3.1 | Intradermal FIX administration enhances inhibitor formation in response to systemic FIX administration

The overall aim of this study was to develop a novel immune tolerance induction protocol based on intradermal delivery of coagulation FIX. The first tolerogenic dose-finding experiment administered 8 intradermal injections of FIX over 4 weeks in the range of doses from 0.01 to 1 IU, followed by 5 weekly IP/i.v. injections (the first injection was delivered intraperitoneally, and the other 4 were administered intravenously) of 1 IU FIX coadministered with antianaphylaxis agents to hemophilia B mice (Figure 1A). All experimental groups developed significantly (8.9-17.6–fold) higher mean FIX inhibitor titers than the control group that received IP/i.v. treatment only. The mean total anti-FIX IgG1 titers were also significantly higher (2.7-3.4–fold) in all ID pretreated groups, but the differences were less pronounced than the differences between inhibitor titers (Figure 1B, Table 1).

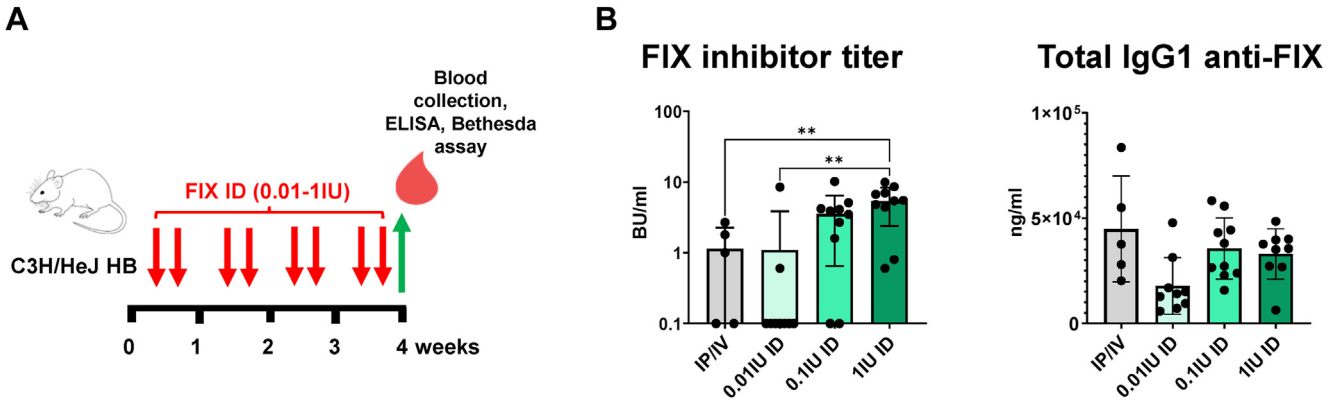


FIGURE 2 Factor IX administration in the skin alone triggers robust FIX inhibitor formation. (A) Experimental timeline. C3H/HeJ hemophilia B (HB) mice received FIX intradermally (ID) (0.01-1 IU) twice weekly for 4 weeks or once weekly intraperitoneal (IP)/intravenous (i.v.) FIX (1 IU) + triprolidine + ABT-491 administration for 6 weeks (n = 5-9 per group). At 4 weeks, blood was collected for enzyme-linked immunosorbent assay (ELISA) and Bethesda assay. (B) Bethesda assay and anti-FIX immunoglobulin G (IgG) 1 ELISA results in mice that received FIX IP/i.v. only (IP/i.v.) or ID (0.01-1 IU) only. Shown are means ± SDs and P values from analysis of variance (*P < .05, **P < .01, ***P < .001, ****P < .0001). BU, Bethesda unit.

