Haemophilia
The Official Journal of the World Federation of Hemophilia,
European Association for Haemophilia and Allied Disorders and
the Hemostasis & Thrombosis Research Society

Haemophilia (2015), **21**, 133–139 DOI: 10.1111/hae.12522

ORIGINAL ARTICLE *Laboratory science*

Immune responses to human factor IX in haemophilia B mice of different genetic backgrounds are distinct and modified by TLR4

B. K. SACK,*† X. WANG,† A. SHERMAN,† G. L. ROGERS† and D. M. MARKUSIC†
**Seattle Biomedical Research Institute, Seattle, WA, USA; and †Department of Pediatrics, University of Florida, Gainesville, FL, USA*

Summary. Our laboratory develops protocols to prevent or reverse ongoing anti-hFIX IgG inhibitors in haemophilia B mice with a *F9* gene deletion on BALB/c and C3H/HeJ backgrounds. C3H/HeJ $F9^{-/Y}$ mice develop high titre anti-hFIX IgG1 inhibitors and anaphylaxis, whereas most BALB/c $F9^{-/Y}$ mice have mild anti-hFIX IgG1 inhibitors and no anaphylaxis. Our aim was to determine if hFIX-specific B- and T-cell responses in BALB/c and C3H/HeJ $F9^{-/Y}$ mice trigger the difference in anti-hFIX immune responses. BALB/c and C3H/HeJ $F9^{-/Y}$ mice were challenged weekly with recombinant hFIX protein. Humoral immune responses were determined by IgG1 and IgG2a anti-hFIX ELISA, Bethesda assay for inhibitors and B-cell ELISpot on bone marrow and spleen cells. T-cell studies measured the T_H1 (IFN-γ) and T_H2 (IL-4) cytokine responses in splenocytes at the mRNA and protein level in response to hFIX protein. Antibody responses were also measured in C3H/HeJ/OuJ $F9^{-/Y}$ mice with restored toll-like receptor 4 (TLR4) function. BALB/c $F9^{-/Y}$ mice have a T_H2 skewed response and a reduction in anti-hFIX secreting plasma cells in the bone marrow. Independent antigen challenge revealed both strains generated equivalent IgG1 antibody titres to an intravenously delivered antigen. C3H/HeJ $F9^{-/Y}$ mice have a mixed T_H1 and T_H2 response (mainly T_H2). Importantly, TLR4 signalling has a modulatory role in the C3H background on the levels of anti-hFIX IgG1 and incidence of anaphylaxis. The background strain strongly impacts the immune response to hFIX, which can be significantly impacted by mutations in innate immune sensors.

Keywords: anaphylaxis, BALBc, C3H/HeJ, factor IX, haemophilia B murine model, immune response

Introduction

Haemophilia B (HB) is an X-linked inherited coagulation disorder that results in the loss of functional coagulation factor IX (FIX). Patients are managed with intravenous (i.v.) injections of plasma derived or recombinant FIX protein. Those with severe disease (<1% residual coagulation factor) are at risk for developing anti-hFIX antibodies that impede coagulation, inhibitors, during treatment. Inhibitors occur in about 1.5–3% of HB patients. High titre inhibitor patients (>5 BU/mL) become unresponsive to coagulation factor protein therapy and have an increased risk of severe bleeds.

Typically patients with high titre inhibitors are managed on demand with bypassing agents, such as activated factor VIIa or FEIBA (Baxter Healthcare Corporation Deerfield, IL USA). These bypassing products are quite expensive and are not generally designated for extended use. Haemophilia A patients with inhibitors can undergo immune tolerance induction (ITI), a protocol that requires daily infusion of high levels of FVIII protein. This protocol can take months to years and is expensive. Unlike haemophilia A, haemophilia B patients with inhibitors are less responsive to ITI and may develop pathogenic antibody responses including anaphylaxis and nephrotic syndrome [1–6].

In most patients, inhibitor development typically occurs within 50 exposure days. Therefore the identification of genetic risk factors that promote inhibitor development would improve the treatment of newly diagnosed haemophilia B patients. For example, pre-

Correspondence: David M. Markusic, PhD, University of Florida, Cancer and Genetics Research Complex, 2033 Mowry Road, Room 206, Gainesville, FL 32610, USA.
Tel.: 352-273-8149; fax: 352-273-8342;
e-mail: dmarkusic@ufl.edu

Accepted after revision 21 July 2014

clinical studies have suggested that prophylactic immune suppression during initial hFIX infusions may prevent inhibitor development [7–9]. These strategies might be particularly beneficial for patients with a high-risk profile for developing inhibitors.

One of the best predictors of inhibitors in haemophilia B is the underlying *F9* mutation [10–13]. However, the fact that only an estimated 1.5-3% of haemophilia B patients develop inhibitors and not all patients with gene deletions develop inhibitors suggests that there are still unidentified susceptibility factors. Lozier *et al.* investigated the immune responses in different strains of wild-type mice immunized with an adenoviral vector expressing *F9* or with hFIX protein [14]. Their study identified a linkage between the major histocompatibility complex II locus H-2 and markers on chromosomes 1 and 10, which are near immunomodulatory genes such as interleukin 10 and interferon-γ [14]. To better model inhibitor development and risk for pathogenic antibody responses, as seen in treated haemophilia B patients, we compared immune responses to hFIX protein (40 IU kg^{-1}) by i.v. delivery in two different strains with a targeted gene deletion for murine *F9* (BALB/c $F9^{-/Y}$ and C3H/HeJ $F9^{-/Y}$).

