



Published in final edited form as:

Mol Immunol. 2014 November ; 62(1): 235–248. doi:10.1016/j.molimm.2014.06.028.

New Insights into Disease-Specific Absence of Complement Factor H Related Protein C in Mouse Models of Spontaneous Autoimmune Diseases

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Abstract

Complement factor H (CFH) protein is an inhibitor of the alternative pathway of complement (AP) both in the fluid phase and on the surface of host cells. Mouse and human complement factor H-related (CFHR) proteins also belong to the fH family of plasma glycoproteins. The main goal of the current study was to compare the presence of mRNA for two mCFHR proteins in spontaneously developing autoimmune diseases in mice such as dense deposit disease (DDD), diabetes mellitus (DM), basal laminar deposits (BLD), collagen antibody-induced arthritis (CAIA) and systemic lupus erythematosus (SLE). Here we report for the first time that the CFHR-C mRNA was universally absent in the liver from three strains of lupus-prone mice and in a diabetic-prone mouse strain. The mRNA levels (pg/ng) for CFH and CFHR-B in MRL-*lpr/lpr*, at 9 wks and 23 wks were 707.2 ± 44.4 , 54.5 ± 5.75 and 729 ± 252.9 , 74.04 ± 22.76 respectively. The mRNA levels for CFH and CFHR-B in NZB/NZW mice, at 9 wks and 54 wks were 579.9 ± 23.8 , 58.8 ± 1.41 and 890.3 ± 135.2 , 63.30 ± 9.2 respectively. CFHR-C protein was absent in the circulation of MRL-*lpr/lpr* and NZB/NZW mice before and after the development of lupus. Similarly, mRNA and protein for CFHR-C was universally absent in liver and other organs and in the circulation of NOD mice before and after the development of DM. In contrast, the mRNAs for CFH, CFHR-B and CFHR-C were universally present in the liver from mice with and without DDD, BLD and CAIA. The levels of mRNA for CFHR-B in mice with and without BLD were ~4

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Disclosure All authors have no competing interests.

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times higher than the mice with lupus. The complete absence of mRNA for CFHR-C in lupus and diabetic-prone strains indicates that polymorphic variation within the mouse CFHR family exists and raises the possibility that such variation contributes to lupus and diabetic phenotypes.

Keywords

Complement factor H related C; Dense deposit disease; diabetes mellitus; basal laminar deposits; collagen-induced arthritis; systemic lupus erythematosus

1. INTRODUCTION

Complement factor H (CFH) is a 155 kDa glycoprotein and consists of 20 short consensus repeat (SCR) domains. It is an essential inhibitor of the alternative pathway (AP) of complement both in the fluid phase and on the surface of host cells. It prevents the formation of AP C3 convertases and also acts as a cofactor for factor I (FI) in the cleavage of C3b (active) into iC3b (inactive). Mouse and human complement factor H-related (CFHR) proteins also belong to the fH family of plasma glycoproteins. Human CFH proteins consist of six CFHR proteins: CFHR1, CFHR2, CFHR3, CFHR4A, CFHR4B and CFHR5 (Estaller et al., 1991; Skerka and Zipfel, 2008). Human CFHR6 is a pseudogene and does not code for a mature protein (personal communication with Dr. Skerka). There is a high degree of sequence homology (36-100%) between human fH and human CFHR proteins in SCRs 18-20 indicating conserved function of this C terminal recognition region (Skerka et al., 2013; Zipfel et al., 2013). This is the region by which human CFHRs and CFH bind to the cell surface and recognize C3b and all five human CFHR proteins binds to C3b and to C3d (Goicoechea de Jorge et al., 2013; Heinen et al., 2009; Hellwage et al., 1999; McRae et al., 2005; Skerka et al., 2013; Tortajada et al., 2013). It has been shown that human CFHR1 protein binds to C3b component of the enzyme, C5 convertase and inhibits the cleavage of C5 into C5a and C5b (Heinen et al., 2009). In addition, CFHR1 also inhibits the formation of C5b-C9 (Membrane Attack Complex) (MAC) (Heinen et al., 2009). Human CFHR2 protein inhibits the AP of the complement (Eberhardt, 2012). Human CFHR3 competes, like CFHR1, with fH for binding to C3b (Fritsche et al., 2010). The exact function of human CFHR4 is unknown, but complement activation by CFHR4A has been documented (Hebecker et al., 2010). CFHR4 also interacts with C-reactive protein (CRP) and is recruited to the surface of necrotic cells (Mihlan et al., 2009). Human CFHR5 has been shown to compete with CFH for binding to C3b and also to iC3b (Goicoechea de Jorge et al., 2013). It can also bind to CRP and then be recruited to sites of tissue damage to inactivate C3b (Park and Wright, 1996). In addition CFHR proteins due to copy number variations in the human cfHR gene cluster are associated with kidney disorders (Chen et al., 2014).

Although the functions of human CFHR proteins have been investigated extensively, the presence and function of mouse CFHR proteins are less well characterized and have not been demonstrated clearly in the pathogenesis of spontaneously occurring autoimmune diseases in mice. The first CFH-related transcripts and genomic sequences from 12 clones that were distinct from CFH in mouse liver were published more than two decades ago (Vik et al., 1990). Later on two CFHR proteins of approximately 38 and 100 kDa were identified

first time in mouse plasma using CFH specific anti-serum and were named as complement FHR-B (CFHR-B) and FHR-C (CFHR-C) proteins, respectively (Hellwage et al., 2006). These two complement FHR proteins in mice were not the breakdown products of CFH, because these were also present in plasma and liver extracts from *fH*^{-/-} mice. This study not only examined the expression of CFHR-B in various tissues by real-time polymerase chain reaction (RT-PCR), but also shows that mouse complement CFHR-B protein interacts with heparin and human C3b (Hellwage et al., 2006). Interestingly, differential expression levels of CFHR-B and *fH* in the liver and kidney were observed indicating their specific role in local complement activation. In general, *fH* deficiency in mice, which leads to absent cell surface and fluid phase complement regulation by *fH*, has been linked to hypocomplementemia and membranoproliferative glomerulonephritis (MPGN) (also termed Dense Deposit Disease [DDD]) (Pickering et al., 2002; Pickering et al., 2006). Mice deficient in CFH develop spontaneous DDD characterized by C3 deposition along the glomerular basement membrane with subsequent development of glomerulonephritis (Pickering et al., 2002). However, specific deficiency of SCR16-20 of mouse CFH, leads to the development of spontaneous atypical hemolytic uremic syndrome (aHUS) (Goicoechea de Jorge et al., 2007). It has been shown *in vitro* that by replacing CFH in serum with a SCR16-20 deletion mutant (Pangburn, 2002) or by blocking CFH binding to cells by use of a recombinant competitive inhibitor containing short consensus repeats 19 and 20 (rfH19-20) of CFH, cell surface protection is impaired and AP activation and amplification is enhanced (Ferreira et al., JI 2006, blood 2009). The role of CFH in rheumatoid arthritis (RA) has been shown in a recent study using collagen antibody-induced arthritis (CAIA) in a mouse model of RA (Banda et al., 2013). The *in vivo* administration of the competitive *fH* inhibitor rfH19-20, but not of control rfH3-5, significantly worsened the arthritis in mice. AMD is the leading cause of blindness in older individuals. The generation of chimeric CFH transgenic mice using human CFH sequences for SCR6-8 flanked by the mouse sequence for SCR1-5 and SCR9-20, lead to the spontaneous AMD-like retinal disease (Ufret-Vincenty et al., 2010). An important role for CFH has been shown in the MRL-*lpr/lpr* mouse model of SLE (Bao et al., 2011). Renal disease was exacerbated in the absence of CFH in this strain.