We next examined a yet lower range of intradermal doses, 10^{-5} to 10^{-3} IU, followed by 1 IP and 5 weekly i.v. injections of 1 IU FIX with antiallergic agents (Figure 1C). Animals in all 3 experimental groups developed FIX inhibitors. The differences in mean FIX inhibitor titers between the experimental and the control group (which received the IP/i.v. treatment only) were not statistically significant, but the highest dose (10^{-3} IU FIX) was the only group without non-responders (Figure 1D, Table 1).

3.2 | FIX injections in the skin alone trigger potent FIX inhibitor formation

The enhancement of immune response to IP/i.v. FIX prompted us to investigate whether intradermal FIX administration alone could elicit FIX inhibitor formation (Figure 2A). We found that 2 (0.1 and 1 IU) out of the 3 evaluated intradermal doses of FIX led to inhibitor formation of similar magnitude to the full-length regimen that included the IP/i.v. treatment. Notably, although the lowest intradermal dose of FIX (0.01 IU) did not seem to produce inhibitor responses in most of the mice (7 out of 9 mice in that group had no detectable FIX inhibitor), the same dose, when followed by the IP/i.v. treatment, resulted in a similar inhibitor response to the 2 higher doses (0.1 IU and 1 IU), suggesting that the lowest dose primed the immune responses, which were further amplified by the IP/i.v. regimen. Also, all ID treated animals had readily detectable anti-FIX IgG1 antibody titers, but the levels were lower than those after the full-length treatment (Figure 2B, Table 2). This suggests that the IP/i.v. regimen after the intradermal pretreatment enhanced mainly the total anti-FIX IgG1 response, with a lesser impact on the neutralizing antibody development.

TABLE 2 Inhibitor and anti–factor IX immunoglobulin G1 titers in hemophilia B mice that received 0.01 to 1 IU of FIX intradermally.

Animal group	1 IU FIX IP/i.v.	0.01 IU FIX ID	0.1 IU FIX ID	1 IU FIX ID
Inhibitor (BU/mL), mean ± SD	1.1 ± 1.1	1.1 ± 2.8	3.6 ± 2.9	5.4 ± 3.0
IgG1 (μg/mL), mean ± SD	44.9 ± 25.2	17.8 ± 13.5	35.6 ± 14.6	33.0 ± 12.0

BU, Bethesda unit; FIX, factor IX; ID, intradermally; IP, intraperitoneal; i.v., intravenous.

3.3 | Intradermal administration of FIX triggers germinal center formation in skin-draining lymph nodes

We next examined which antigen-presenting cells respond to intradermal FIX administration and asked whether it triggers germinal center (GC) formation in skin-draining lymph nodes. To that end, we analyzed frequencies of conventional dendritic cells (DCs) type 1 (cDC1; $CD11c^+MHC\text{-}II^+CD11b^-CD8\alpha^+$) and type 2 (cDC2; $CD11c^+MHC\text{-}II^+CD11b^+CD8\alpha^-$), dermal DCs $CD207^-$ ($CD11c^+MHC\text{-}II^+CD11b^{+/-}CD207^-$) and $CD207^+$ ($CD11c^+MHC\text{-}II^+CD11b^{low}CD207^-$), and Langerhans cells ($CD11c^+MHC\text{-}II^+CD11b^+CD207^+EpCAM^+$). Twenty-four hours after administration, we did not find significant differences between FIX- and PBS-injected mice, but mean Langerhans cell and cDC2 frequencies trended 2.1- and 1.9-fold higher, respectively, in animals that received FIX (Figure 3A).

We also analyzed frequencies of follicular helper T (Tfh; $CD4^+PD\text{-}1^+CXCR5^+$) and GC B cells (GC B; $CD19^+GL7^+CD95^+$) in inguinal (skin-draining) lymph nodes from animals treated ID with FIX or PBS (negative control) twice per week for 4 weeks. We found 1.8-fold and 3.7-fold higher mean frequencies of Tfh and GC B cells, respectively, in lymph nodes from animals that received FIX (Figure 3B).