Recent studies have indicated links between innate immunity and coagulation [15,16]. Absence of toll like receptor 4 (TLR4), an innate immune sensor of bacterial lipopolysaccharide (LPS), has been described as sensitizing mice to food antigen allergy and inducing anaphylaxis, whereas restoration of TLR4 provides protection [17]. C3H/HeJ $F9^{-/Y}$ mice are deficient in TLR4 and are prone to developing fatal anaphylactic responses to intravenously delivered FIX protein[13,18,19]. The availability of a congenic stain, C3H/OuJ that has functional TLR4, allowed us to determine if restoring TLR4 function will have an impact on the severity and frequency of anaphylactic responses to intravenously delivered FIX protein.

Materials and methods

Animal studies

BALB/c, C3H/HeJ and C3H/OuJ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Haemophilia B mice with targeted deletion of murine *F9* ('null mutation') have been bred on BALB/c and C3H/HeJ backgrounds for >10 generations [20]. Crossing female C3H/HeJ $F9^{-/Y}$ with male C3H/OuJ mice generated C3H/HeJ/OuJ $F9^{-/Y}$ F1 mice in which all male mice were $F9^{-/Y}$ and heterozygous for toll-like receptor 4 (TLR4). Animals were housed under special 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.

Immunization protocols for haemophilia B mice were performed as described unless otherwise stated [13]. Mice were immunized weekly with 1 IU recombinant hFIX protein starting with an intraperitoneal injection, followed by five weekly i.v. injections via the tail vein. When indicated C3H/HeJ $F9^{-/Y}$ mice were given 150 μg antihistamine (triprolidine; Sigma, St Louis, MO USA) and 50 μg platelet activating factor (PAF) antagonist CV-3988 (Sigma) along with hFIX protein to prevent anaphylactic responses. Blood for plasma analyses from haemophilia B mice were collected in citrate buffer (0.38%) by tail transection [20]. One week following a final intravenous immunization, hFIX mice were killed and spleen and bone marrow cells were collected for analysis. Wild-type BALB/c and C3H/HeJ mice received 2 mg keyhole limpet hemocyanin (KLH) antigen (Sigma) by intravenous injection [21]. These BALB/c and C3H/HeJ mice were bled from the retro-orbital plexus using heparinized capillaries for plasma antibody analyses.

Antibody measurements

Inhibitory antibody titres were determined by Bethesda assay [20]. One Bethesda unit is the reciprocal of the dilution of plasma that neutralizes 50% of FIX in a normal plasma sample which it is mixed with *in vitro*. Immunocapture assays to determine titres of hFIX and KLH specific IgG1 and IgG2a were as described [21,22] using purified mouse IgG1 and IgG2a as standards.

B- and T-cell assays

B-cell ELISpots were performed on total splenocytes and bone marrow cells as previously described [13,23,24]. For *in vitro* restimulation studies, isolated splenocytes were cultured in RPMI 1640 media (containing 55 μM β-mercaptoethanol, glutamine and antibiotics) with or without 10 μg mL^{-1} hFIX for 48 h (at 37°C, 5% CO_2). Transcript levels of cytokines in these cells were measured by quantitative RT-PCR using an SA Bioscience array [13].

Il-6 ELISA

10^6 total splenocytes isolated from C3H/HeJ, C3H/OuJ and C3H/HeJ/OuJ $F9^{-/Y}$ mice were cultured in 100 μL RPMI 1640 media for 48 h in the presence and absence of 10 μg mL^{-1} of LPS-*Salmonella minnesota* (InvivoGen, San Diego, CA, USA), a TLR4-specific activator. A mouse IL-6 ELISA Ready-Set-Go! kit (eBioscience, San Diego, CA, USA) was used to measure secreted IL-6 in cell culture media as instructed.

IFN-γ and IL-4 ELISpot

ELISpot assays were performed for hFIX-specific IL-4 and IFN-γ responses using mouse IL-4 (SEL404) and

IFN-γ development module (SEL485) according to manufacturer's protocol (R&D system, Minneapolis, MN, USA). Splenocytes were isolated from primed BALB/c and C3H/HeJ haemophilia B mice. 10^6 splenocytes were cultured in 200 μL of RPMI 1640 with 10% FBS, 1% penicillin/streptomycin, 15 mM Hepes (pH7.2) and 55 μM 2-beta-mercaptoethanol, with or without the stimulation of 10 μg mL^{-1} hFIX protein for 14 to 16 h (IFN-γ) or 48 h (IL-4) at 37°C in a 5% CO_2 incubator. Staphylococcal Enterotoxin B (1 μg 100 μL^{-1}; Sigma-Aldrich, St. Louis, MO, USA), and PMA-Phorbol 12-myristate 13-acetate (0.05 g mL^{-1})/Ionomycin (1 μg mL^{-1}; Sigma-Aldrich), were used as positive controls. Spots were analysed and counted with the CTL-ImmunoSpotH S5 UV analyser (Cellular Technology, Shaker Heights, OH, USA).