Taken together, CFH is important in the pathogenesis of DDD, aHUS and AMD and influences tissue injury in rheumatoid arthritis and SLE. The roles of the FHR proteins remain unclear so here we investigated the presence of mRNAs for CFH as well as two mouse CFHR proteins B and C in spontaneous mouse models of autoimmune disease. We surprisingly found that the expression level of mRNA and protein for CFHR-C was missing not only in the liver, but also in the circulation, of MRL-*lpr/lpr*, LG/J and NZB/NZW lupus-prone and of NOD diabetic-prone mice. However, the mRNA for CFHR-C was present in the liver and also in other organs in mice with DDD, BLD and RA. This study regarding the complete absence of CFHR-C in lupus-prone and diabetic-prone mice compared with DDD, BLD, and RA suggests that it plays an important role in the pathogenesis of spontaneously occurring autoimmune diseases in mice.

2. Materials and Methods

2.1 Mice

Six to 44-week-old $fH^{-/-}$ and WT C57BL/6 mice were used for the DDD study. Our laboratory has maintained a colony of $fH^{-/-}$ C57BL/6 homozygous mice with the F10 progeny used for this study and older $fH^{-/-}$ mice develop DDD. DDD was renamed as membranoproliferative glomerulonephritis type II (MPGN-II) (Smith et al., 2011). For the current study, 6-week-old male $fH^{-/-}$ mice without DDD and 40-week-old male $fH^{-/-}$ mice with DDD were used.

Four to 20-week-old female non-obese diabetic (NOD) mice (Jackson Laboratories, Bar Harbor) were used for DM studies. NOD mice spontaneously develop clinical symptoms of diabetes at ~12 weeks of age. The clinical symptoms of diabetes before and after the development of DM in NOD mice were confirmed by measuring blood glucose levels using Ultima Blood Glucose Monitor (ReliON, Betoville, AR) and Abbot Glucose testing strips. Thus, glucose levels were used to differentiate between non-diabetic and diabetic mice.

Ninety-week-old Chimeric $cfhTg/mCfh^{-/-}$ mice were used for AMD studies. Dr. Ufret-Vincenty has maintained a colony of $cfhTg/mCfh^{-/-}$ mice. We obtained frozen organs such as liver and knee joints from $cfhTg/mCfh^{-/-}$ mice and also from age- and sex-matched littermate B6 control mice. Serum was also obtained from 90-week-old $cfhTg/mCfh^{-/-}$ and B6 mice. Serum was also obtained from 90-week-old $cfhTg/mCfh^{-/-}$ and B6 mice. These transgenic mice were chimeric (containing human fH SCR domains 6-8, flanked by murine 1-5 and 9-20) and spontaneously develop basal laminar deposits (BLD) under the retinal pigment epithelium by 12-months of age, which could be considered a model for early stages of AMD (Ufret-Vincenty et al., 2010). Mice cannot develop AMD because they do not have a macula (personal communication with Dr. Ufret-Vincenty). 50% of the AMD cases are associated with the domain 7 “H402 risk variant” of CFH and these chimeric mice are based on this model (Ufret-Vincenty et al., 2010). Liver from chimeric mice was also used as a negative control to confirm the absence of mouse CFH according to previously described methods and primers (Ufret-Vincenty et al., 2010).

CAIA (a.k.a. RA) is a mouse model of human RA. Eight-week-old C57BL/6 WT male mice (Jackson Laboratories) were used for the RA studies described below. Additionally, 8-week to 54-week-old MRL- lpr/lpr (MRL/MpJ-Fas^{lpr}/J), NZB/NZW and LG/J mice were used for the current studies. All strains of lupus-prone mice i.e. MRL- lpr/lpr , and NZB/NZW females were directly purchased at the age of 8 weeks from the Jackson Laboratories. The MRL- lpr/lpr strain of mice started showing signs of lupus at around 18 weeks. However, the NZB/NZW strain of mice started showing the signs of lupus at around 44 weeks. We also obtained livers from 8-week-old LG/J female mice directly from Jackson Laboratories. MRL- lpr/lpr mice were originated in a colony from LG/J mice due to a spontaneous mutation. MRL- lpr/lpr , the most commonly studied mouse model of the SLE, develops an autoimmune disease that reflects pathologies of human SLE, including lymph node enlargement, increased IgG levels, anti-nuclear antibody production, proteinuria, and kidney failure caused by inflammation of the glomeruli (Perry et al., 2011). Urine was also

collected before and after the development of disease and we examined the signs of lupus development in MRL-*lpr/lpr* and NZB/NZW by testing for proteinuria.

Age-matched and sex-matched C57BL/6 mice (Jackson Laboratories) were used as wild type (WT) controls for DDD, BLD and RA studies. All animals were kept in a barrier animal facility with a climate-controlled environment having 12-h light/dark cycles. Filter-top cages were used with 3 mice in each cage. During the course of this study, all experimental mice were fed breeder's chow provided by the Center for Laboratory Animal Care, University of Colorado School of Medicine. All animal studies were approved by the University of Colorado IACUC.

2.2 Induction of collagen antibody-induced arthritis

We used CAIA, a mouse model of RA, due to the non-availability of a spontaneous mouse model of arthritis at the time these studies were conducted. CAIA was induced in WT C57BL/6 mice by using a cocktail of 4 mAb to bovine CII (ArthroGen-CIA, Chondrex) suspended in sterile Dulbecco's PBS according to our published studies (Banda et al., 2013; Banda et al., 2006). All mice received i.p. injections of 4 mg/mouse of ArthroGen (a mixture of 5 clones) on day 0 and 50 µg/mouse of LPS from *E. coli* strain 0111B4 on day 3 to synchronize the development of arthritis. All mice started to develop arthritis at day 4 and were sacrificed at day 10, including 4 age-matched WT mice that were not treated with ArthroGen-CIA. Severity of clinical disease (CDA) in WT mice was examined according to our published studies (Banda et al., 2013; Banda et al., 2006). At day 10, all mice with and without RA, were sacrificed and above-mentioned tissues were removed and snap frozen prior to the extraction of RNA.

2.3 Surgical removal and processing of various organs for RNA extraction

Various organs including brain, liver, lung, heart, spleen, kidney, knee joint and skin from mice before and after the development of DDD, DM, BLD, CAIA and SLE were surgically removed after sacrificing mice via anesthesia followed by cervical dislocation. Fat and muscle were carefully removed from each organ. All organs were rinsed three times in 1xPBS to remove blood and/or debris and then snap frozen in liquid nitrogen. Total RNA was extracted from all organs using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's suggested protocol.

2.4 Expression levels of mRNAs for CFH, CFHR-B and CFHR-C

The main focus of current studies was on liver. Therefore, RNA was extracted using liver from *fH*^{-/-}, MRL-*lpr/lpr* (MRL/MpJ-Fas^{lpr}/J), NZB/NZW, LG/J, *cfhTg/mCfh*^{-/-} and WT C57BL/6 mice. RNA was also extracted from various other tissues including brain, lung, heart, spleen, kidney, knee joint and skin. All RNA samples were pretreated with DNAase. cDNA was synthesized from all RNA samples and also analyzed for 18srRNA. All qRT-PCR data were analyzed using 18srRNA cDNA based standard curve. All tissue samples were run in duplicate from each mouse. All qRT-PCR reactions were run in a 96-well plate using a 7900HT- Real Time PCR System (Applied Biosystems). RNA samples for CFH, CFHR-B and CFHR-C were analyzed according to published methods by qRT-PCR using 40 cycles (Schmittgen and Livak, 2008). A Standard curves were made using liver from WT

C57BL/6 mice with no disease and were used as a positive control to calculate the mRNAs for CFH, CFHR-B and CFHR-C. Gene-specific primers and probes for qRT-PCR for fH, CFHR-B and CFHR-C were designed by using exon # 3, exons # 4/5 and exon # 11, respectively. Specifically, exon # 11 from the CFHR-C nucleotide data base sequence (Accession numbers: Variant 1: NM_001029977; Variant 2: NM_001160303 and Variant 3: NM_001160304) was used to design primers for CFHR-C, because it has three variants and all share identical nucleotide sequences in this region. Moreover, there was no homology in nucleotide sequences between CFHR-B and CFHR-C in exon # 11 and this strategy was used to design primers so that the mRNA for both can be detected precisely. The reason we have selected primers from a single exon for CFHR-C because all three variants happened to be present in this exon. All RNA samples were pre-treated with DNAase to avoid contamination from genomic DNA and also during cDNA preparation an internal control without RT enzyme was used. No amplification was seen in samples without RT compared with RT. The liver and knee joints from *cfhTg/mCfh*^{-/-} chimeric mice contains mRNA for CFH when primers from exon #3 were used. The sequences of primers and probes used to determine mRNAs for CFH, CFHRB and for CFHR-C levels have been listed in table 1.