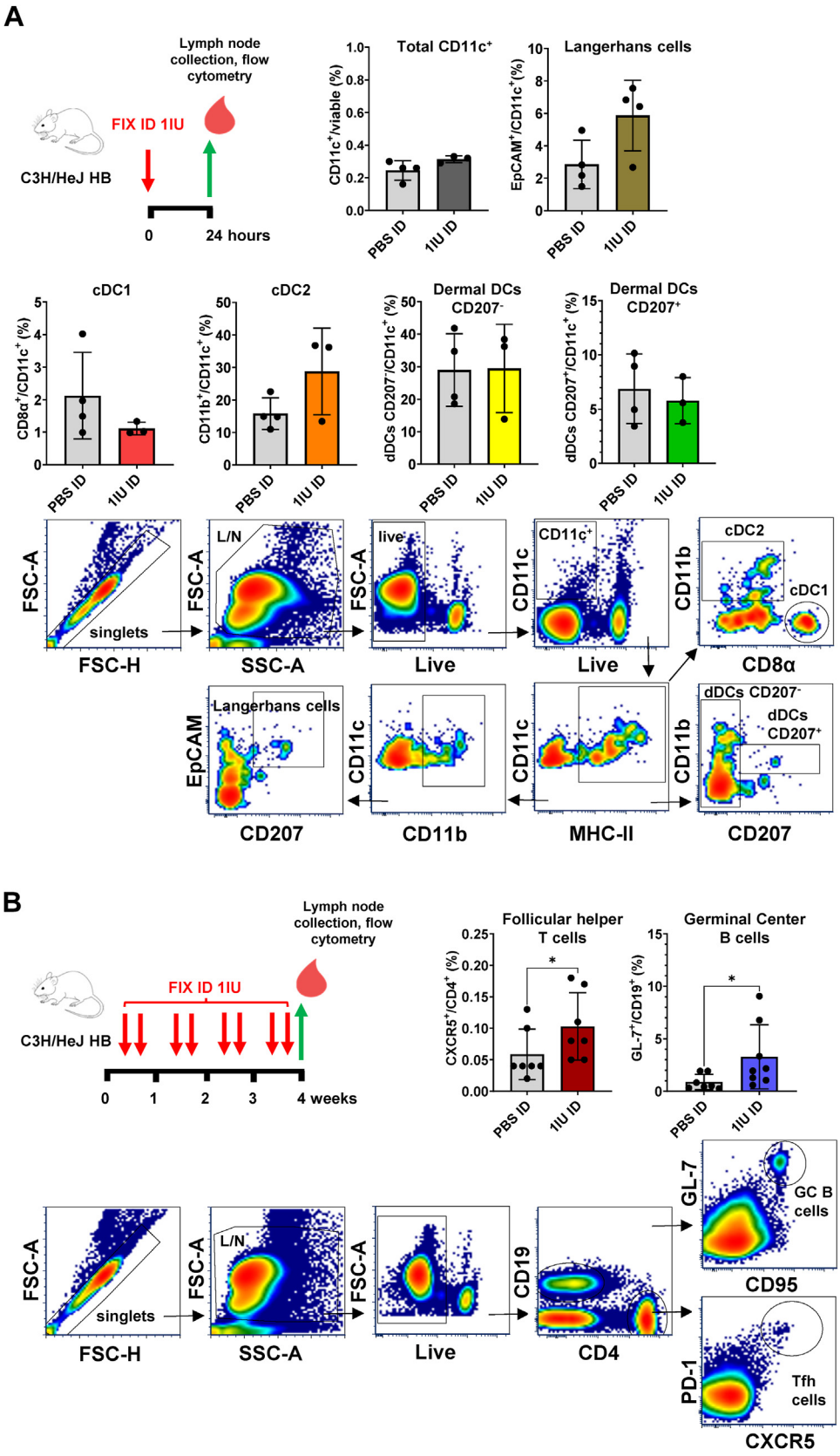


FIGURE 3 Factor IX administration in the skin induces expansion of follicular helper T cells and formation of germinal centers in skin-draining lymph nodes. (A) Flow cytometry analysis of skin antigen presenting cells after a single intradermal injection of FIX in C3H/HeJ hemophilia B (HB) mice. Twenty-four hours after administration, inguinal lymph nodes were collected for processing and frequency analysis of live $CD11c^+$ cells, conventional dendritic cells (DCs) type 1 (cDC1; $CD11c^+MHC\text{-}II^+CD11b^-CD8\alpha^+$), conventional DCs type 2 (cDC2;

