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ORIGINAL ARTICLE

Nuclear factor (NF)-κB and its associated pathways are major molecular regulators of blood-induced joint damage in a murine model of hemophilia

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Summary. *Background:* The present study was designed to investigate the molecular signaling events from onset of bleeding through the development of arthropathy in a murine model of hemophilia A. *Methods and results:* A sharp-injury model of hemarthrosis was used. A global gene expression array on joint-specific RNA isolated 3 h post-injury revealed nuclear factor-kappa B (NF-κB) as the major transcription factor triggering inflammation. As a number of genes encoding the cytokines, growth factors and hypoxia regulating factors are known to be activated by NF-κB and many of these are part of the pathogenesis of various joint diseases, we reasoned that NF-κB-associated pathways may play a crucial role in blood-induced joint damage. To further understand its role, we screened NF-κB-associated pathways between 1 h to 90 days after injury. After a single articular bleed, distinct members of the NF-κB family (NF-κB1/NF-κB2/RelA/RelB) and their responsive pro-inflammatory cytokines (IL−1β/IL−6/IFNγ/TNFα) were significantly up-regulated (> 2 fold, $P < 0.05$) in injured vs. control joints at the various time-points analyzed (1 h/3 h/7 h/24 h). After multiple bleeds (days 30/60/75/90), there was increased expression of NF-κB-associated factors that contribute to hypoxia (HIF-1α, 3.3−6.5 fold), angiogenesis (VEGF-α, 2.5−4.4 fold) and chondrocyte damage (matrix metalloproteinase−13, 2.8−3.8 fold) in the injured joints. Micro RNAs (miR) that are known to regulate NF-κB activation (miRs-9 and 155), inflammation (miRs-16, 155 and 182) and apoptosis (miRs-19a, 155 and 186) were also differentially expressed (−4 to +13-fold) after joint bleeding, indicating that the small RNAs could modulate the arthropathy phenotype. *Conclusions:* These data suggest that NF-κB-associated signaling pathways are involved in the development of hemophilic arthropathy.

Keywords: arthropathy, hemophilia, mouse model, NF-κB, Synovitis.

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Introduction

Hemophilia is an X-linked bleeding disorder caused by a deficiency of human coagulation factor (*F) VIII* and *FIX* [1]. The severity of the disease has been classified by the level of the missing coagulation factor: severe (< 1%), moderate (1−5%) or mild (5−40% of normal levels) [2]. A majority of patients with severe hemophilia have 15−35 spontaneous bleeds per year, approximately 90% of which occur in large joints [3]. Joint disease is therefore the major cause of morbidity and often results in irreversible arthropathy after multiple bleeds. This can then lead to physical disability and loss of full participation in society. Intensive prophylactic replacement therapy with clotting factor concentrates (CFCs) has been shown to maintain near normal joints. In spite of such replacement, breakthrough bleeds occur in such patients [4,5]. They are also common among those (approximately 25−30%) who develop inhibitors and the many patients around the world who do not have access to adequate CFC [6,7].

It has also been observed that there is heterogeneity not only in bleeding frequency among patients with severe hemophilia but also in the response of the joint to the bleeds that occur. While some patients develop significant arthropathy with just a few bleeds, others may not damage their cartilage even with several bleeds [5]. These dif-

ferences are likely to be related to the variations in the inflammatory response to bleeding in different individuals. Better understanding of the pathways involved in this process therefore could be very useful in designing potential interventions. Analysis of samples of human joints from patients with hemophilia removed during arthroplasty surgeries has confirmed that repeated episodes of joint bleeding results in synovitis, followed by destruction of the cartilage and the underlying bone [8]. However, the ability to examine early stages of blood-induced arthritis in patients is limited by access to samples. The response in the joint to the first episode of bleeding has also been described in animal models and *in vitro* studies [9–13]. Blood, or its breakdown products such as iron (hemosiderin), has been implicated as the trigger for the inflammatory response [9,10] and the following events after a joint bleed has been evaluated in some detail [11,14–22]. However, the molecular mechanisms in the development of hemarthropathy are not precisely known, with only limited data available regarding this aspect. In a study by Hakobyan *et al*., synovial cells obtained from a murine hemarthrosis model and cultured with ferric citrate had increased expression of a p53 supressor protein, *mdm2*, shown to induce synovial proliferation [10].

In the present study, we have used a murine model of hemophilic arthropathy to investigate the molecular signaling events from onset of bleeding through to the development of arthropathy. Our preliminary genome-wide expression studies in this model demonstrated a strong activation of the nuclear factor kappa B (NF-κB)-mediated inflammatory factors. A number of genes are known to be translated after activation of this transcription factor, NF-κB, including various cytokines, chemokines, growth factors, cell adhesion molecules and hypoxic factors [23–25]. Because many of these genes are part of the pathogenesis of various joint diseases [26–34] and based on our preliminary micro-array data, we hypothesized that NF-κB-associated factors are mediators of blood-induced joint damage in hemophilia. To further understand how the transcriptional potential and activity of this factor is regulated upon bleeding, we carried out a targeted screening of NF-κB and its associated molecular pathways between 1 h and 90 days after injury. Our studies in this model demonstrate that these pathways play a significant role in blood-induced inflammatory, hypoxic, angiogeneic and chondro-degenerative processes in the affected joint.

Methods

Animals

Hemophilia A mice $^{(F8-/-\ E16\ FVIII\ B6;\ 129s4\text{-}f8\ tm1kaz)}$ and hemostatically normal C57BL/6 mice were obtained from Jackson's Laboratory (Bar Harbor, ME, USA). The hemophilia A mice supplied by Jackson's Laboratory are from a mixed C57BL/6:129 background. All the experiments were reviewed and approved by the institutional review board and the animal care and ethics committee (Christian Medical College, Vellore, India).

Hemarthrosis model

A sharp-injury model of hemarthrosis was developed as described previously [9,35]. Briefly, groups (n = 14 animals at each time-point) of 8 to 16-week-old hemophilia A ($F8^{-/-}$) or normal C57BL/6 mice (n = 7 animals at defined time-points) were anesthetized. The right knee joint capsule of each mouse was punctured with a 30-g needle below the patella to induce hemorrhage. The left knee joint served as the control. Injury was repeated serially every 14 days to recapitulate the effects of single (day 1 and day 14) and multiple (day 30/60/75/90) (re) bleeding episodes (Fig. S1). Hemophilic mice were then euthanized at the above-mentioned time-points for gross-examination, histology (n = 4 animals) and evaluation of molecular (n = 10) and biochemical mediators (n = 5) of joint damage. Normal mice that served as controls were studied at the representative time points (1 h, 3 h, 30 days, 60 days) by histological analysis or the molecular signaling pathways. Mice were treated as humanely as possible. If any of the injured mice were found to be in distress as determined by the in-house veterinarian, analgesics (meloxicam, 1−2 mg kg^{-1}, s.c.) were administered. Animals in significant distress were immediately euthanized.