2.5 Western blot analysis of serum for detecting CFH and CFHR-C

To examine for the presence of CFH and CFHR-C proteins, sera from WT, *fH*^{-/-}, NOD, *cfhTg/mCfh*^{-/-} and MRL-*lpr/lpr* mice were electrophoresed on 8% Tris-glycine gel running buffer (Life Technologies) under reducing conditions with 1xTris-glycine buffer. Serum samples were diluted 1:240 in 1x SDS buffer. After transfer, the PVDF membrane was blocked with 5% milk in 1x PBS/0.5% Tween 20 solution for 2 h. To detect CFH and CFHR-C simultaneously the blots were then incubated for 24 h at 4° C with goat anti-human CFH polyclonal Ab (dilution 1:1000) (Quidel), followed by a rabbit anti-goat HRP-conjugated secondary antibody (dilution 1:2000) (Santa Cruz Biotechnology). The blots were washed three times for 10 min each in 1x PBS/0.5% Tween 20 solution. The blots were then developed for 3 min by using a 1:1 mixture of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). It was noticed that the band of CFHR-C was too close to a non-specific band when using 10% NuPAGE Bis-Tris gel. Thus, to clearly separate the band of CFHR-C we used 8% Tris-glycine gel. Nonetheless, most of the anti-CFH antibodies bind to non-specific bands. In spite of multiple attempts we failed to find an antibody that specifically recognizes the mouse CFHR-B protein (expected MW of ~40 kDa). We cannot rule out the possibility that the anti-CFH antibody used in this study also recognizes the CFHR-B band. A sharp band of 155 kDa shows the presence of CFH and a sharp band of 98-100 kDa shows the presence of CFHR-C. The band of CFHR-C was normally less intense than the CFH band and it indicated that less amount of this protein was naturally present in the circulation (not shown). For western blot sera from WT and *fH*^{-/-} mice have been used as a positive and negative controls respectively.

2.6 Statistical analyses

All *p* - values were calculated using Student's t test with the GraphPad Prism^R 4 statistical program. The PCR data were first analyzed with the Kolmogorov-Smirnov test for normal distribution. The qRT-PCR data in histograms are shown as the mean + SEM, with *p* < 0.05 considered significant using an unpaired, two-tailed t-test.

3. Results

3.1 mRNA levels in the liver and various organs of mice with and without DDD

To determine the expression of mRNAs for CFH, CFHR-B and CFHR-C in liver and various organs from aged *fH*^{-/-} with DDD and WT (*fH*^{+/+}) mice with no DDD, total RNA was analyzed by qRT-PCR (Fig. 1). The mRNA levels for CFH, CFHR-B and C have been expressed in pg/ng 18S rRNA throughout this study. The mRNAs for CFH and CFHR-B were present in all tissues of WT mice with no DDD (Fig.1A, 1B). The levels of mRNAs for CFHR-B and CFHR-C in liver of mice with no DDD were 613.1 ± 36.1 and 183.2 ± 25.9 respectively (Fig.1B, 1C). The mRNA expression for CFH was absent in mice with DDD, as expected, not only in the liver but also in the brain, lung, heart, spleen, kidney, knee joint and skin (Fig.1D). The levels of mRNAs for CFHR-B and CFHR-C in liver of mice with DDD were 141.4 ± 13.8 and 107.1 ± 11.2 respectively (Fig.1E, 1F). The levels of mRNA for CFHR-B and CFHR-C in mice with DDD in liver were significantly ($P < 0.0001$) lower than the WT mice with DDD. The levels of mRNA for CFHR-C in spleen of mice with DDD vs. in the spleen of mice with no DDD were 24.1 ± 2.83 and 4.74 ± 10.60 and these differences were significant ($P < 0.011$) (Fig. 1C, 1F). In contrast, no major differences were seen in kidney (Fig.1C, 1F). Overall, mRNAs for CFHR-B and CFHR-C were present in the liver and also in other organs in mice with and without DDD.

3.2 mRNA levels in the liver and various organs of mice with and without DM

Total RNA was analyzed by qRT-PCR, as mentioned above, to determine the expression of mRNAs for CFH, CFHR-B and CFHR-C in liver and various organs from female NOD mice with and without DM (Fig. 2). The glucose levels before and after the development of DM were 114.2 ± 6.46 and >500 respectively. The mRNA for CFH was present not only in the liver of NOD mice with and without DM but it was also present in various organs such as brain, lung, heart, spleen, kidney, knee joint and skin (Fig. 2A, 2D). The mRNAs for CFHR-B were present in all tissues of NOD mice with and without DM (Fig. 2B, 2E). The levels of mRNAs for CFH, CFHR-B and CFHR-C in liver of mice with no DM were 851.0 ± 49.9 , 137.9 ± 15.0 and 0 ± 0 respectively (Fig. 2A, 2B, 2C). In liver, CFH and CFHR-B were significantly ($P < 0.05$) higher than brain, lung, heart, spleen, kidney, knee joint and skin in mice with and without DM (Fig. 2A, 2B, 2D, 2E). The levels of mRNAs for CFH, CFHR-B and CFHR-C in liver of mice with DM were 554.5 ± 212.24 , 137.4 ± 66.6 and 0 ± 0 respectively (Fig. 2D, 2E, 2F). Thus, mRNA for CFHR-C was universally absent not only in the liver, but also in all other organs of the NOD mouse with and without DM (Fig. 2C, 2F). No significant differences were seen in the mRNAs for CFH and CFHR-B in the liver of mice with and without diabetes (Fig. 2A, 2D). However, the differences in the mRNAs levels were significant ($p < 0.034$) for only CFH but not for CFHR-B in kidney of mice with and without DM. The presence of mRNAs for CFH and CFHR-B in liver of mice with and without DM and the universal absence of CFHR-C in liver of diabetic NOD-prone mice and also in various organs of these mice indicated that it has important relevance in the pathogenesis of diabetes.

3.3 mRNA levels in the liver and knee joints of mice with and without BLD

The mRNA levels were determined only from the liver and knee joints of *CfhTg/mCfh*^{-/-} mice with BLD and B6 mice with no BLD (Fig. 3). The mRNA levels from brain, lung, heart, spleen, kidney and skin were not determined because of the non-availability of enough *CfhTg/mCfh*^{-/-} mice at the time these studies were conducted. The levels of mRNAs for CFH, CFHR-B and CFHR-C in liver of mice with no BLD were 465.8 ± 1.7 , 246.0 ± 15.9 and 70.1 ± 5.9 respectively (Fig. 3A, 3B, 3C). The mRNA for CFH was completely absent in the liver of mice with BLD (Fig.3A), but the mRNA levels for CFHR-B and CFHR-C were 270.5 ± 2.3 and 68.1 ± 2.3 respectively (Fig. 3E, 3F). CFH was absent in the knee joints of mice with BLD (Fig. 3D) but the levels for CFHR-B and CFHR-C were 0.40 ± 0.06 and 0.11 ± 0.08 respectively (Fig. 3E, 3F). Nonetheless, liver was positive for the human/mouse fH chimeric transgene as confirmed by using specific primers and probes (data not shown) (Ufret-Vincenty et al., 2010). There was no statistically significant difference in the mRNA levels for CFHR-B ($p < 0.15$) (Fig.3B, 3E) and CFHR-C ($p < 0.75$) in the liver of mice with and without BLD (Fig. 3E, F). The mRNA for CFHR-B and CFHR-C were present but very low in the knee joints of mice with and without BLD (Fig. 3E, F). No statistically significant differences were seen in the levels of mRNA for CFHR-B ($p < 0.18$) and CFHR-C ($p < 0.38$) in the knee joints of mice with and without BLD. In these studies, age-matched and sex-matched littermate non-chimeric WT C57BL/6 mice without BLD were used. These results showed that CFHR-B and CFHR-C mRNAs were present in the liver but very low knee joint of mice with and without BLD.