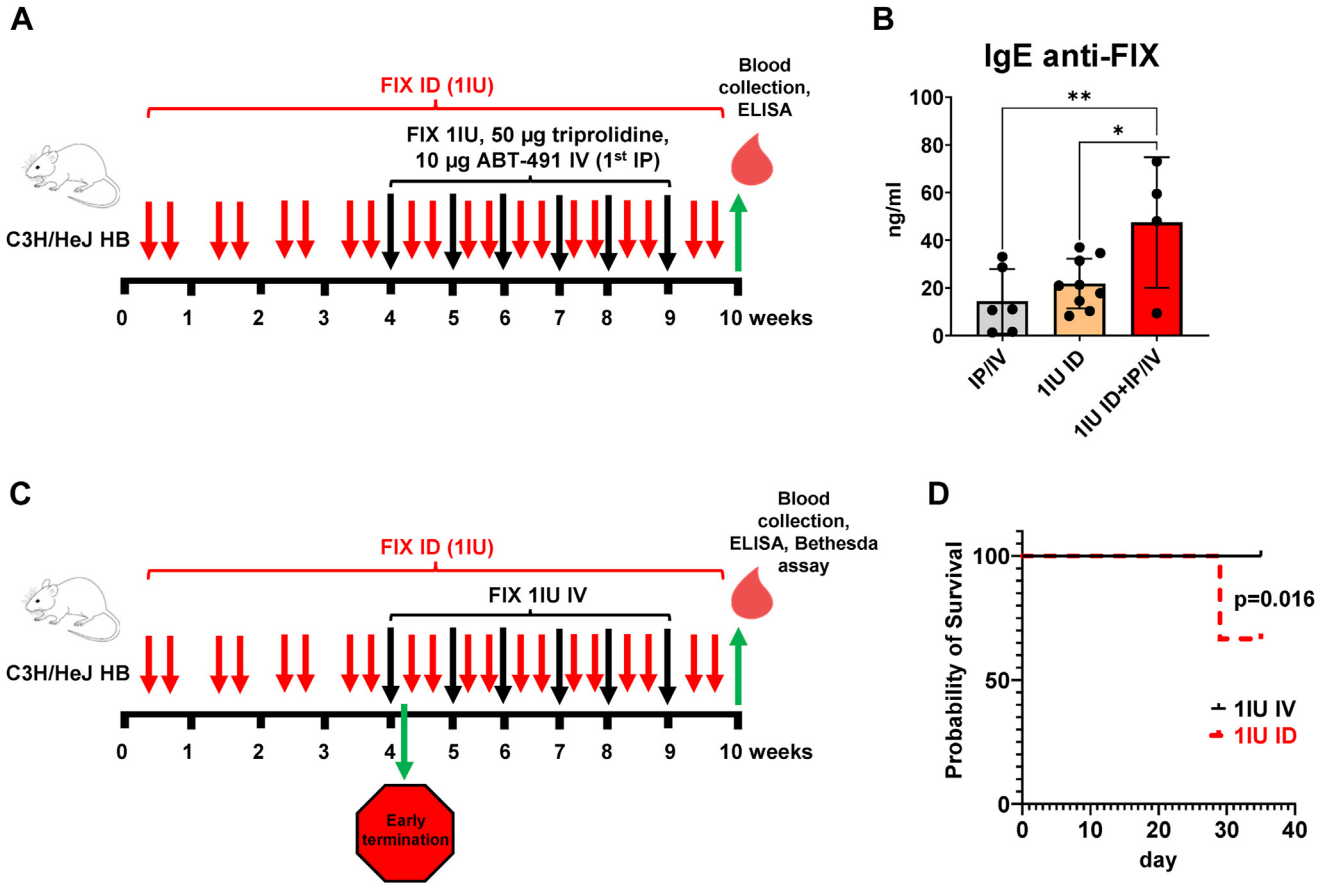


FIGURE 4 Intradermal factor IX administration sensitizes hemophilia B (HB) mice to systemic FIX administration. (A) Experimental timeline. C3H/HeJ HB mice received FIX intradermally (ID) (1 IU) twice weekly for 4 weeks before initiation of once weekly intraperitoneal (IP)/intravenous (i.v.) FIX (1 IU) + triprolidine + ABT-491 administration for 6 weeks with intradermal injections continued throughout (n = 4-9 per group). At 10 weeks, blood was collected for enzyme-linked immunosorbent assay (ELISA). (B) Anti-FIX immunoglobulin E (IgE) ELISA results in mice that received FIX IP/i.v. only (IP/i.v.), ID only or ID + IP/i.v. (C) Experimental timeline. C3H/HeJ HB mice received FIX ID (1 IU) twice weekly for 4 weeks before a single i.v. dose of FIX (1 IU) injection, after which the experiment was stopped (n = 15 per group). (D) Survival of C3H/HeJ HB mice upon receiving 1 IU FIX i.v. with (1 IU ID) or without (1 IU i.v.) intradermal pretreatment. Shown are means ± SDs and P values from analysis of variance or log-rank test (*P < .05, **P < .01, ***P < .001, ****P < .0001).

3.4 | Repeat FIX injections in the skin do not trigger anaphylaxis but sensitize hemophilia B mice to systemic FIX administration

Interestingly, none of the ID treated mice died of anaphylaxis despite receiving 8 intradermal injections of FIX without antiallergic agents, which were injected only with the IP/i.v. FIX. Intravenous administration of FIX alone in hemophilia B mice on C3H/HeJ background results in fatal IgE-dependent anaphylaxis beginning after fourth injection with ~20% mortality and rising with subsequent injections in