Histology

The knee joints of hemophilia A animals were examined for macroscopic evidence of hemorrhage on days 1, 14, 30, 60, 75 and 90. For histology, knee joint tissues from at least four animals were fixed and stained with hematoxylin and eosin or Prussian blue (iron stain) and examined by light microscopy. Histological scoring was carried out as previously described [9]. A similar strategy was used for scoring of joints from hemostatically normal C57BL/6 mice at the representative time-points of 3 h and 24 h and on days 30 and 60. Vascularity was excluded from this scoring as it was not discriminatory in both the injured and control joints, perhaps because synovium is a highly vascular tissue [36]. The pathologist performing the histological scoring was blinded to the experimental conditions.

Joint-specific RNA isolation and its validation

The control or injured joint tissue from each of the time-points (1 h/3 h/7 h/24 h and days14/30/60/75/90) was pooled and further processed as described earlier [37] to isolate the total RNA (NucleoSpin® kit; Macherey-Nagel, GmbH, Germany). cDNA was synthesized by reverse-transcription (RT) (Life Technologies, Carlsbad, CA,

USA). Qualitative PCR amplification of the collagen type 2 A (COL2A) gene was performed (Table S1) to confirm the specificity of the RNA extracted from joints. For all the molecular analyses described below RNA was isolated from the pooled joint tissue of at least five animals and selected targets verified independently in another set of five animals.

Gene expression microarray

Total RNA samples isolated from joint tissues, as described above, 3 h after the injury to the hemophilia A mice, from both their injured and control joints, were compared by a gene expression microarray analysis performed using an Illumina mouse gene microarray (n = 34 000 genes) platform, with each sample being a biological replicate (n = 5 per group; Mouse-WG6 expression BeadChip; Illumina, San Diego, CA, USA). Genes that were > 1.5-fold expressed with a P-value of less than 0.05 were considered as true differentials. Transcription factor binding analysis of pro-inflammatory and inflammation genes differentially expressed was carried out using the Mapper 2 tool (http://genome.ufl.edu/mapper/run/?init = db).

Targeted pathway-specific RT-PCR array

Approximately 1 μg of total RNA was reverse transcribed using the first-strand RT kit (Qiagen, SABiosciences, Frederick, MD, USA). The expression of 84 key genes related to NF-κB-mediated signal transduction was profiled by using the mouse NF-κB signaling pathway RT²*Profiler*™ PCR Array. cDNA from 3-h to 60-day time-points was analyzed for the expression of 84 genes related to the T-cell and B-cell response by the mouse T-Cell and B-Cell activation RT² *Profiler*™ PCR Array. All experiments were performed using ABI 7500 real-time PCR equipment. Data normalization and differential gene expression were computed using SABiosciences web-based analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Targeted microRNA RT-PCR profiler array

Joint RNA from the 3-h time-point was reverse transcribed using the miScript^2 RT kit (Qiagen, SABiosciences). Mouse inflammatory response and autoimmunity miRNA RT-PCR Array (Qiagen, SABiosciences) was used to profile the expression of 84 microRNAs (miRNA) related to inflammation and immune responses.

Quantitative real-time PCR analysis

The expression of key genes such as hypoxia-inducing factor (HIF)-1α, HIF-2α, matrix metallo proteinase (MMP)-3, MMP-13, vascular endothelial growth factor (VEGF)-α, which were not covered by targeted arrays, and control genes collagen (COL)2A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table S1) were measured in RNA isolated from joints after single (1 h/3 h/7 h/day 14) or multiple injuries (days 30/60/75/90). PCR was performed using the manufacturer's protocol (DyNAmo™ HS SYBR® Green qPCR Kit; Thermo Scientific, Rockford, IL, USA). The relative gene expression between injured and control joints was measured by the comparative threshold cycle (ΔΔCt) method.

Validation studies using TaqMan® real-time PCR assay

The differential expression of genes such as RelA, HIF-1α, MMP-13 and interleukin (IL)-6 was further confirmed by custom-designed TaqMan® primers and probes (Table S2) on these RNA samples and normalized to an endogeneous control (GAPDH) to generate reproducible quantitative RNA expression data from independent experiments at each of the time-points. PCR was performed as described by the manufacturer (Eurogentec, Seraing, Belgium).

Immunoblotting

Approximately 50 μg of total protein isolated from pooled injured or normal joint tissue of hemophilia A mice (n = 5) was analyzed for the levels of NF-κB proteins (p65 and p52/p100), HIF-1α, phosphorylated inhibitory kinase Bα (P-IκBα), total IκBα and GAPDH proteins by western blot analysis using antibodies from Cell Signaling Inc. (Danvers, MA, USA). The intensity of the protein bands was measured with Quantity 1-D analysis software (BioRad, Hercules, CA, USA) and normalized to GAPDH protein levels used as loading controls.

Detection of cytokines in synovial fluid by ELISA

Synovial fluid from the joints of hemophilia A mice was collected as described earlier [38]. Briefly, a piece of filter paper of diameter approximately 2 mm was placed in the knee joint until saturated with synovial fluid. The filter paper was transferred to a sterile 1.5-mL tube. Synovial fluid was eluted from this filter paper by addition of 50 μL of the extraction buffer with protease inhibitor (PBS containing complete protease inhibitor cocktail with EDTA; Cell Signaling) followed by centrifugation at 600 ***g*** at 37 °C for 1 h. Eluates were stored at −80 °C until analysis. In addition, serum from whole blood samples from these mice was also collected and stored at −80 °C until analysis. For each of the time-points analyzed, the synovial fluid and serum were pooled from five mice after extraction. ELISA was performed in these pooled samples, using the multi-analyte ELISArray that detects 12 murine inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17α, interferon (IFN)γ, tumor necrosis factor (TNF)α,

and granulocyte or granulocyte/monocyte colony stimulating factors (G-CSF and GM-CSF) (Qiagen, SABiosciences).

Statistical analysis

Relative gene expression in RT-PCR profiler arrays was analyzed by the SABiosciences web-based software at www.sabiosciences.com/pcr/arrayanalysis.php. Briefly, the fold-change (2^(- Delta Delta Ct)) from the normalized gene expression (2^(- Delta Ct)) in the test sample (injured joints) divided by the normalized gene expression (2^(- Delta Ct)) in the control sample (uninjured joints) was calculated. Fold-change values greater than one indicate a positive or an up-regulation, while values less than 1 represent down-regulation of test genes. A 2-fold regulation is considered to be statistically significant ($P < 0.05$) by the software. All other quantitative data are presented as mean values plus or minus their standard deviation. Data were compared by analysis of variance tests using Microsoft Excel (2010 version). *P*-values less than < 0.05 were considered to be statistically significant.