Statistics

All statistical analysis was carried out using Prism software using Student's two-tailed t-test. A $P < 0.05$ was considered statistically significant.

Results

Immune responses to intravenous challenge of hFIX protein in F9$^{-/Y}$ mice is strain dependent

C3H/HeJ *F9*$^{-/Y}$ mice develop high titre IgG antibodies and fatal anaphylactic reactions in a dose-dependent manner after weekly intravenous injection of hFIX protein, whereas BALB/c *F9*$^{-/Y}$ mice are hyporesponsive [13]. To investigate why the two strains respond differently to hFIX protein we conducted studies to compare B and T responses directed against hFIX protein. All mice were challenged with six weekly exposures of 1 IU hFIX protein (40 IU kg^{-1}). C3H/HeJ *F9*$^{-/Y}$ mice developed fatal anaphylactic reactions starting after the third hFIX injection (Fig. 1a) and required co-administration of antihistamine and platelet activating factor antagonist CV3988 to survive the challenge protocol. Similarly to previously published studies on food allergy [25], none of the BALB/c *F9*$^{-/Y}$ mice showed any signs of anaphylaxis and all mice survived the challenge protocol (Fig. 1a). To determine if this could be due a deviated immune response against hFIX protein in BALB/c F9$^{-/Y}$ mice, we assayed for T_H1 (IgG2a) and T_H2 (IgG1)-associated anti-hFIX immunoglobulin (IgG). IgG1 subclass was predominantly formed by either strain, albeit at significantly lower titres in BALB/c *F9*$^{-/Y}$ mice (Fig. 1b). C3H/HeJ *F9*$^{-/Y}$ mice had significantly higher levels of IgG2a, indicating an additional contribution of T_H1 responses (Fig. 1b, c). All animals that had measurable IgG1 were also positive for inhibitors in a Bethesda assay with BALB/c *F9*$^{-/Y}$ mice again developing significantly lower titres (Fig. 1d).

To compare the B-cell response between the strains, spleen and bone marrow cells were analysed by ELISpot for the presence of anti-hFIX IgG1 secreting B and plasma cells (PC). While we observed no significant difference in the frequencies of anti-hFIX IgG1 secreting cells in splenocytes (Fig. 2a), there was a significant elevation in hFIX antibody secreting cells in the bone marrow of C3H/HeJ *F9*$^{-/Y}$ mice (Fig. 2b). As long-term PC survival is dependent on signalling in the bone marrow, these data suggest that BALB/c mice may lack the ability to generate long-term anti-hFIX secreting PC following intravenous hFIX protein challenge [26]. To test if BALB/c mice in general have a failure to form antibodies against an intravenously

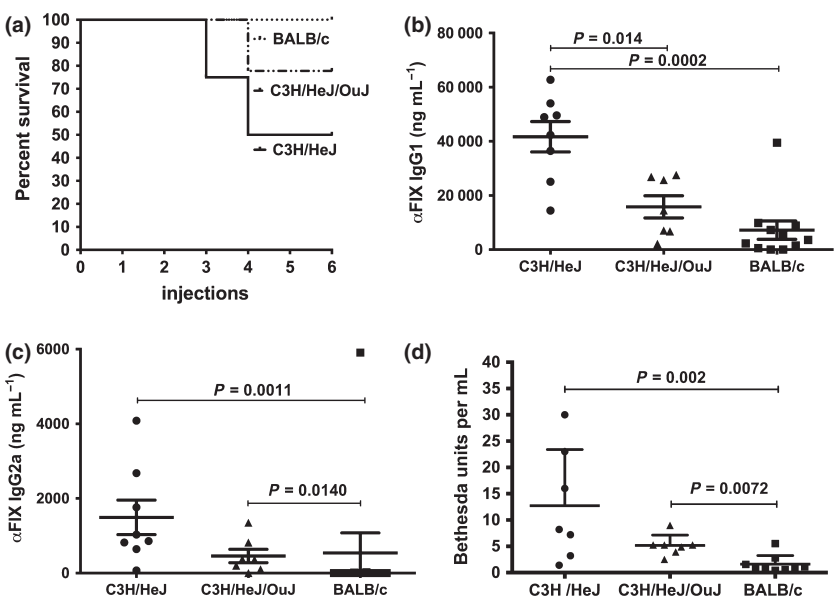

Fig. 1. Genetic background impacts hypersensitivity and antibody responses against recombinant hFIX protein. (a) Kaplan–Meier survival plot for BALB/c *F9*$^{-/Y}$ ($n = 9$), C3H/HeJ/OuJ *F9*$^{-/Y}$ ($n = 9$), and C3H/HeJ *F9*$^{-/Y}$ ($n = 16$) mice. C3H/HeJ *F9*$^{-/Y}$ mice received antihistamine and PAF antagonist on the fourth, fifth and sixth exposures to hFIX protein. (b) Anti-hFIX IgG1 titres (ng mL^{-1}) 1 week following last hFIX protein injection. (c) Anti-hFIX IgG2a titres (ng mL^{-1}) 1 week following last hFIX protein injection. (d) Inhibitor (Bethesda) titres 1 week following last hFIX protein injection. Statistical analysis was performed using Student's *t*-test with $P < 0.05$ considered significant. Calculated *P* values are included on plots.