the surviving mice. Therefore, prolonged studies in this mouse line necessitate the use of antiallergic agents [15]. We measured anti-FIX IgE titers in animals that received FIX intraperitoneally/intravenously only, ID only, or ID + intraperitoneally/intravenously (Figure 4A). We found that ID + intraperitoneally/intravenously treated mice had 3.3- and 2.1-fold higher mean anti-FIX IgE titers than intraperitoneally/intravenously and ID treated animals, respectively. Surprisingly, mice that received intradermal injections only had 1.5-fold higher mean anti-FIX IgE titers than mice that received the IP/i.v. treatment only, although the difference was not statistically significant (Figure 4B). Nevertheless, the 0% mortality rate in ID treated animals suggested that intradermal FIX delivery might have a protective effect against

$CD11c^+MHC\text{-}II^+CD11b^+CD8\alpha^-$), dermal DCs $CD207^-$($CD11c^+MHC\text{-}II^+CD11b^{+/-}CD207^-$), dermal DCs $CD207^+$ ($CD11c^+MHC\text{-}II^+CD11b^{low}CD207^-$), and Langerhans cells ($CD11c^+MHC\text{-}II^+CD11b^+CD207^+EpCAM^+$). (B) Flow cytometry analysis of follicular helper T (Tfh) cell and Germinal Center B (GC B) cell frequencies after four weeks of twice-weekly intradermal injections of FIX in C3H/HeJ HB mice. At 4 weeks, inguinal lymph nodes were collected for processing and frequency analysis of Tfh ($CD4^+PD\text{-}1^+CXCR5^+$) and germinal center B (GC B; $CD19^+GL7^+CD95^+$) cells. Density plots show gating schemes. All frequency graphs show means ± SD and P values from analysis of variance (*P < .05, **P < .01, ***P < .001, ****P < .0001). ID, intradermally; PBS, phosphate-buffered saline.