Results

Validation of an in vivo model of hemarthrosis

To investigate the histopathological changes following a single joint hemorrhage, an animal model of severe hemarthrosis was adapted [9] and further developed to recapitulate multiple-articular bleeds. Briefly, groups of hemophilia A mice were injured once on their right knee joint, while the left knee joint served as the control (Fig. S1). To study the short-term effect of blood in the joints, animals were euthanized on days 1 and 14 after a single injury. To study the long-term effects of joint bleed, the injury was serially repeated at 14-day intervals (to mimic repeated bleeding) and mice were euthanized on days 30, 60, 75 and 90. Gross examination of the injured joint demonstrated hemorrhage and erythematous swelling, but this did not occur in control joints (Fig. S2). Furthermore, the histological joint scores employed [9] demonstrated a gradual but significant increase in these scores from a single-bleed (day 1) to multiple bleeding episodes (days 30–90), over a maximum possible score of 7, when compared between injured and control joints (Fig. 1). These data indicate that the histomorphologic changes classically observed in patients with hemophilic arthropathy are evident in this murine model of hemarthrosis. Histological studies performed at representative time-points of 3 h, 24 h (single injury), 30 days and 60 days (multiple injuries) in hemostatically normal mice showed no such increase in the scores (Fig. S3). A similar phenomenon has been previously established for a single joint puncture in hemostatically normal mice [39].

Global gene expression analysis identifies NF-κB as the major transcription factor driving cellular inflammatory response immediately after a single massive hemarthrosis

The RNA sample isolated from the joint tissue was first profiled for the chondrocyte-specific COL2A gene. The

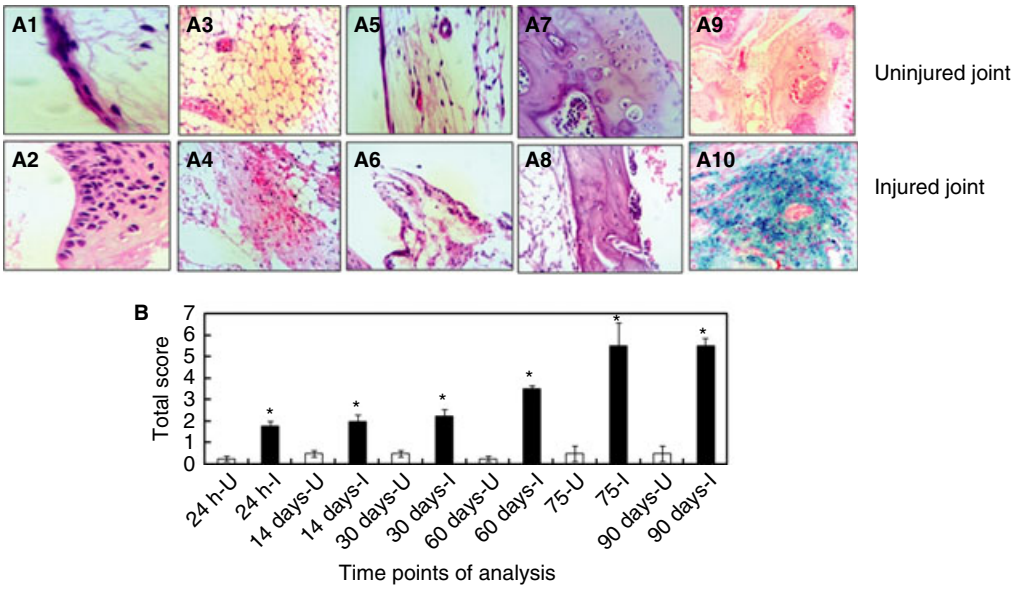

Fig. 1. Investigation of the histopathological changes following major joint hemorrhage in a hemophilia A mouse model of hemarthrosis. Representative histological images from joint tissue of a multiple-injury model. Significant amount of hyperplasia (A2) residual RBC (A4), villus formation (A6), cartilage erosion (A8) and hemosiderin deposition (A10) is seen in joints injured (I) at multiple time-points vs. uninjured control (U) joint tissue from the same animals (A1, A3, A5, A7 and A9). (B) Graphical representation of the total histological joint scores at the different time-points analysed. The data are +/− SD from two independent experiments and at least four animals each. * $P < 0.05$ and considered statistically significant as compared with control joint.

amplification of COL2A only in the joint tissue sample and not in other tissues confirmed the specificity of the material isolated (Fig. S4). Microarray analysis revealed approximately 3766 genes that were differentially expressed in the injured vs. control joints (Fig. 2). As blood-induced inflammation is expected to predominate at this time-point (3 h) [9,40], it was studied further. A transcription factor binding analysis carried out on these inflammatory genes (Table S3) (Fig. S5) demonstrated that a majority ($n = 28/28$, 100%) were predicted to have NF-κB binding sites on their promoter compared with other transcription factors such as EVi-1 (64%), SRF (68%) and SP1 (49%). This suggested that NF-κB may be the prime transcription factor driving pro-inflammatory mediators after the first articular bleed.

A NF-κB-associated inflammatory state predominates for the first 24 h in the single-injury hemarthrosis model

To further understand how the transcriptional potential and activity of NF-κB is regulated upon bleeding, we did a targeted screening of its associated pathways between 1 h to 90 days after injury. We first profiled the expression of 84 key genes related to NF-κB mediated signal transduction. Our data show that distinct members of the NF-κB family (RelA, RelB, NF-κB1, NF-κB2) (Fig. 3A) and NF-κB activators (TLRs, ICAM, Table S4) were activated between 1 h and 3 h. NF-κB-associated inflammatory genes (IL-1, IL-6, TNFα, IFNγ, etc.) were significantly activated (> 2-fold, $P < 0.05$) as early as 1 h post-injury (Fig. 3B, Table S4). Elevated levels of IL-6 were sustained for 24 h after a single articular bleed (Fig. 3 B and E). This phenomenon was further confirmed at the protein level. Western blot analysis demonstrated that the levels of NF-κB proteins p65 and p52/p100 were significantly up-regulated for the first 24 h after injury, with peak levels seen at 24 h for p65 (2.8-fold) and p52/p100 (9.5-fold) (Fig. 4 A). Concurrently, inhibitory kinase (Iκ)bα is also phosphorylated at 1 h and 3 h after injury, which indicates the activation and translocation of cytoplasmic NF-κB into the nucleus to turn on pro-inflammatory genes (Fig. S6). When the levels of these inflammatory cytokines were measured in the synovial fluid at the same 3-h time-point, pro-inflammatory cytokines such as IL-1β (154.4 pg mL^{-1} vs. 4.29 pg mL^{-1}), IL-6 (48.8 pg mL^{-1} vs. undetectable levels) and TNFα (1.45 pg mL^{-1} vs. undetectable levels) were significantly elevated in the injured joints (Fig. 5 A, B and C, respectively) compared with the control joints. These cytokines were below the detectable limit in the serum from whole blood samples analyzed at the representative time-points of 3 h or 60 days (data not shown). This indicates that the change in levels of the cytokines in synovial fluid did not just reflect the mere presence of blood in the joint cavity, as has been previously described [38]. It is also clear from available data (Fig. 3 A, B, D and E), that the inflammatory changes resulting from a single bleeding episode do not last for 14 days, possibly due to the synovial reabsorption of residual blood. It is also possible that a wound-healing response is initiated, as demonstrated by an increase in early growth response protein (EGR)-1 transcript levels at the 14-day time-point (Table S4) in these mice, which do not bleed naturally into the joint