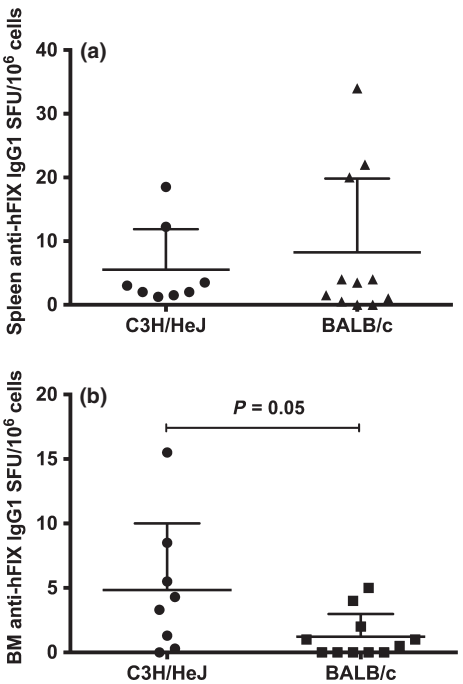

Fig. 2. Comparison of B cell response against recombinant hFIX protein. Summary of B-cell ELISpot spot forming units (SFU) per 1×10^6 cells of anti-hFIX antibody secreting cells from spleen (a) and bone marrow cells (b) isolated from immunized mice. Statistical analysis was performed using Student's *t*-test with $P < 0.05$ considered significant. Calculated *P* values are included on plots.

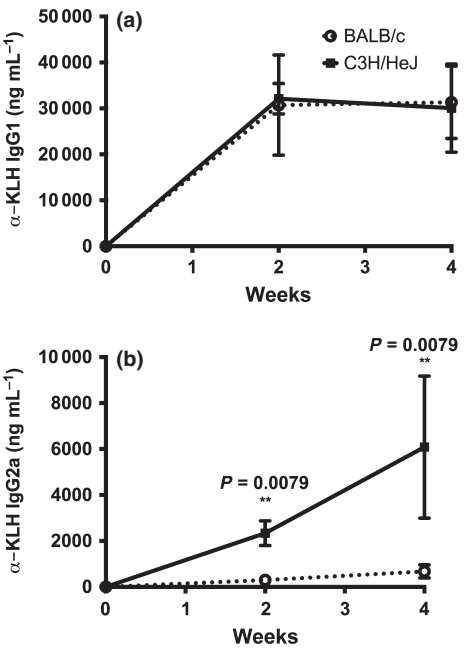

Fig. 3. Comparison of IgG1 (ng mL^{-1}) and IgG2a antibody responses against a T-cell-dependent antibody-inducing antigen, keyhole limpet haemocyanin (KLH). BALB/c and C3H/HeJ mice ($n = 5$ per group) were i.v.-injected with 2 μg KLH and bled two and 4 weeks later to measure circulating anti-KLH (a) IgG1 and (b) IgG2a. Statistical analysis was performed using Student's *t*-test with $P < 0.05$ considered significant. Calculated *P* values are included on plots.

delivered antigen, we challenged wild type BALB/c and C3H/HeJ mice with keyhole limpet hemocyanin (KLH). Mice on both backgrounds produced equivalent levels of anti-KLH IgG1 antibodies (Fig. 3a), while BALB/c mice had a significant reduction (~10 fold) in IgG2a, indicative of a T_H2 biased immune response. Together these data suggest that the hyporesponsiveness of BALB/c mice towards hFIX protein does not reflect a general unresponsiveness to exogenous proteins.

No difference in T-cell responses to hFIX in the BALB/c and C3H/HeJ F9$^{-/Y}$ mice

As antibody responses against hFIX protein are T-cell dependent, we investigated if there were differences in the T-cell responses to hFIX protein. Splenocytes from hFIX-immunized BALB/c and C3H/HeJ *F9*$^{-/Y}$ mice were stimulated with full-length hFIX protein and analysed for the production of T_H1 and T_H2-associated cytokines by ELISpot. While not reaching statistical significance, the data showed a trend towards higher IL-4 spot forming units in BALB/c *F9*$^{-/Y}$ mice (Fig. 4a). The majority of BALB/c *F9*$^{-/Y}$ mice were negative for IFN-γ, whereas most of the C3H/HeJ *F9*$^{-/Y}$ mice had some level of IFN-γ (Fig. 4b). This skewing of T cell responses to T_H1 in C3H/HeJ *F9*$^{-/Y}$ mice correlated with previous observations in the anti-hFIX antibody classes (Fig. 1b, c). In another set of experiments, splenocytes from immunized BALB/c and C3H/HeJ *F9*$^{-/Y}$ mice were restimulated *in vitro* with hFIX protein and mRNA was extracted to assess changes in T_H1, T_H2, and Treg-related gene expression. In agreement with our IL-4 ELISpot data, both strains showed an up-regulation in IL-4 mRNA (Fig. 4c). BALB/c *F9*$^{-/Y}$ mice had a significant up-regulation in IL-10 mRNA compared to C3H/HeJ *F9*$^{-/Y}$ mice, but was below our threshold to be considered relevant. In summary, the data show no significant difference in the T-cell responses to hFIX protein between the two strains of *F9*$^{-/Y}$ mice.