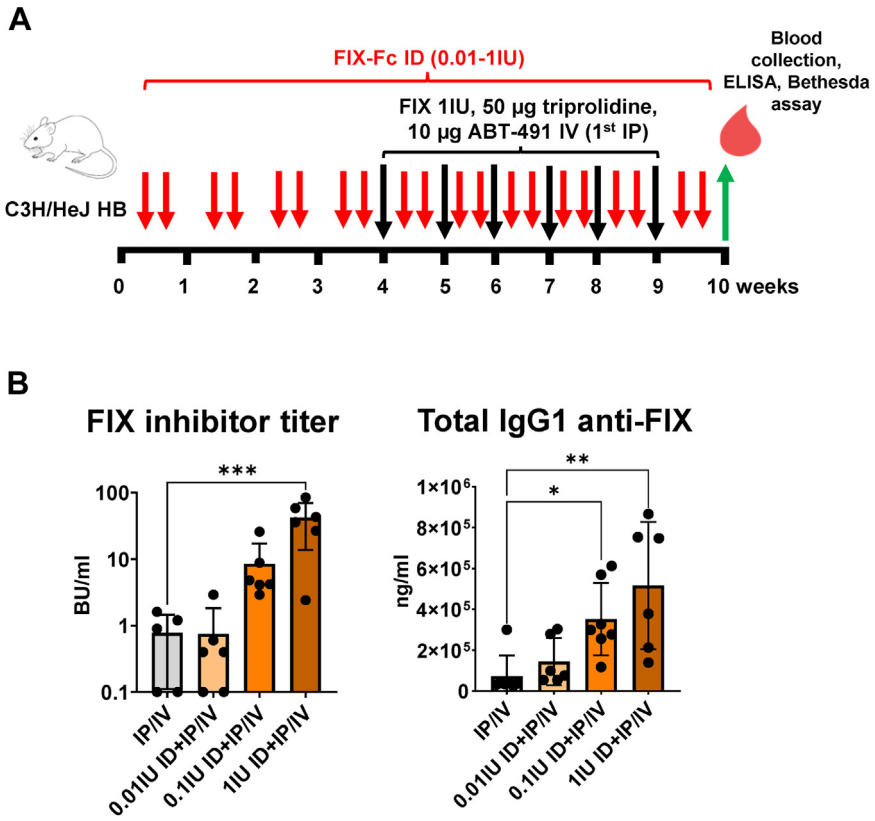


FIGURE 5 Factor IX–Fc does not modulate FIX inhibitor formation. (A) Experimental timeline. C3H/HeJ hemophilia B (HB) mice received FIX-Fc intradermally (ID) (0.01-1 IU) twice weekly for 4 weeks before initiation of once weekly intraperitoneal (IP)/intravenous (i.v.) FIX (1 IU) + triprolidine + ABT-491 administration for 6 weeks with intradermal injections continued throughout (n = 5-6 per group). At 10 weeks, blood was collected for enzyme-linked immunosorbent assay (ELISA) and Bethesda assay. (B) Bethesda assay and anti-FIX immunoglobulin G (IgG) 1 ELISA results in mice that received FIX IP/i.v. only (IP/i.v.) or FIX-Fc intradermally (0.01-1 IU)+FIX IP/i.v. Shown are means ± SDs and P values from analysis of variance (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$). BU, Bethesda unit.

anaphylaxis, regardless of IgE formation. To test this hypothesis, hemophilia B mice received 8 twice-weekly intradermal doses of 1 IU FIX followed by i.v. or i.v. only, with neither group receiving anti-allergic agents at any time (n = 15/group) (Figure 4C). After the first i.v. injection, 33.3% and 0% of animals died in the ID treated and i.v. only groups, respectively, prompting early termination of the experiment for humane reasons (Figure 4D). This outcome shows that along with priming or eliciting FIX inhibitor formation, intradermal administration of FIX sensitizes hemophilia B mice to systemic delivery of FIX.