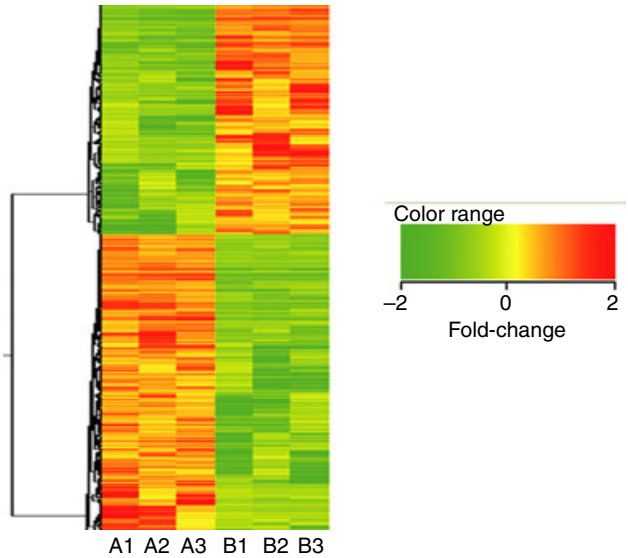

Fig. 2. Hierarchical clustering of differentially regulated genes in joints of the hemarthrosis model of hemophilia A mice 3 h after injury. The heat map illustrates the gene array run from joint total mRNA. Each sample is represented by a block: either from uninjured joints (A1–A3) or from injured joints (B1–B3). Relative down-regulation of expression in injured joints compared with uninjured joints is represented by green, while relative up-regulation is in red.

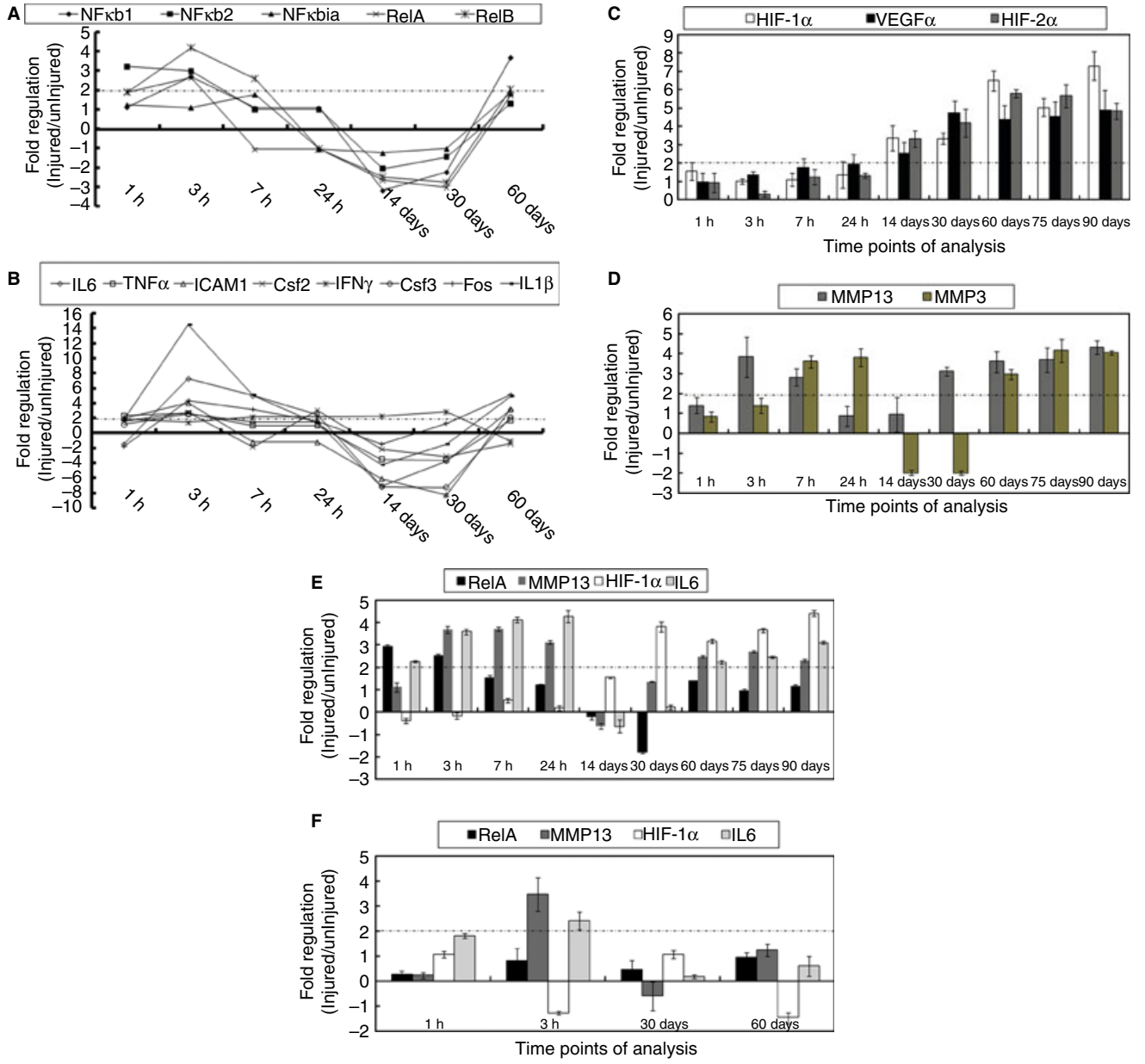

Fig. 3. Profiling of NF-κB and its associated genes at different time-points in injured vs. control joints of the hemarthrosis model by real-time PCR by SYBR green and TAQMAN chemistry. (A) Regulation of NF-κB subunits. (B) Regulation of NF-κB responsive inflammatory genes. (C) Induction of HIF-1α, VEGF-α and HIF-2α mRNA expression following injury. (D) Induction of MMP3 and MMP13 mRNA expression following injury. NF-κB-mediated signal transduction was profiled using the mouse NF-κB signaling pathway RT²*Profiler*™ PCR Array [SABiosciences] (A and B). Key genes related to NF-κB signaling were analysed by SYBR green RT-PCR (C and D). (E) Validation analysis for key targets using TAQMAN probes as described in materials and methods. (F) TAQMAN PCR analysis of key genes at different time-points of injury in samples isolated from knee joints of hemostatically normal C57BL/6 mice. The data for C to E is +/- SD of two independent experiments and six PCR replicates at each time-point. The data for F are +/- SD of one experiment (n = 5 animals) and three PCR replicates at each time-point. The horizontal dotted line denotes 2-fold up-regulation of genes analyzed and at a level of statistical significance ($P < 0.05$) compared with uninjured control joints.