Role of TLR4 signalling in modulating pathogenic immune responses against recombinant hFIX protein therapy

Food allergy-induced anaphylaxis studies showed that C3H/HeJ mice are highly susceptible and C3H/OuJ mice are highly resistant [17]. Introducing a similar defective TLR4 allele into BALB/c mice, who are normally resistant, failed to promote anaphylaxis [17,27], suggesting that TLR4 only modulates hypersensitivity in a high-responder strain. To address the role of TLR4 signalling in hFIX-mediated anaphylaxis we bred female C3H/HeJ *F9*$^{-/Y}$ with male C3H/OuJ mice to generate male mice with a gene deletion for *F9* and

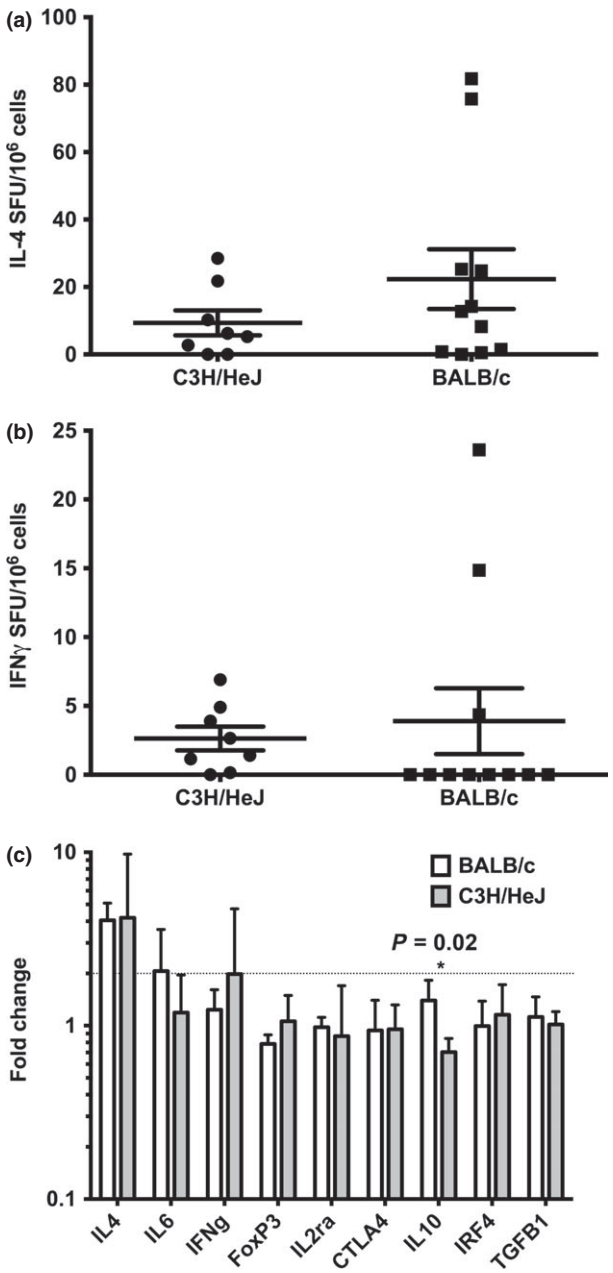

Fig. 4. Comparison of T-cell responses against hFIX protein. IL-4 (a) and IFN-γ (b) ELISpot assays using splenocytes isolated from immunized mice of indicated strain. All samples were run in duplicate with unstimulated, 10 μg mL^{-1} hFIX, and SEB or PMA/Ionomycin stimulated. Data are presented as (SFU_{hFIX} − $SFU_{unstimulated}$ per 1×10^6 cells). (c) Cytokine responses specific to hF.IX for BALB/c (white) and C3H/HeJ (grey) $F9^{-/Y}$ mice. Splenocytes (1×10^5 per well) were cultured *in vitro* without or with hFIX protein (10 mg mL^{-1}) and harvested 48 h later for mRNA extraction and transcriptional analysis via qPCR array for indicated genes. Data are presented as fold change compared to unstimulated cells.

heterozygous for TLR4. To determine if TLR4 signalling was restored in these F1 mice, we compared the secretion of IL-6 by splenocytes from wild-type C3H/HeJ, C3H/OuJ and F1 C3H/HeJ/OuJ $F9^{-/Y}$ mice following *in vitro* stimulation with a TLR4-specific LPS

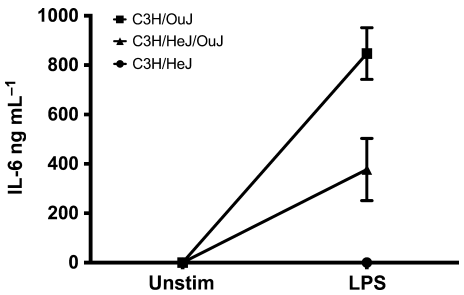

Fig. 5. Verifying reconstitution of TLR4 signalling in C3H/HeJ/OuJ $F9^{-/Y}$ mice. 1×10^6 splenocytes from C3H/HeJ ($n = 2$), C3H/OuJ ($n = 2$), and C3H/HeJ/OuJ $F9^{-/Y}$ ($n = 4$) were cultured *in vitro* in triplicate for 48 h either unstimulated or stimulated with 10 μg mL^{-1} LPS-SM, a specific TLR4 activator. Cell culture media was collected, pooled and analysed for IL-6 (ng mL^{-1}) secretion into media using a murine IL-6 ELISA kit.