3.5 | Fc fusion does not modulate FIX inhibitor formation

Since the Fc moiety in coagulation factor-IgG Fc fusion proteins used in the clinic has been suggested to show tolerogenic properties, a FIX-Fc fusion protein was tested in the same range of doses and dosing scheme as FIX in the initial dose-finding experiment (0.01-1 IU), except the IP/ i.v. FIX regimen consisted of 6 injections (Figure 5A). Surprisingly, the FIX-Fc protein seemed even more immunogenic than the standard half-life molecule, with the highest-dose group developing 54.5-fold higher mean inhibitor titers than the control group, which received FIX IP/i.v. only (Figure 5B, Table 3).

4 | DISCUSSION

We attempted to develop a new immune tolerance protocol based on intradermal administration of FIX, building on our previous success with oral tolerance toward FIX in mice and dogs with hemophilia B [8,15]. If effective, intradermal tolerance induction would have the advantage of delivering existing well-defined pharmaceuticals to a more accessible skin compartment than peripheral veins, especially in pediatric patients. Disappointingly, FIX administration in the skin of hemophilia B mice was equally or more immunogenic than i.v.

TABLE 3 Inhibitor and anti–factor IX immunoglobulin G1 titers in hemophilia B mice that received 0.01 to 1 IU of FIX-Fc intradermally + intraperitoneally/intravenously.

Animal group	1 IU FIX IP/i.v.	0.01 IU FIX-Fc ID + IP/i.v.	0.1 IU FIX-Fc ID + IP/i.v.	1 IU FIX-Fc ID + IP/i.v.
Inhibitor (BU/mL), mean ± SD	0.8 ± 0.7	0.8 ± 1.1	8.4 ± 8.7	41.9 ± 28.1
IgG1 (µg/mL), mean ± SD	72.9 ± 100.8	144.5 ± 115.5	351.4 ± 177.0	516.3 ± 311.7

BU, Bethesda unit; FIX, factor IX; ID, intradermally; IP, intraperitoneal; i.v., intravenous.

administration, with higher FIX inhibitor and total anti-FIX IgG1 titers in most ID treated animals across a wide range of doses. Even the lowest dose of 0.01 IU FIX, which is 100-fold lower than clinically used FIX doses, triggered inhibitor development in some animals and anti-FIX IgG1 in all animals. The dose of 0.01 IU is approximately 50 ng of FIX protein, which is far below typical doses required to induce FIX inhibitor formation by i.v. administration in mouse models. Successful immunization of mice against FIX by i.v. administration often requires combining IP and i.v. injections (as in this study) and/or addition of adjuvants. When injected intravenously at a dose of 300 ng (which is equivalent to clinically relevant FVIII protein doses), FIX does not trigger anti-FIX antibody development in mice [17]. Also, in all intradermal + IP/i.v. experiments, intradermal pretreatment drove most inhibitor development, without significant increase after initiation of the IP/i.v. regimen, while anti-FIX IgG1 continued to rise. These findings suggest that FIX administration in the skin is extremely immunogenic and skews the immune response to neutralizing anti-FIX antibody formation. These properties may warrant extra caution in FIX protein replacement therapy to minimize the incidence of i.v. infiltration. Further validation of our results would require studies in larger animal models, preferably involving alloimmune responses to alloantigenic FIX instead of xenoantigens. Recent studies have shown that FVIII inhibitor development proceeds through activation of Tfh cells and formation of GCs, moving away from the traditional T helper cell type 2–based model [17,18]. While immune responses to intravenously delivered antigens ensue in the spleen, administration of the same antigens in the skin induces responses in skin-draining lymph nodes [17]. Here, we find that FIX administration in the skin triggers robust expansion of Tfh and GC B cells in inguinal lymph nodes. Among several antigen-presenting cell types operating in the skin that we evaluated early after FIX administration, we detected only nonsignificantly elevated frequencies of Langerhans cells and cDC2s. Langerhans cells are immune skin sentinels that deliver antigens to skin-draining lymph nodes, and both Langerhans cells and cDC2s have been implicated in Tfh cell–mediated antibody responses [19,20].