3.4 mRNA levels in the liver and various organs of mice with and without CAIA

The arthritis in WT mice was confirmed visually and Clinical disease scores were used to assess the severity of arthritis according to our published studies (Banda et al., 2013; Banda et al., 2006). All mice with CAIA were sacrificed at day 10. Age- and sex-matched WT mice with no arthritis were also sacrificed at the same time. The mRNA levels of CFH in the liver of mice with and without CAIA were 573.95 ± 61.37 and 375.63 ± 35.51 , respectively (Fig. 4A, 4D). The increases in the mRNA levels of fH in the liver of mice with disease were significant ($p < 0.04$). The levels of mRNAs for CFHR-B and CFHR-C in liver of mice with no CAIA were 498.42 ± 46.12 and 100.33 ± 9.60 , respectively (Fig.4B, 4C). The levels of mRNAs for CFHR-B and CFHR-C in liver of mice with RA were 607.62 ± 50.10 and 86.81 ± 9.36 , respectively (Fig.4E, 4F). Interestingly, there was an increase in the mRNA levels of CFHR-C in the spleen of mice with RA compared with mice with no CAIA (Fig. 4f, c).

3.5 mRNA levels in the liver and various organs of mice with SLE

Three different strains of lupus-prone mice were used to determine the mRNA expression of CFH, CFHR-B and CFHR-C in the liver. The mRNA for CFH and CFHR-B in liver of MRL-*lpr/lpr* before the development of lupus were 707.2 ± 44.4 and 54.5 ± 5.75 respectively (Fig.5A, 5B). The mRNA for CFH and CFHR-B in liver of MRL-*lpr/lpr* after the development of lupus were 729.3 ± 252.9 and 74.0 ± 22.76 respectively (Fig. 5D, 5E). These differences in the mRNA levels, before and after the development of lupus for both CFH and CFHR-B, were not statistically significant ($p < 0.91$) and ($p < 0.37$), respectively.

However, the mRNA for CFHRC was completely absent in the liver of these mice before and after the development of lupus (Fig. 5C, 5F). The CFHR-C mRNA was again absent in all other organs including brain, lung, heart, spleen, kidney, knee joint and skin before (Fig. 5C, 5c) and after the development of lupus (Fig. 5F, 5f).

Next we examined the mRNA for CFH, mCFHR-B and CFHR-C in NZB/NZW mice, which is a different strain of lupus-prone mice with similar disease phenotype (Fig. 6). The mRNA for CFH and CFHR-B in liver of NZB/NZW before the development of lupus were 579.9 ± 23.8 and 58.87 ± 1.41 , respectively (Fig. 6). The mRNA for CFH and CFHR-B in liver of NZB/NZW after the development of lupus were 890.3 ± 135.2 and 63.3 ± 9.2 , respectively (Fig. 6). These differences in the mRNA levels, before the development of lupus in NZB/NZW mice for both CFH and CFHR-B, were barely statistically significant ($p < 0.05$). However, again the mRNA for CFHR-C was completely absent in the liver of these mice before and after the development of lupus (Fig. 6).

Finally, we examined for the mRNA levels in the liver from LG/J mice with no lupus. LG/J is the mouse line from which MRL-*lpr/lpr* lupus-prone mice was originated that also develop lupus, but slower than the MRL-*lpr/lpr* and NZB/NZW mice. We found that the mRNAs for CFH and CFHR-B in liver of these mice before the development of lupus were 654.6 ± 33.4 and 119.6 ± 3.8 , respectively (Fig. 6). Surprisingly, the mRNA for CFHR-C was again totally absent in the liver of LG/J mice (Fig. 6). The complete universal absence of CFHRC mRNA in liver of all three strains of lupus-prone mice and also in various organs of MRL-*lpr/lpr* mice, despite the presence of mRNAs for CFH and CFHR-B in liver, indicate that CFHRC is might have an important relevance to lupus pathogenesis.

3.6 Absence of CFHR-C protein in the serum from mouse with and without DM and SLE

When analyzed by western blot, the serum from mice with DDD, BLD, and CAIA all showed the presence of CFHRC (Fig. 7A, 7B, 7C). We also examined sera from NOD mice before (4 weeks) and after (20 weeks) the development of DM (Fig. 7D). To confirm the absence of CFHR-C protein in circulation, serum from MRL-*lpr/lpr* mouse was also examined by western blot analysis before (9 weeks) and after (23 weeks) the development of lupus (Fig. 7E). We used a specific antibody as mentioned in methods that recognized both CFH and CFHR-C simultaneously. A band of CFH at 155 kDa was present using sera from WT and MRL-*lpr/lpr* mice (Fig. 7E, lanes 1, 2 & 3). It was absent in the serum from *fH*^{-/-} mouse as expected (Fig. 7E, lane 4). A band of ~98-100 kDa of CFHR-C was present only in the sera from WT and *fH*^{-/-} mice (Fig. 7, lanes 1 & 4) and it was completely absent in the sera from MRL-*lpr/lpr* mice before and after the development disease (Fig. 7, lanes 2 & 3). Qualitatively, by Western blot analysis, the protein expression of CFHR-C in the sera from WT and *fH*^{-/-} mice was low and these results are consistent with the low mRNA levels of this protein in liver as well as in various tissues from WT and *fH*^{-/-} mice (Fig. 7). The complete absence of CFHR-C protein in sera from MRL-*lpr/lpr* mice (Fig. 7E, lanes 2 & 3) was consistent with the universal absence of mRNA for CFHR-C in lupus-prone mice (Fig. 5C & F; Fig. 6). A band of CFHR-C was also absent in the sera from NOD mice with and without DM (Fig. 7D, lanes 2 & 3). We did not carry out a western blot analysis for CFHR-B, because specific antibody to detect it was not available, even though we screened over

four anti-CFH antibodies for this purpose. We did not analyze cell lysates from various organs to detect intracellular CFHR-C protein, because the anti-CFH antibody we used for western blot analysis failed to recognize a clean band instead multiple non-specific bands in the SDS-PAGE gels (not shown).

4. Discussion

Although the functions of human CFHR proteins have been investigated extensively, their biological role remains uncertain. FHR1, FHR2 and FHR5 may act as competitive inhibitors of CFH, a process termed CFH de-regulation (Goicoechea de Jorge et al., 2007). The characterization of mouse CFHR proteins is incomplete. The main goal of this study was to define if levels (through measurement of mRNA and where possible, protein quantification in plasma) of CFH, CFHR-B and CFHR-C differed amongst strains with different pathologies. Most of the complement proteins are synthesized in the liver so we assessed expression in liver tissue in addition to other organs.