[28]. As expected C3H/HeJ splenocytes were unresponsive to LPS stimulation (Fig. 5). Both C3H/OuJ and C3H/HeJ/OuJ splenocytes secreted IL-6 only in the presence of LPS, with splenocytes from C3H/HeJ/OuJ mice secreting approximately one half the level of C3H/OuJ mice (Fig. 5) confirming partial restoration of TLR4 function in the F1 offspring. When challenged with hFIX protein, without antihistamine and PAF antagonist, only two of nine F1 C3H/HeJ/OuJ $F9^{-/Y}$ mice died from anaphylaxis, which is a substantial reduction compared to C3H/HeJ $F9^{-/Y}$ mice (Fig. 1a). Anti-hFIX IgG1, IgG2a and Bethesda titres were intermediate when compared with C3H/HeJ $F9^{-/Y}$ and BALB/c $F9^{-/Y}$ mice (Fig. 1b–d). Consistent with previous studies using C3H/HeJ and C3H/OuJ mice in food allergy models [29], we show that TLR4 is also critical in down-modulating IgG1, IgG2a and hypersensitivity responses directed against hFIX protein. Nonetheless, 70% of C3H $F9^{-/Y}$ formed high-titre inhibitors (>5 BU) regardless of the TLR4 mutation, whereas only 1 of 8 BALB/c mice had >5 BU, indicating that additional genetic factors modulate the response.

Discussion

Identifying genetic susceptibility factors for inhibitor formation and pathogenic antibody responses against recombinant hFIX protein would be of significant clinical benefit. Because of the relatively low numbers of haemophilia B patients with inhibitors, it has been difficult to study contributing factors. Therefore, we and others have generated murine haemophilia B models with human *F9* mutations to study the immunogenicity of hFIX protein [10,12,20]. Our group was the first to describe fatal anaphylaxis to intravenously delivered hFIX protein in null mutant (but not missense and late stop *F9* mutations) C3H/HeJ $F9^{-/Y}$ mice [7,13,18,19]. These mice provide a robust and consistent model of inhibitor and anaphylaxis development that is much higher than that observed

in human haemophilia B patients, which have heterogeneous *F9* mutations and immune profiles, and thus provides an ideal platform to develop protocols that can prevent or eliminate inhibitors [13,30].

The formation of an antibody response against a recombinant protein, such as hFIX, is dependent on multiple factors. Antigen presenting cells can modulate the $CD4^+$ T-cell response by presenting antigen in the context of varying co-stimulation during T-cell receptor (TCR) engagement [31]. $CD4^+$ T cells can in turn provide help to B cells to stimulate their expansion and differentiation into antibody-secreting PC. In this study, we investigated if differences in B- and T-cell responses to hFIX protein contributed to either the low-responding BALB/c $F9^{-/Y}$ or high-responding C3H/HeJ $F9^{-/Y}$ mice.

Similar to previously published data on food allergy, we found anaphylactic responses to hFIX in C3H/HeJ $F9^{-/Y}$, but not BALB/c $F9^{-/Y}$ mice[25]. The only significant difference in B- and T-cell responses was a reduction in antibody secreting PC in the bone marrow of BALB/c $F9^{-/Y}$ mice. An inability to generate sufficient anti-hFIX producing PC would explain the reduced systemic anti-hFIX levels in the BALB/c strain. The underlying cause for the diminished B cell response is less clear. Consistent with immune responses to pathogens in the two strains, BALB/c mice showed a stronger T_H2 bias in their response to hFIX. Furthermore, IL-4 ELISpot data and cytokine gene expression profiles suggest a similar strength of T_H2 responses for both strains, so that the comparatively low formation of the T_H2-dependent IgG1 anti-hFIX in BALB/c mice unlikely is due to lack of T help (although we cannot rule out reduced levels of cytokine secretion)[32].

A similar hypo-responsiveness against FVIII in haemophilia A BALB/c mice has been previously reported [8,32]. Taken together these studies suggest that BALB/c mice have a reduced response to intravenously delivered coagulation factors. However, the robust anti-KLH IgG1 response (Fig. 3a) suggests that these differential responses are antigen-dependent rather than reflecting a general defect in responding to intravenous proteins.