[41]. RelA levels did not alter significantly after single joint injury in hemostatically normal C57BL/6 mice, indicating that the profile change is linked to the hemophilia phenotype and not solely due to the sharp injury (Fig. 3F). However, IL6 and MMP13 levels were elevated (> 2 fold) 3 h after the first injury but this was not sustained in multiple injury models (days 30 or 60). This correlates with previous observations that a single bleed in hemostatically normal mice does not show the joint pathology observed in human hemarthropathy, which is seen only in hemophilia mice [39]. Collectively, these data strongly suggest that NF-κB and its associated factors are acute phase modulators of inflammation following hemarthrosis in hemophilia mice.

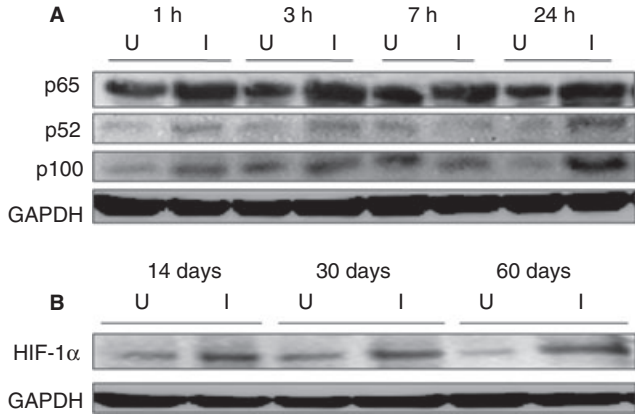

Fig. 4. Immunoblot for p65, p52/p100 and HIF-1α proteins. Induction of (A) p65, p52/p100 and (B) HIF-1α following injury of the knee joints. Uninjured (U) and injured (I) samples for each time-point.

Hemarthrosis in a multiple injury model induces NF-κB-associated hypoxic, pro-angiogeneic and chondro-degenerative processes in affected joints

In multiple injury models, with repeated bleeding into the joints between day 30 (two episodes) and day 90 (six episodes), the levels of NF-κB or inflammatory mediators progressively increased in injured joints, although they did not mimic the peak levels seen after the first bleeding episode. This was demonstrated by a significant increase in RelB (2-fold, Fig. 3A) and IL-1β (5-fold) or IL-6 transcript (2.2–3-fold, Fig. 3B and 3E) or protein levels (56.2 pg mL^{-1} vs. 0.95 pg mL^{-1}, 22 pg mL^{-1} vs. undetectable levels, Fig. 5A and 5B) between days 30 and 60. A reduced activation is possibly due to a primed wound-healing response operational after the first bleeding episode as mentioned above, but this process seems to be countered gradually with repeated bleeding in multiple injury models. We then studied the role of various NF-κB-associated factors such as HIF-2α, HIF-1α, MMP-3, MMP-13 and VEGF-α that are known to cause cartilage damage [33,34,42–48]. Figure 3(C) demonstrates the increase in hypoxia-inducible factors (3.3–6.5-fold for HIF-1α and HIF-2α, $P < 0.05$) and a simultaneous up-regulation of the pro-angiogeneic factor, VEGF-α (2.5–4.4-fold, $P < 0.05$). The levels of both these factors seem to increase with the number of joint bleeds. This increase in HIF-1α after multiple bleeds was further confirmed by an independent set of experiments using TAQMAN real-time PCR (Fig. 3E) or by immunoblotting (Fig. 4B).

As hypoxia regulating factors are known to affect chondrocyte turnover through their direct activity on MMPs [49], we then studied the modulation of MMPs in multiple-injury models. The expression levels of MMP-3 (3.6–3.8 fold, $P < 0.05$) and MMP-13 (2.1–3.8 fold, $P < 0.05$) were increased as early as 3 h after injury (Fig. 3D, E), and this was maintained upon repeated bleeding between days 60 and 90 (Fig. 3D, E). Concomitantly, the RelA, IL-6, MMP-13 and HIF-1α gene expression was not significantly different in the joints of the hemostatically normal mouse model of multiple hemarthrosis (Fig. 3F).

Interestingly, our studies also demonstrated a shift from the inflammatory response seen at earlier time-points after a single-bleed to a more adaptive immune response in multiple-injury models. As can be seen in Fig. 5, at 3 h post-injury, the levels of proinflammatory cytokines TNFα (1.45 pg mL^{-1} vs. undetected), IL-1β (154.4 pg mL^{-1} vs. undetected) and IL-6 (48.8 pg mL^{-1} vs. undetected) were significantly higher in injured joints compared with the 60-day time-point. The concentration of other immunomodulatory cytokines like IL-12 (2.2 pg ml^{-1}) and IL-4 (4 pg mL^{-1}), which play a major role in the adaptive immune response, [50,51] was significantly higher at the 60-day time-point in injured joints compared with their undetectable levels in uninjured joints, 3 h post-injury. A similar outcome was seen at the transcript measurements by the targeted mouse T-cell and B-cell activation PCR Array carried out at 3-h and 60-day time-points (Table S5). Further studies need to be carried out to study the role of these adaptive immune effectors in the development of arthropathy.