Our unexpected finding was the complete absence of CFHR-C in NOD, LG/J, MRL-*lpr/lpr* and NZB/NZW strains. CFHR-C was detectable in C57BL/6 and CFH-deficient animals inter-crossed onto the C57BL/6 genetic background. This suggested that there could have been recombination within the murine FHR locus that has resulted in a copy number variation in which there is deletion of the CFHR-C gene and that this allele is present in homozygosity in NOD, LG/J, MRL-*lpr/lpr* and NZB/NZW strains. Deletion of the human FHR genes, FHR3 and FHR1, is a common polymorphism resulting in an allele in which there is complete absence of the genes for these two CFHRs (Malik et al., 2012). Notably the FH-deficient mouse strain (Pickering et al., 2002) was generated using gene-targeting techniques in 129/Sv embryonic stem cells so the original 129/Sv targeted locus contains an intact CFHR-C gene both before and after targeted mutation of the CFH gene.

Here, the data show an important and surprising finding that CFHR-C was universally absent in three different strains of lupus-prone and in one strain of diabetic-prone mice. We provided evidence by comparing the mRNA levels in liver and in other vital organs of mice with and without lupus. CFHR-C protein was also absent in the circulation of MRL-*lpr/lpr* mice and NOD mice, with and without lupus and diabetes, respectively. However, the mRNA for CFHR-C was present in the liver and other vital organs of mice with and without DDD, BLD and CAIA. CFHR-C protein was also present in the circulation of mice with and without DDD, BLD and CAIA. In mouse models of all autoimmune diseases tested in this study the mRNA for fH in brain, liver, lung, heart, spleen, kidney, knee joints and skin was consistently more than the mRNA for CFHR-B and the latter was more than the mRNA for CFHR-C. We found a significant increase in the levels of mRNA for CFHR-C in the spleen of mice with DDD and CAIA compared with the mRNA levels in the spleen of mice with no DDD and CAIA, which indicated its pro-inflammatory role. CFHR-B mRNA was 4-fold higher in mice with BLD vs. mice with lupus. Finally, the liver of mice with DDD, BLD, DM, CAIA and SLE had the highest, and skin had the lowest, levels of CFH, CFHR-B and CFHR-C mRNA.

The molecular weight of mouse CFHR-C protein was ~98-100 kDa and thus consistent with that found in a previous study (Hellwage et al., 2006). The sequence of CFHR-C and its variants has also been published previously (Hellwage et al., 2006). Our study indicates that genetic deficiency of CFHR-C in lupus-prone mice might play a critical role in the pathogenesis of lupus. The mRNA for CFHR-C was also universally absent in the diabetic-prone NOD mice before and after the development of diabetes. Older MRL-*lpr/lpr* and NOD mice develop lupus and diabetes, respectively. We thought that the complete absence of CFHR-C in mice susceptible to two different autoimmune diseases that occur spontaneously might be a coincidence, but we found that the strains of lupus and diabetic-prone mice from the Jackson database were different. MRL-*lpr/lpr* and LG/J mice belong to group 1 of the mouse family tree, while NOD and NZB/NZW mice belong to group 2 and group 3, respectively (Petkov et al., 2004). LG/J mice, which do not have CFHR-C mRNA in liver, are actually the parent strain of MRL-*lpr/lpr* mice. These results indicate that CFHR-C might be a “common risk factor” for two different autoimmune diseases that develop spontaneously in MRL-*lpr/lpr*, NZB/NZW, LG/J and NOD mice at different ages due to specific mutations. We do not know the exact function of CFHR-C and it could be a pro-inflammatory protein. Our study also indicated that CFH and CFHR-B proteins could not substitute for CFHR-C, because the mRNA for both of these proteins was present in the liver and also in various organs of lupus- and diabetic-prone mice. We have not examined the presence or absence of complement regulatory proteins, because it was not the focus of current study.

There are three known variants of CFHR-C in mouse and *Fhrc* gene codes for all three variants (Hellwage et al., 2006; Vik et al., 1990). In order to show the specificity and sensitivity of the qRT-PCR, the primers and probes to examine the mRNA levels of all variants of CFHR-C have been carefully designed so as to include all three variants, but to exclude mouse CFHR-B. Furthermore, the specificity of primers and probes has also been confirmed by using RNA from various organs from *fH*^{-/-} mice. Liver samples from *fH*^{-/-} mice contain the mRNAs for CFHR-B and CFHR-C, but completely lack the mRNA for fH as expected since we used primers that targeted CFH exon 3 and this has been deleted in the FH-deficient mouse. Mouse CFH exon 3 mRNA was present in *CfhTg/mCfh*^{-/-} chimeric mice (data not shown), which develop BLD spontaneously due to the expression of a CFH chimeric protein. We confirmed by using different primer sets that these transgenic mice have exon 3 RNA because the transgene present which contain mouse exon 3 as only SCR 6 7 8 are human. The reason fH protein is also detected in the serum from *CfhTg/mCfh*^{-/-} mice in western blot analysis (Fig. 7C) is because these mice express a human/mouse transgenic CFH and the anti-CFH antibody used for the western blots cross-reacts with both mouse and human CFH.

C3 glomerulonephritis (C3GN) is subtype of C3 glomerulopathy that results from diverse abnormalities of the AP of complement (Sethi et al., 2012). In this study, in addition to the presence of H402 and V62 alleles of CFH, other abnormalities such as CFH autoantibodies, mutations in CFH, FI, CFHR genes as well as copy number variations in the human cFHR gene cluster were reported (Sethi et al., 2012). A mutation in hCFHR5 has also been associated with C3 glomerulonephritis (C3GN) in Greek Cypriots (Gale and Maxwell,

2013). In addition in two related patients diagnosed for C3G with DDD pattern, a chromosomal deletion of a 25 kbp fragment links the CFHR2 and CFHR5 genes and results in expression of a hybrid CFHR2-CFHR5 protein that in patient plasma deregulates the fluid phase C3 convertase (Chen et al., 2014). One study has linked variants of genes encoding fH and five CFH-related proteins (CFHR1-CFHR5) within the chromosome 1q32 locus linked to SLE (Zhao et al., 2011). We hypothesized that there must be a sequence homology among human CFHR1-CFHR5 and mouse CFHR-C, because the mRNA as well as the protein was universally absent in lupus-prone mice. Therefore, we examined the sequence similarities between hCFHR5 and mouse CFHR-C variants using available bioinformatics information. The sequence homology between human CFHR5 and all three variants of mouse CFHR-C was modest at ~47%. Other human CFHR proteins, such as human CFHR2 and human CFHR3, have an identical sequence homology with mouse CFHR-C and this sequence homology was ~45% to 47% respectively. Furthermore, the sequence similarity among all three variants of mouse CFHR-C with all three variants of human CFHR4 ranged from 33% to 47.7%. SLE has been associated with a single nucleotide polymorphism (SNPs) in CFH-CFHRs regions in various ethnic groups (Zhao et al., 2011). This study has shown that human CFHR3-CFHR1 deletion led to absence of human CFHR1 and human CFHR3 proteins and it was the causal variant for high risk of SLE within the SLE-associated block. Interestingly, CFHR1-CFHR3 deletions in humans has also been associated with AMD (Hageman et al., 2005), the auto immune form, DEAP-HUS of atypical hemolytic uremic syndrome (aHUS) (Pickering et al., 2007; Zipfel et al., 2010) and MPGN II (Abrera-Abeleda et al., 2006; Pickering et al., 2007). Complete absence of fH in mouse plasma has been reported to cause DDD (Appel et al., 2005). Bioinformatics data related to the moderate sequence homologies between human CFHR proteins and mouse CFHR-C show that the later may be a “common moderate risk factor” in autoimmune diseases that develop spontaneously, but specific mutations are necessary for certain autoimmune disease phenotypes.