As C3H/HeJ mice lack functional TLR4, we investigated the role of TLR4 signalling on shaping the anti-hFIX immune response. Our F1 C3H/HeJ/OuJ $F9^{-/Y}$ mice with partially restored TLR4 function (Fig. 5) had a significant reduction in anaphylaxis associated mortality: 78% survival versus 40% (Fig. 1a). The 40% survival rate for C3H/HeJ $F9^{-/Y}$ mice was dependent on prophylactic intervention with antihistamine and PAF antagonist, whereas 78% of the C3H/HeJ/OuJ $F9^{-/Y}$ mice survived the challenge protocol without any prophylaxis. This indicates that TLR4 acts as negative modulator of anamnestic responses against hFIX protein in C3H mice. TLR4 has originally been identified as an innate sensor of LPSs, components of the cell walls of Gram-negative bacteria. More recently, it has become clear that TLR4 is also a critical link between the innate immune and coagulation systems, serving as a receptor for fibrinogen bound to a *Plasmodium* metabolite and for blood-born adenovirus decorated with factor X [15,16]. Interestingly, TLR4 activation in B cells is not a signal for antibody formation [33]. The absence of TLR4 signalling in C3H/HeJ mice results in a reduced ability to clear Gram-negative bacterial infections, such as *Klebsiella oxytoca* [34,35], which resides in the gut of healthy mice with functional TLR4. Such lesions may act to prime the immune system, although further studies are required to examine this. Multiple food allergy studies show that functional TLR4 protects against IgE-mediated anaphylaxis [17,29] and TLR4 deficiency exacerbates autoimmune disease in colitis and type 1 diabetes[36,37]. We have now extended these findings to hFIX replacement therapy. The mechanism of the allergic response to hFIX, characterized by an enhanced response in the absence of TLR4, is distinct from that in allergic airway disease, where sensing of proteolytic cleavage products of fibrinogen by TLR4 is required [16].

Relevance to human haemophilia B patients

The most relevant finding of our study was the role of TLR4 signalling on both the magnitude of anti-hFIX antibodies and hypersensitivity. It is not known if polymorphisms or mutations with reduced functional activity in TLR4 or other innate immune sensors have an impact on the risk of inhibitors and hypersensitivity in haemophilia B patients as compared to our murine model [38]. Nonetheless, these data provide novel links between genetic factors, environmental stimuli and inhibitor formation/anaphylaxis.

Conclusions

Genetic background plays a significant role in shaping the immune response against recombinant hFIX protein. Comparing BALB/c and C3H/HeJ mice with the same *F9* gene deletion showed that BALB/c mice are hyporesponsive to hFIX protein, fail to produce bone marrow-resident PC and are biased towards generating a T_H2 skewed immune response. C3H/HeJ mice are hypersensitive to hFIX protein and show a mixed T_H1 and T_H2 immune response. TLR4 was identified as a negative modulator of hypersensitivity in the C3H background. We would offer caution in the design and interpretation of studies when conducted in strains with limited immune responses.

Acknowledgements

This research was funded through the Bayer Hemophilia Awards Program (BHAP) to DMM, which supports Basic and Clinical Research and Education in haemophilia.

Author contribution

DMM, BKS, XW and AS performed the research and conducted data analysis. DMM and GLR designed research and wrote the manuscript. DMM supervised the research project.

Disclosures

The authors stated that they have no interests which might be perceived as posing a conflict or bias.