Hemarthrosis modulates the miRNAome of target joints

As most NF-κB-associated inflammatory cytokines are subjected to miRNA regulation [52], we wished to study the potential effect of the very first bleed on these miRNA regulatory networks. As seen from Tables 1 and S6, many key miRNAs were differentially expressed (–4 to +13-fold, $P < 0.05$) between injured and control joints. These include miRs with defined roles in NF-κB activation (miR155, 9, 16, 181b) [52–57], inflammation (miR155, 182, 16, 29a, 181a) [58–61] and apoptosis (miR155, 186, 19a, 23a) [62–64] (Table 1). This indicates that small RNAs could modulate the inflammatory network and contribute to the arthritis phenotype in hemophilia. However, further detailed studies are needed to

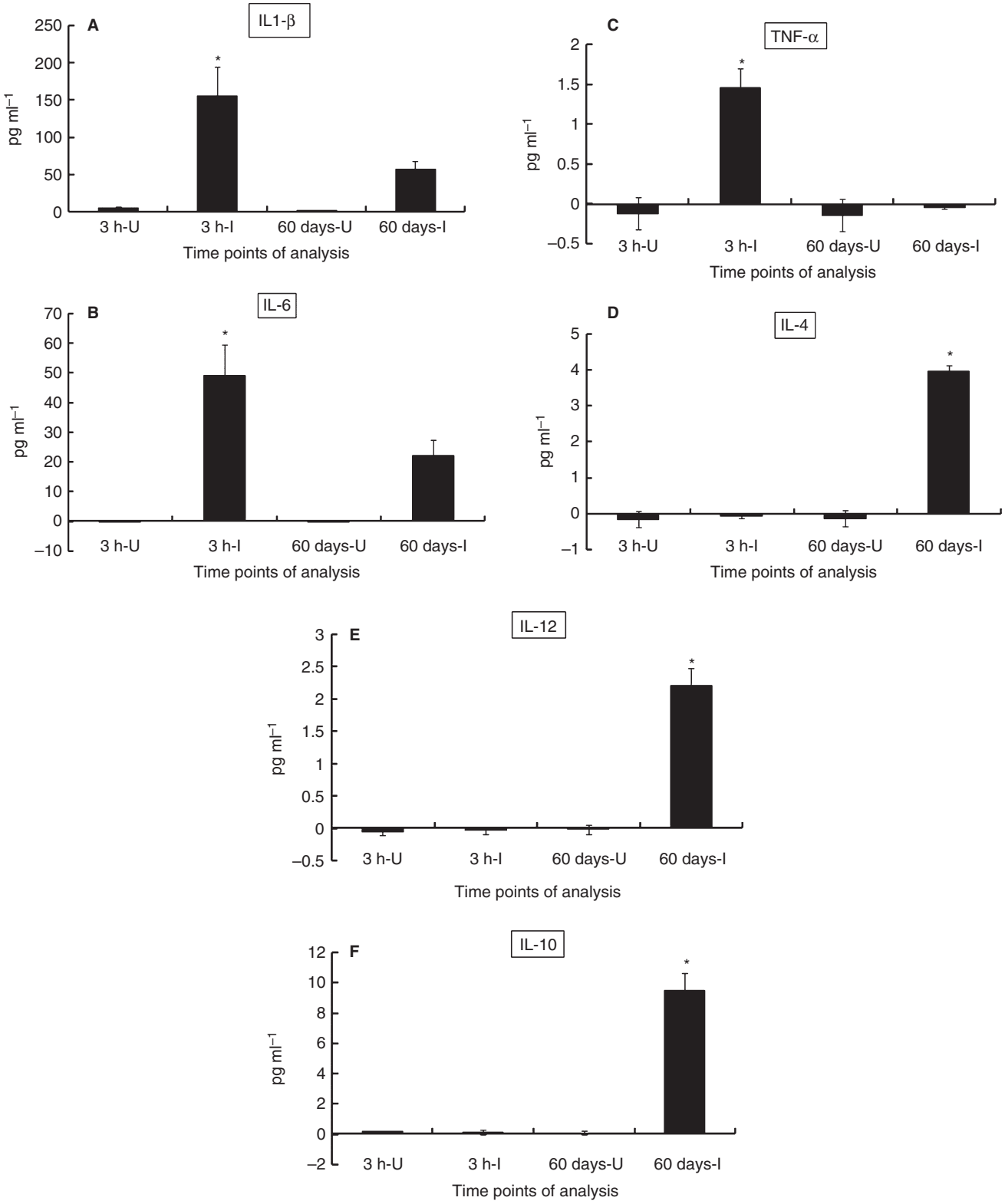

Fig. 5. Analysis of cytokine levels from synovial fluid of injured (I) and uninjured (U) knee joints at 3 h and 60 days post-injury. Synovial fluid was assayed for 12 murine proinflammatory cytokines as described in the 'Methods'. (A to F) shows the concentration of IL-1β, IL-6, TNFα, IL-4, IL-12 and IL-10, respectively. The data are +/- SD from triplicates of synovial fluid pooled from five mice joints at each time-point. *$P < 0.05$ considered as statistically significant as compared with uninjured control joint.

Table 1 Differential regulation of proinflammatory and proapoptotic miRNAs 3 h post injury. Mouse inflammatory response & autoimmunity miRNA PCR array was used to profile the expression of 84 key miRNAs related to inflammation and immune response in RNA samples collected from injured or un-injured (control) joints 3 h post-injury

mi-RNA	Fold change vs. control	Predicted function
155	+6.5	NF-κB activation
9	+11	
16	+5.4	
181b	+4.2	
let7a	−2.4	
155	+6.5	Inflammation and immune response
16	+5.4	
181a	+6	
182	+2.12	
29a	+4.3	
155	+6.5	Apoptosis
19a	−2.8	
186	+2.4	
23a	+5.78	

confirm their specific role in the development of hemophilic joint disease.

Discussion

The present understanding on the pathogenesis of blood-induced joint damage is largely based on a series of clinical observations [12,13,15]. The limited data available from various *in vitro* and *in vivo* experiments [9–13,65] suggest that the mechanism of blood-induced joint damage includes both inflammatory (synovium-mediated) and/or degenerative (cartilage-mediated) components. Iron deposition from joint bleeding, deep in synovial tissue, appears to contribute significantly to this process [40,65–67]. The presence of iron is also correlated with increased c-myc expression, as well as over-expression of a p53 suppressor, mdm2 [10,11]. The increased inflammatory response is probably due to the monocytes/macrophages recruited to the area along with accompanying inflammatory cytokines (IL-6, IL-1β, TNFα) [38]. The actual mediators of synovial cell proliferation and synovial hypertrophy are unknown, but a role for angiogeneic factors (VEGF-α, MMP-9) in this process has been proposed [68]. It is not yet clear, what are the molecular factors that are activated upon exposure of blood/iron in the synovium and which drive the sustained inflammatory state in the synovial lining. We also do not understand the ensuing mediators of (synovial) inflammation-driven expression of downstream catabolic factors that result in articular damage. Even though the joint damage includes both inflammatory and degenerative components, it is also not clear whether these processes are interlinked by a common pathway. Understanding these processes is crucial to give us insights into the pathogenetic mechanisms and also reveal targets for intervention.