We found that the mRNA for mouse CFHR-B was significantly lower in the liver of DDD-prone $fH^{-/-}$ mice compared with the liver of DDD-resistant mice (Fig. 1B, 1E). In contrast, the mRNA for CFHR-C was significantly higher in spleen of DDD-prone mice compared with the spleen of DDD-resistant mice (Fig. 1C, 1F). The liver and spleen differ anatomically as well as functionally, but these vital organs might play differential roles in locally regulating the CFHR proteins (Fig. 1E, 1F). It has previously been reported that $fH^{-/-}$ and $fB^{-/-}$ mice develop DDD because of the lack of CFH while also maintaining a very low level of C3 in the circulation (Pickering et al., 2002). We have reported that $fH^{-/-}$ mice are resistant to the development of RA and these mice maintain very low levels of C3 in their circulation (Banda et al., 2013). Interestingly, our studies show that CFHR-C protein was still present in the circulation of $fH^{-/-}$ mice at a level almost equivalent to that of the WT mice. The presence of CFHR-C in DDD-prone $fH^{-/-}$ mice separates these mice from lupus-prone, MRL-*lpr/lpr* and DM-prone, NOD mice. The mRNA of CFHR-C was also present in lung, heart, spleen, and knee joint of $fH^{-/-}$ mice with DDD, but absent in brain indicating the unique nature of this protein as it may not cross blood-brain barrier in certain autoimmune diseases. In contrast, the mRNA and protein for CFHR-C was present in the sera from DDD-prone, $fH^{-/-}$, AMD-prone, *CfhTg/mCfh*^{-/-}, and RA-susceptible WT mice.

One main reason for the complete absence of CFHR-C in lupus- and diabetic-prone mice could be that spontaneous lupus and DM develops in strains of mice that are different from those that develop DDD, BLD, and CAIA. DDD, BLD and CAIA develop in mice on C57BL/6 background, which are deficient in CFH, chimeric for CFH, or have normal levels of CFH, respectively. Therefore, the complete absence of CFHR-C mRNA and protein in lupus- and DM-prone mice could be due to two reasons. First, the presence or absence of CFHR-C may be strain-specific and this is the most likely explanation for these disease-specific differences. Second, DDD and BLD develop in older CFH-deficient or CFH-chimeric mice, respectively, on a C57BL/6 background, even in the absence of genetic mutations, while lupus and DM develops spontaneously due to specific genetic mutations in aged MRL and NOD mice, respectively. The lower level of mRNA for fH only in the kidney but not in the liver of mice with DM showed that there is an organ-specific dysregulation of the AP in diabetic prone mice. Furthermore no differences were seen in the mRNA of CFHRB in the kidney and liver of mice with and without DM confirm the hypothesis that various CFHR proteins play a distinct and an organ-specific role in regulating the AP in various autoimmune diseases. Although CFHR-C is universally absent in both MRL-*lpr/lpr* and NOD mice, the outcome of the autoimmune diseases are different due to specific genetic mutations in lupus-prone mice. Therefore, we conclude that the complete absence of CFHR-C in lupus- and DM-prone mice may be a “common risk factor” that may play a vital role in the pathogenesis of these two spontaneously occurring autoimmune diseases in mice. At present we do not know whether complete absence of CFHR-C promotes or protects mice from developing lupus and DM. It has been reported that treatment of pre-diabetic NOD mice (i.v. injection) with heat-killed *Mycobacterium bovis* (bacillus Calmette-Guerin (BCG)) protects them from DM, but leads to the development of SLE-like disease (Baxter et al., 1994b). This study led to the possibility that genetic susceptibility for DM and SLE might be conferred by single collection of genes in NOD mice, because BCG treatment of normal non-autoimmune C57BL/6 mice did not cause the development of SLE (Baxter et al., 1994a). To further explore the role of CFHR-C in the pathogenesis of DDD studies are in progress to cross-breed *fH*^{-/-} mice with NOD mice to generate *fH*^{-/-}/NOD mice. This double KO mouse will be deficient not only in CFH, but also in CFHR-C. We hypothesize that *fH*^{-/-}/NOD will develop rapid and severe DDD vs. *fH*^{-/-} alone due to the lack of CFHR-C. Similarly, loss of fH in *fH*^{-/-}/MRL-*lpr* mice has been shown to accelerate the development of lupus nephritis (Bao et al., 2011). This study also reported high mortality rate in *fH*^{-/-}/MRL-*lpr* mice due to renal failure. Nonetheless, this study did not explore the role of CFHR-C. Thus, it remains possible that the high mortality rate observed due to renal failure in the *fH*^{-/-}/MRL-*lpr* mice is due to the probable absence of CFHR-C in these mice.

Further studies are needed in lupus- and DM-prone mice to verify that *in vivo* administration of recombinant CFHR-C or of plasmid coding for CFHR-C could significantly attenuate the severity of these diseases in terms of less proteinuria, lupus nephritis, double stranded DNA auto-antibodies, and glucose levels. Furthermore, the 47% homology between all three CFHR-C variants and the huCFHR5 gene indicate that CFHR-C might be playing a role in murine lupus, because a mutation in huCFHR5 that leads to a reduced amount of the protein has been linked with C3 glomerulopathy in a Greek Cypriot family (Gale et al., 2010).

AP	alternative pathway
LP	lectin pathway
MAC or C5b-9	membrane attack complex
CFH	complement factor H
CFHR	complement factor H related proteins
mouse CFHR-C	mouse complement factor H related C
mouse CFHR-B	mouse complement factor H related B
human CFHR-1	human complement factor H related-1
human CFHR-2	human complement factor H related -2
human CFHR-3	human complement factor H related-3
hCFHR-4	human complement factor H related-4
human CFHR-5	human complement factor H related-5
DDD	dense deposit disease
BLD	basal laminar deposits
AMD	macular degeneration
DM	diabetes mellitus
SLE	systemic lupus erythematosus
CAIA	collagen antibody-induced arthritis
CII	type II collagen
DAS	disease activity score
RA	rheumatoid arthritis

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Highlights

- CFHR-C was universally absent in the circulation of lupus- and diabetic- prone mice.
- CFHR-C was present in the circulation of DDD, BLD and CAIA susceptible mice.
- Increase in the mRNA of CFHR-C in spleen of mice with DDD and CAIA indicated its pro-inflammatory role.
- CFHR-B mRNA was 4 fold higher in mice with BLD vs. mice with lupus.
- Liver has the highest and skin has the lowest levels of CFH, CFHR-B and CFHR-C mRNA.