References

1 Warrier I, Ewenstein BM, Koerper MA *et al.* Factor IX inhibitors and anaphylaxis in hemophilia B. *J Pediatr Hematol Oncol* 1997; **19**: 23–7.
2 Warrier I. Management of haemophilia B patients with inhibitors and anaphylaxis. *Haemophilia* 1998; **4**: 574–6.
3 Warrier I, Lusher JM. Development of anaphylactic shock in haemophilia B patients with inhibitors. *Blood Coagul Fibrinolysis* 1998; **9**(Suppl 1): S125–8.
4 Thorland EC, Drost JB, Lusher JM *et al.* Anaphylactic response to factor IX replacement therapy in haemophilia B patients: complete gene deletions confer the highest risk. *Haemophilia* 1999; **5**: 101–5.
5 Jadhav M, Warrier I. Anaphylaxis in patients with hemophilia. *Semin Thromb Hemost* 2000; **26**: 205–8.
6 DiMichele DM. Immune tolerance in haemophilia: the long journey to the fork in the road. *Br J Haematol* 2012; **159**: 123–34.
7 Nayak S, Cao O, Hoffman BE *et al.* Prophylactic immune tolerance induced by changing the ratio of antigen-specific effector to regulatory T cells. *J Thromb Haemost* 2009; **7**: 1523–32.
8 Sack BK, Merchant S, Markusic DM *et al.* Transient B cell depletion or improved transgene expression by codon optimization promote tolerance to factor VIII in gene therapy. *PLoS ONE* 2012; **7**: e37671.
9 Moghimi B, Sack BK, Nayak S, Markusic DM, Mah CS, Herzog RW. Induction of tolerance to factor VIII by transient coadministration with rapamycin. *J Thromb Haemost* 2011; **9**: 1524–33.
10 Cao O, Hoffman BE, Moghimi B *et al.* Impact of the underlying mutation and the route of vector administration on immune responses to factor IX in gene therapy for hemophilia B. *Mol Ther* 2009; **17**: 1733–42.
11 DiMichele D. Inhibitor development in haemophilia B: an orphan disease in need of attention. *Br J Haematol* 2007; **138**: 305–15.
12 Sabatino DE, Armstrong E, Edmonson S *et al.* Novel hemophilia B mouse models exhibiting a range of mutations in the Factor IX gene. *Blood* 2004; **104**: 2767–74.
13 Markusic DM, Hoffman BE, Perrin GQ *et al.* Effective gene therapy for haemophilic mice with pathogenic factor IX antibodies. *EMBO Mol Med* 2013; **5**: 1698–709.
14 Lozier JN, Tayebi N, Zhang P. Mapping of genes that control the antibody response to human factor IX in mice. *Blood* 2005; **105**: 1029–35.
15 Doronin K, Flatt JW, Di Paolo NC *et al.* Coagulation factor X activates innate immunity to human species C adenovirus. *Science* 2012; **338**: 795–8.
16 Millien VO, Lu W, Shaw J *et al.* Cleavage of fibrinogen by proteinases elicits allergic responses through toll-like receptor 4. *Science* 2013; **341**: 792–6.
17 Berin MC, Zheng Y, Domaradzki M, Li XM, Sampson HA. Role of TLR4 in allergic sensitization to food proteins in mice. *Allergy* 2006; **61**: 64–71.
18 Verma D, Moghimi B, LoDuca PA *et al.* Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. *Proc Natl Acad Sci USA* 2010; **107**: 7101–6.
19 Markusic DM, Herzog RW, Aslanidi GV *et al.* High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Mol Ther* 2010; **18**: 2048–56.
20 Mingozzi F, Liu YL, Dobrzynski E *et al.* Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *J Clin Invest* 2003; **111**: 1347–56.
21 Plitnick LM, Herzyk DJ. The T-dependent antibody response to keyhole limpet hemocyanin in rodents. *Methods Mol Biol* 2010; **598**: 159–71.
22 Dobrzynski E, Fitzgerald JC, Cao O, Mingozzi F, Wang L, Herzog RW. Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells. *Proc Natl Acad Sci USA* 2006; **103**: 4592–7.
23 Hausl C, Maier E, Schwarz HP *et al.* Long-term persistence of anti-factor VIII antibody-secreting cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 840–5.
24 Wang L, Cao O, Swalm B, Dobrzynski E, Mingozzi F, Herzog RW. Major role of local immune responses in antibody formation to factor IX in AAV gene transfer. *Gene Ther* 2005; **12**: 1453–64.
25 Morafo V, Srivastava K, Huang C-K *et al.* Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *J Allergy Clin Immunol* 2003; **111**: 1122–8.
26 Winter O, Dame C, Jundt F, Hiepe F. Pathogenic long-lived plasma cells and their survival niches in autoimmunity, malignancy, and allergy. *J Immunol* 2012; **189**: 5105–11.
27 Vogel SN, Wax JS, Perera PY, Padlan C, Potter M, Mock BA. Construction of a BALB/c congenic mouse, C.C3H-Lpsd, that expresses the Lpsd allele: analysis of chromosome 4 markers surrounding the Lps gene. *Infect Immun* 1994; **62**: 4454–9.
28 Kawai T, Akira S. Signaling to NF-kappaB by toll-like receptors. *Trends Mol Med* 2007; **13**: 460–9.
29 Bashir ME, Louie S, Shi HN, Nagler-Anderson C. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *J Immunol* 2004; **172**: 6978–87.
30 Nayak S, Sarkar D, Perrin GQ *et al.* Prevention and reversal of antibody responses against factor IX in gene therapy for hemophilia B. *Front Microbiol* 2011; **2**: 244.
31 Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 2011; **34**: 637–50.
32 Qadura M, Waters B, Burnett E *et al.* Immunoglobulin isotypes and functional anti-FVIII antibodies in response to FVIII treatment in Balb/c and C57BL/6 haemophilia A mice. *Haemophilia* 2011; **17**: 288–95.
33 Wang X, Moghimi B, Zolotukhin I, Morel LM, Cao O, Herzog RW. Immune tolerance induction to factor IX through B cell gene transfer: TLR9 signaling delineates between tolerogenic and immunogenic B cells. *Mol Ther* 2014; **22**: 1139–50.
34 Bleich A, Kirsch P, Sahly H *et al.* Klebsiella oxytoca: opportunistic infections in laboratory rodents. *Lab Anim* 2008; **42**: 369–75.
35 MacArthur CJ, Pillers DA, Pang J, Degagne JM, Kempton JB, Trune DR. Gram-negative pathogen Klebsiella oxytoca is associated with spontaneous chronic otitis media in toll-like receptor 4-deficient C3H/HeJ mice. *Acta Otolaryngol* 2008; **128**: 132–8.
36 Gulden E, Ihira M, Ohashi A *et al.* Toll-like receptor 4 deficiency accelerates the development of insulin-deficient diabetes in non-obese diabetic mice. *PLoS ONE* 2013; **8**: e75385.
37 Gonzalez-Navajas JM, Fine S, Law J *et al.* TLR4 signaling in effector $CD4^+$ T cells regulates TCR activation and experimental colitis in mice. *J Clin Invest* 2010; **120**: 570–81.
38 Schwartz DA, Cook DN. Polymorphisms of the toll-like receptors and human disease. *Clin Infect Dis* 2005; **41**(Suppl 7): S403–7.