Our objective in this study was to profile the molecular mediators from the onset of bleeding to the development of arthropathy, data hitherto not available. Thus, we first validated the single-hemarthrosis mice model described earlier [9,35] and further developed a multiple-injury hemarthrosis model of hemophilia A. A global gene expression analysis, 3 h post-injury in the single hemarthrosis model, identified NF-κB-associated inflammatory and immunoregulatory networks as being predominantly activated. To further understand their role, we studied the targeted expression of approximately 350 key genes related to NF-κB-signal transduction, its associated inflammatory/immuno-regulatory pathways and micro RNAs in the single hemarthrosis (1 h/3 h/7 h/24 h/day14) or multiple hemarthrosis models (days 30/60/75/90). Distinct members of the NF-κB (NF-κB1/NF-κB2/RelA/RelB), NF-κB-responsive inflammatory cytokine genes (IL-1b/IL-6/IFNγ/TNFα) were significantly up-regulated in injured vs. control joints after a single articular bleed. In multiple injury models, NF-κB-associated factors, which contribute to hypoxia (HIF-1α), angiogenesis (VEGF-α) and chondrocyte damage (matrix metalloproteinase-13), were significantly ($P < 0.05$) elevated in injured joints. The differential expression of some key targets was further confirmed to be reproducibly regulated by using different real-time PCR chemistries and at the protein level by western blotting or ELISA. Taken together, these data strongly suggest that NF-κB-associated pathways regulate the molecular pathogenesis of arthropathy in hemophilia. The documented role of NF-κB in many of the joint diseases, including rheumatoid arthritis and osteoarthritis, supports this further [69,70].

Based on our observations from this study and a literature review on the pathogenesis of various joint diseases [21,25,27,29,30,33,34,38,40,43–45,66,67,71–81], we propose a speculative model for the role of NF-κB and its associated factors in the development of hemarthropathy (Fig. 6). NF-κB is possibly activated in the joint tissue post-bleeding by three possible mechanisms. 1. Bleeding into the joint increases the iron stores that exceed the reabsorption capacity of synovium. These iron stores (hemosiderin) can generate free radicals as well as act as a pro-inflammatory trigger by activating NF-κB as shown earlier in arthritis patients [71]. 2. Re-bleeding may increase the net amount of pro-inflammatory microparticles in the joint cavity, which can then stimulate NF-κB and associated pathways as described in diseases such as systemic lupus erythematosus and rheumatoid arthritis [25,72,73]. 3. The macrophages that infiltrate the synovium in response to iron overload can activate NF-κB through inflammatory cytokines, a phenomenon described earlier in various inflammatory disease states [74,75]. Interestingly, in experimentally induced hemarthrosis models, Ovlisen *et al.* have demonstrated elevated levels of a macrophage chemoattractant protein (MCP)-1 in the synovial fluid [38], further supporting our hypothesis. The

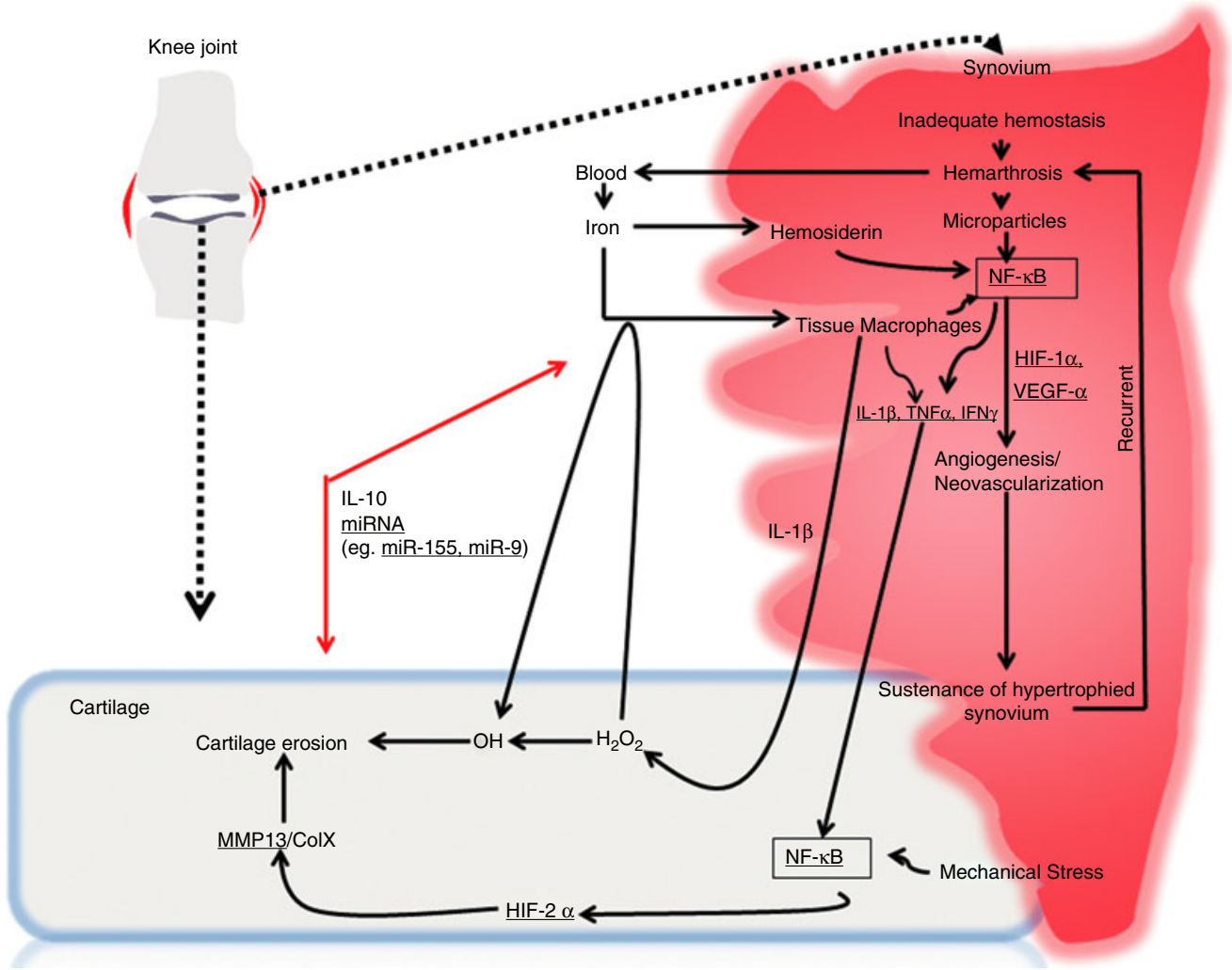

Fig. 6. Proposed mechanism of blood-induced joint damage in hemophilia by NF-κB-associated pathways. The molecular mediators identified in this study are underlined in the figure. The rest of the information presented is based on a comprehensive literature review of the pathogenesis of various joint diseases. For further details please refer to the 'Discussion' section in the text.

transcription factor, NF-κB, activated by any/all of these processes can then subsequently act on its associated factors, such as pro-inflammatory cytokines IL-1β and TNFα, to exacerbate synovitis, as shown in animal models of arthritis [29]. It is noteworthy that elevated levels of IL-1β in the synovial fluid from the hemarthrotic joints of mice [38] and IL-1β and TNFα in cultured hemosiderotic synovial tissue from a hemophilic patient have been demonstrated earlier [40,77].