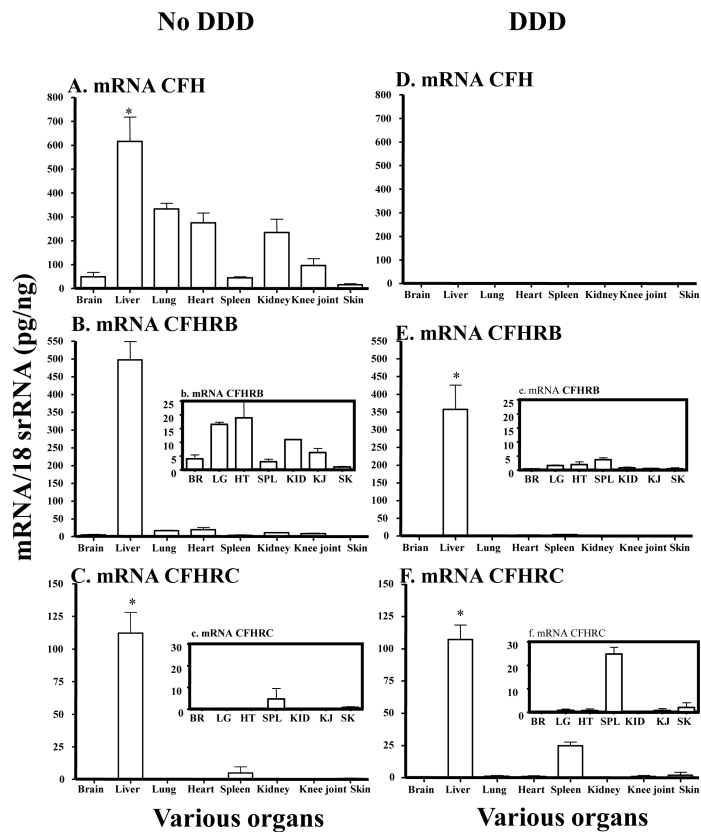
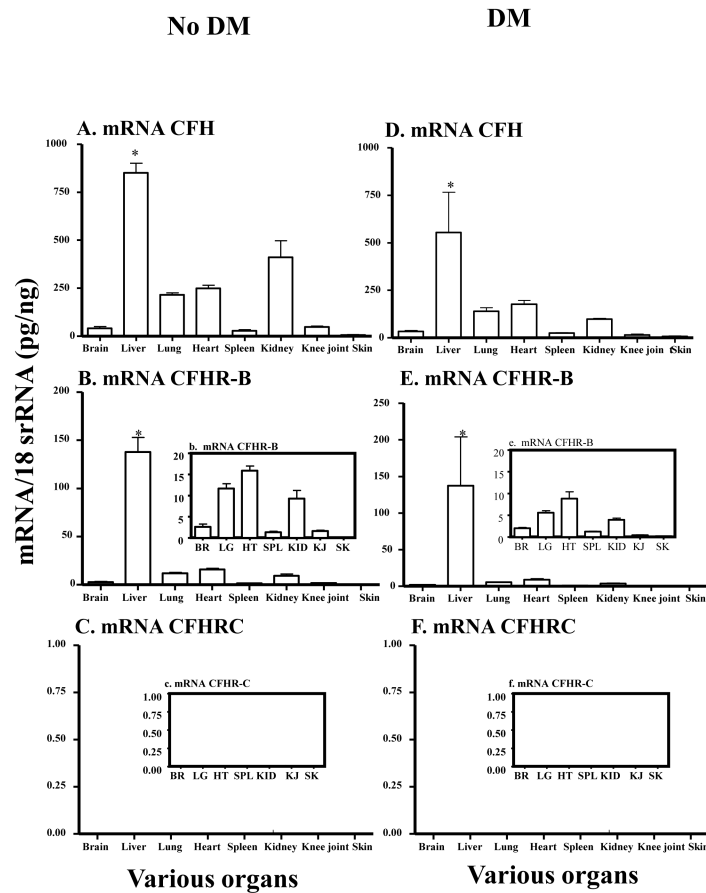


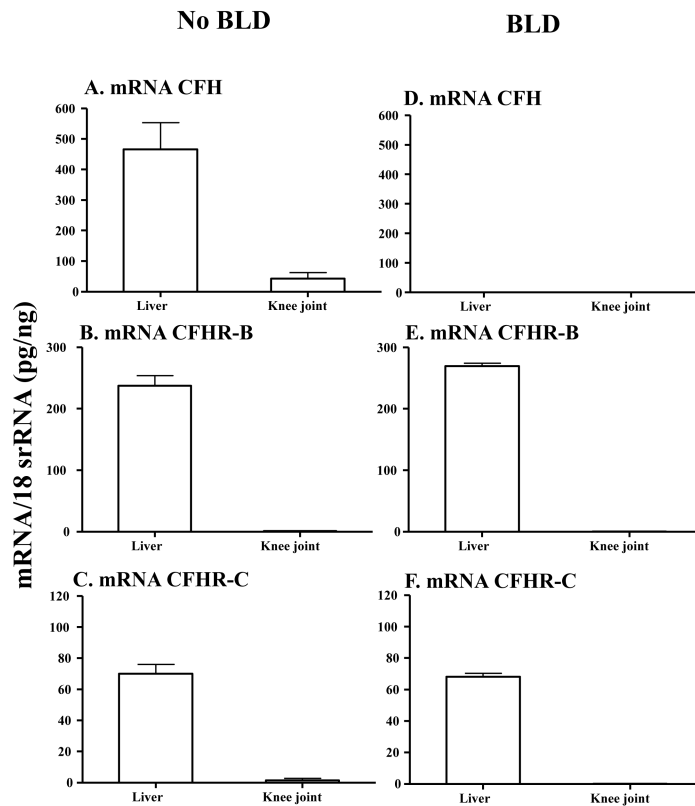
FIGURE 1. mRNA expression level analysis of CFH, CFHR-B and CFHR-C in various organs in mice with and without DDD. Aged $fH^{-/-}$ mice, but not $fH^{+/+}$, were susceptible to DDD. Total RNA was extracted from brain, liver, lung, heart, spleen, kidney, knee joint and skin from $fH^{-/-}$ and $fH^{+/+}$ with and without DDD. All RNA samples were analyzed in duplicate by qRT-PCR. The mRNA for CFHR-B and CFHR-C were not only universally present, but were present at very high levels in the liver of mice with and without DDD vs. brain, lung, heart, spleen, kidney, knee joint and skin. **A.** CFH mRNA in various organs of $fH^{+/+}$ mice with no DDD. **B.** CFH mRNA in various organs of $fH^{-/-}$ mice with DDD and no CFH was present as expected. **b.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in brain, lung, heart, spleen, kidney, knee joint and skin. **C.** CFHR-B mRNA in various organs of $fH^{+/+}$ mice with no DDD. The mRNA was detectable in all organs. **c.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B only in SPL and SK. **D.** The CFH mRNA in various organs of $fH^{-/-}$ mice with DDD was absent, as expected. **E.** CFHR-B mRNA in various organs of $fH^{-/-}$ mice with DDD. **e.** A small histogram embedded in the main histogram shows that it was also detectable in LG, HT, SPL, KID, KJ and SK. **F.** CFHR-C mRNA in various organs of $fH^{-/-}$ mice with DDD. **f.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-C in LG, HT, SPL, KJ and SK. All data presented in these graphs, as Mean \pm SEM, were from $fH^{+/+}$ mice with no DDD (n = 5) and $fH^{-/-}$ mice with DDD (n = 5) and these experiments were repeated three times. BR = brain, LG = lung,

HT = heart, SPL = spleen, KID = kidney, KJ = knee joint and SK = skin. *p-value < 0.05 compared to liver

**FIGURE 2.**

mRNA expression levels of CFH, CFHR-B and CFHR-C analysis of various organs of NOD mice without and with diabetes. Eighteen-week-old, but not 4-week-old NOD mice were susceptible to the DM. Total RNA was extracted from brain, liver, lung, heart, spleen, kidney, knee joint and skin from NOD mice with and without DM. All RNA samples were analyzed in duplicate by qRT-PCR. The mRNA for CFH and CFHR-B were universally present in liver, brain, lung, heart, spleen, kidney, knee joint and skin of mice with and without DM. However, CFHR-C mRNA was universally absent in all organs. **A.** CFH mRNA levels in various organs of NOD mice with no DM. **B.** CFHR-B mRNA level in various organs of NOD mice with no DM. **b.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in BR, LG, HT, SPL, KID, and KJ. **C.** No detection of CFHR-C mRNA in various organs including liver of NOD mice with no DM. **c.** A small histogram embedded in the main histogram showing universal absence of mRNA for CFHR-C in BR, LG, HT, SPL, KID, KJ and SK in mice without DM. **D.** CFH mRNA levels in various organs of NOD mice with DM was detectable in all organs. **E.** CFHR-B mRNA levels in various organs of NOD mice with DM. **e.** A small histogram embedded in the main histogram showing presence of detectable levels of mRNAs for CFHR-B in BR, LG, HT, SPL, KID, KJ and SK in mice with DM. **F.** No detection of CFHR-C mRNA in various organs including liver of NOD mice with DM. **f.** A small histogram embedded in the main histogram showing complete absence of mRNAs for