None of the animals treated with ID only had fatal anaphylaxis despite receiving multiple ID injections without antiallergic agents and formation of anti-FIX IgE. However, a single i.v. injection of FIX after intradermal pretreatment killed 33.3% of mice. Also, only ID + intraperitoneally/intravenously treated animals reached IgE titers exceeding 50 ng/mL, which predicted fatal reactions in our previous studies [15]. This suggests that anaphylaxes after a single i.v. dose in this study were mediated by IgG, independent from IgE [21]. In clinical FIX inhibitor cases, both IgE, IgG1, and complement activation have been implicated in anaphylaxis [22–24].

We also found that FIX-Fc administered to the skin was markedly more immunogenic than standard half-life FIX. This finding contradicts previous preclinical observations that Fc fusion might have immunomodulatory properties in protein replacement therapy for hemophilia [25]. In clinical studies, neither FVIII-Fc nor FIX-Fc has shown significantly different immunogenicity from the standard half-life FVIII and FIX [26–28]. The discrepancy between our study and others might be due to the incompatibility of human IgG Fc and mouse FcγRs, which are 60% to 70% identical to their human FcγRs counterparts. However, human IgGs bound to mouse FcγRs show remarkably similar binding strengths between human and mouse FcγR orthologs, suggesting similar biological activities of human IgGs in mice [29]. On the other hand, our finding might be specific to the skin compartment because i.v. administration of FVIII-Fc induces immune tolerance in hemophilia A mice [30].

Finally, our results may indicate that the skin compartment is generally not amenable to immune tolerance induction or therapeutic delivery of clotting factors due to high immunogenicity of this administration route. Despite its promise for food allergy, intradermal antigen delivery not only fails to reduce the immune response to factor replacement therapy but further increases it. Several clinical studies administering FVIII or FIX subcutaneously have been discontinued over the last few years following increased antidrug antibody formation in participants despite demonstrated safety of at least 2 of those products when delivered i.v. [31–33]. Interestingly, it is thought that peanut allergy may be triggered by initial exposure to peanut allergens in the skin rather than through the oral route [34]. Thus, the skin immune system has the capacity to promote or reduce immune responses, likely depending on the circumstances of antigen exposure and doses. Recent clinical experience and our findings argue that introduction of coagulation factor antigens to the skin is risky, and therefore, future studies exploring exposure of skin-associated lymphoid tissue to drug candidates that contain FVIII or FIX should do so with caution.

FUNDING

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ETHICS STATEMENT

The study protocols were approved by the Institutional Animal Care and Use Committee at Indiana University.

AUTHOR CONTRIBUTIONS

R.K., T.B.B., and A.S. performed experiments. R.K. and A.S. designed the experiments. R.K. and R.W.H. analyzed and interpreted the data and wrote the manuscript. R.K. supervised the study.

RELATIONSHIP DISCLOSURE

R.K. is serving on a scientific advisory board of BioMarin and received research funding from Bayer. R.W.H. is serving on the scientific advisory boards and committees of Regeneron Pharmaceuticals, Pfizer, BioMarin, Spark Therapeutics, Hoffman-La Roche, and Prevail Therapeutics. A.S., T.B.B., and S.A. declare no competing financial interests.

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