Prolonged activation of NF-κB and other inflammatory mediators can subsequently lead to the induction of HIF-1α and its target genes, such as the pro-angiogenic VEGF [43], as part of a stress response mechanism initiated in the presence of low oxygen tensions. Of these, NF-κB is known to directly control the expression of HIF-1α and various HIF target genes as shown earlier [27,43]. Conversely, hypoxic conditions are also known to induce NF-κB [27]. Significantly, Acharya *et al.* [68] have shown in human synovial cells a role for HIF-1α transcripts in VEGF-mediated neo-angiogeneic processes in hemophilic joint disease. Thus, while a combination of NF-κB-mediated inflammatory processes exacerbates synovitis, the activity of NF-κB-associated factors such as HIF-1α and VEGF-α may contribute to neo-vascularization to support the sustenance of hypertrophied synovium. As the neovessels are unstable and are prone to damage, they contribute to recurrent hemarthrosis. This establishes a vicious cycle of bleeding, NF-κB-associated inflammation, hypoxia and angiogenesis and results in synovial hyperplasia. It is known that hyperplasia can drive other degenerative joint diseases such as osteoarthritis [30]. Thus, prolonged NF-κB activation and/or additional biomechanical injury can activate HIF-2α, possibly through the classical (p65) NF-κB subunit [33]. HIF-2α is a transcriptional factor that can directly act on chondro-degenerative enzymes, MMP-13 and ColX, both of which are known to be early terminal signals of stressed cartilage in osteoarthritis models [30,46,47,76]. The expression of these MMPs would lead to the breakdown of extra-

cellular matrix, and pave the way for the growing vasculature, induced by VEGF-α, and complete the process of endochondral ossification and cartilage damage. The macrophage infliltrates may also directly activate the generation of free radicals through IL-1β, as proposed earlier in arthritis models, thereby initiating the extracellular matrix breakdown [40,66,67,77–79]. IL-10 has been shown to have potential as an anti-inflammatory agent in this process, based on data from cultured articular cartilage tissue from hemophilic patients [80]. Similarly, several miRNAs that are known to modulate NF-κB or their downstream targets with wide-ranging functions from inflammation to apoptosis as proposed earlier in several inflammatory conditions [52,82,83] could alter the arthritis phenotype in hemophilia.

In conclusion, we have identified that NF-κB and its associated pathways contribute to articular damage in a murine model of hemarthrosis. However, further confirmatory studies by specific blocking of NF-κB alone or in combination with other key targets such as HIF-1α, VEGF-α or MMP-13, or modulating the expression of miRNAs (e.g. miR-155, miR-9) in the hemarthrosis models, are necessary to gain further mechanistic insights into this phenomenon. Another alternative is to probe functional polymorphisms in cytokine, hypoxic and angiogeneic genes regulated by NF-κB promoter binding in a series of hemophilia patients we have described earlier and are clinically well characterized with respect to their joint outcome [84].

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Addendum

G.R. Jayandharan designed research; D. Sen, A. Chapla, N. Walter, V. Daniel performed research; D. Sen, A. Srivastava, G.R. Jayandharan analyzed data; D. Sen, A. Srivastava, G.R. Jayandharan wrote the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Experimental design for the induction of hemarthrosis in a murine model of hemophilia A. Experiments were performed in *factor 8*-deficient ($F8^{-/-}$) mice according to the schedule shown. The first injury occurred on day 0. Mice were then euthanized at 1 h, 3 h, 7 h, 24 h or 14 days to study the effect of single-articular bleed for a period of 14 days. The multiple-injury models had at least two and up to six episodic bleedings between days 30 and 90, respectively. On all the animals, the right knee joint was injured whereas the left knee joint served as the uninjured control. At least 14 animals per time-point were used for a combination of histological, molecular and biochemical studies.

Figure S2. Gross changes in knee joints of $F8^{-/-}$ mice 4 days (A,B) or 14 days (C,D) following injury. (A,C) Right injured knee joint and (B,D) left uninjured knee joint.

Figure S3. Investigation of the histopathological changes following major joint hemorrhage in hemostatically normal C57BL/6 mice. Graphical representation of the total histological joint scores at the different time-points analyzed.

Figure S4. Validation of RNA isolated from the joint tissue of the hemarthrosis model. Reverse-transcription PCR amplification of joint-specific COL2A gene was seen only in the the RNA isolated at different time-points from the injured right-knee joint (R) or from uninjured left-knee joints (L) but not from other tissues.

Figure S5. Hierarchical clustering of differentially expressed inflammatory mediators in joints of the hemarthrosis model of hemophilia A mice, 3 h after injury. Heat maps illustrate the results of a gene array run from joint total mRNA. Each sample is represented by a block: (A) from uninjured joints (samples 1–3) and (B) from injured joints (samples 1–3). Relative down-regulation of expression in injured joints compared with uninjured joints is represented by green, while relative up-regulation is in red (see scale on Figure).

Figure S6. Immunoblot for phosphorylated IκBα protein. Total protein was isolated from knee joint tissues at each time-point. Western blotting was done for phosphorylated (p) IκBα protein according to the manufacturer's protocol (Cell Signaling). GAPDH was used as a loading control. Uninjured (U) and injured (I) samples for each time-point. Protein lysates were pooled from mice ($n = 5$) for each time-point.

Table S1. Primer sequences used for SYBR green based real-time PCR amplification of target genes.

Table S2. Primer sequences used for *TaqMan*®-based real-time PCR validation of target genes.

Table S3. List of inflammatory mediators found to be differentially expressed after 3 h in injured vs. control

knee joints by micro-array analysis.

Table S4. List of NF-κB-related genes differentially expressed in injured vs. control knee joints of the murine model of hemarthrosis at different time-points analyzed.

Table S5. RT-PCR profiling of murine T-cell and B-cell activation markers between injured and control joints. Total RNA from injured joints and control joints at 3-h and 60-day time-points was profiled for the expression of 84 genes related to T-cell and B-cell responses by the mouse T-Cell and B-Cell activation RT² *Profiler*™ PCR Array (SABiosciences). Data are shown for immunomodulators significantly ($*P < 0.05$) different between the two groups.

Table S6. List of miRNA differentially expressed in injured vs. control knee joints 3 h post-injury in the hemarthrosis model (excluding those functionally annotated in Table 1).

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