CFHRC in BR, LG, HT, SPL, KID, KJ and SK in mice with DM. All data presented in these graphs, as Mean \pm SEM, were from NOD mice with no DM (n = 5) and NOD mice with DM (n = 5). The q-RTPCR was repeated two times. BR = brain, LG = lung, HT = heart, SPL = spleen, KID = kidney, KJ = knee joint and SK = skin. *p-value < 0.05 compared to liver

**FIGURE 3.**

mRNA expression levels of CFH, CFHR-B and CFHR-C analysis from liver and knee joint of *CfhTg/mCfh*^{-/-} mice without and with BLD. Only liver and knee joint from 12-month-old *CfhTg/mCfh*^{-/-} chimeric mice (with BLD) and age-matched littermate B6 mice (with no BLD) were analyzed. All RNA samples were analyzed in duplicate by qRT-PCR. **A.** CFH mRNA was present in liver and knee joints of B6 mice with no BLD using primers from exon #3. **B.** CFHR-B was present in liver and knee joints of B6 mice with no BLD. **C.** CFHR-C was present in liver and knee joints of B6 mice with no BLD. **D.** No mouse fH mRNA was detected in liver and knee joint of *CfhTg/mCfh*^{-/-} chimeric mice with BLD using primers described previously (Ufret-Vincenty et al., 2010) but mRNA for CFH was present using primers from exon #3 (data not shown). **E.** CFHR-B mRNA in liver and knee joints of *CfhTg/mCfh*^{-/-} mice with BLD. **F.** CFHR-C mRNA in liver and knee joint of *CfhTg/mCfh*^{-/-} mice with BLD mice. All data presented in these graphs, as Mean ± SEM, were from *CfhTg/mCfh*^{-/-} mice with BLD (n = 5) and B6 mice (n = 5) with no BLD. These experiments were repeated three times. *p-value < 0.05 compared to liver

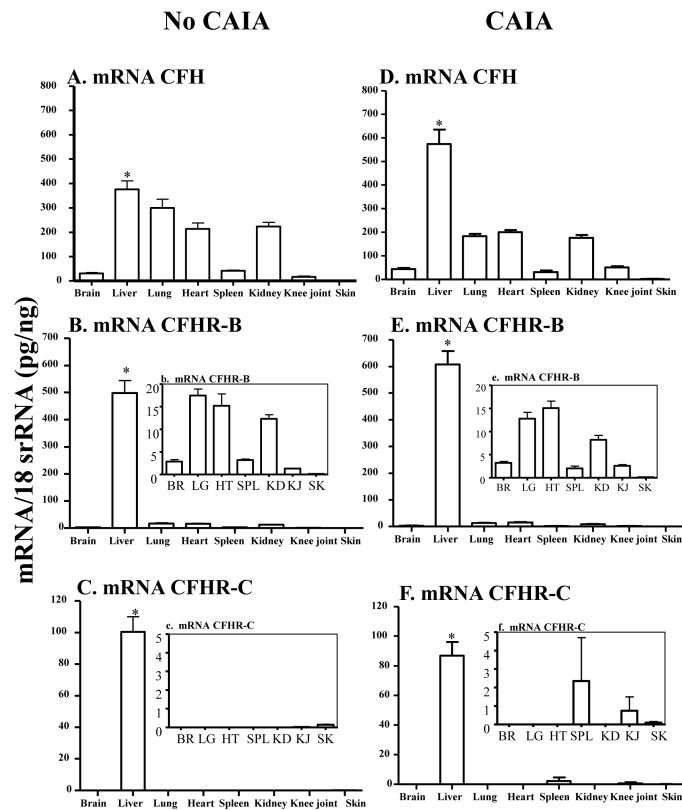


FIGURE 4. mRNA expression levels of CFH, CFHR-B and CFHR-C analysis of various organs in mice with and without CAIA. CAIA was induced in C57BL/6 mice as described in Materials and Methods. All mice with CAIA were sacrificed at day 10. Age-matched WT mice were also sacrificed in parallel at day 10 without the induction of CAIA. Total RNA was extracted from brain, liver, lung, heart, spleen, kidney, knee joint and skin from WT mice with and without arthritis. **A.** CFH mRNA various organs of mice with no CAIA. **B.** CFHR-B mRNA in various organs of mice with no CAIA. **b.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in BR, LG, HT, SPL, KID, and KJ but not in SK. **C.** CFHR-C mRNA in various organs of mice with no CAIA. **c.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B only in SK. **D.** CFH mRNA in various organs of mice with CAIA. **E.** CFHR-B mRNA in various organs of mice with CAIA. **e.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in BR, LG, HT, SPL, KID, KJ and SK. **F.** CFHR-C mRNA in various organs of mice with CAIA. **f.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in SPL, KJ and SK. All RNA samples were analyzed in duplicate by qRT-PCR. All data presented in these graphs, as Mean \pm SEM, were from WT mice with no CAIA (n = 4) and WT mice with CAIA (n = 5). BR = brain, LG = lung, HT = heart, SPL = spleen, KID = kidney, KJ = knee joint and SK = skin. *p-value < 0.05 compared to liver

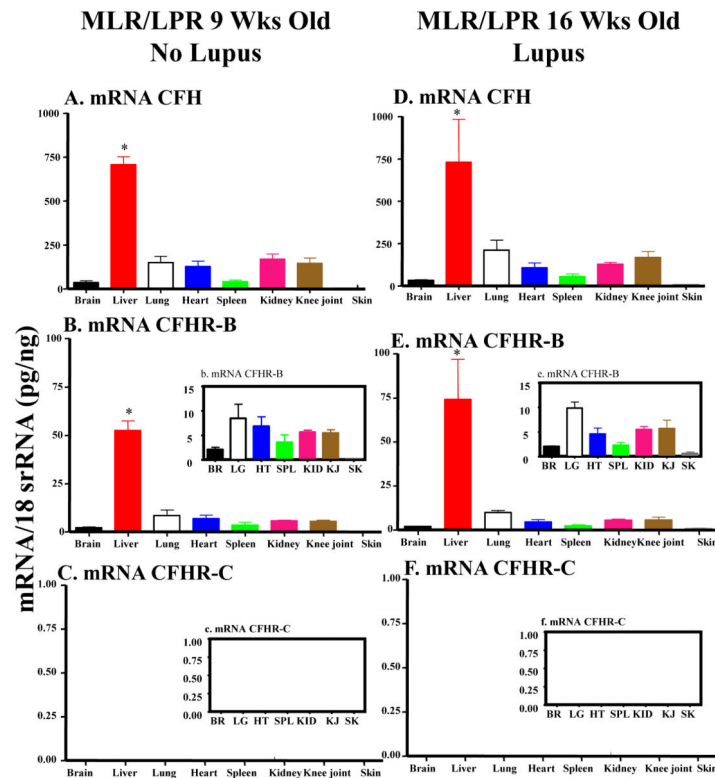
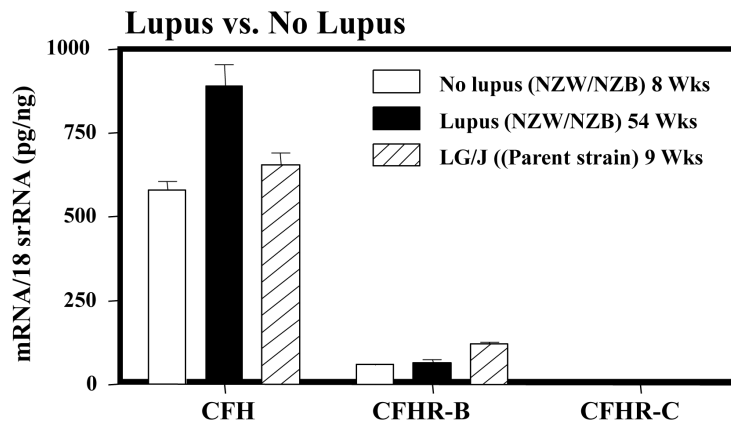
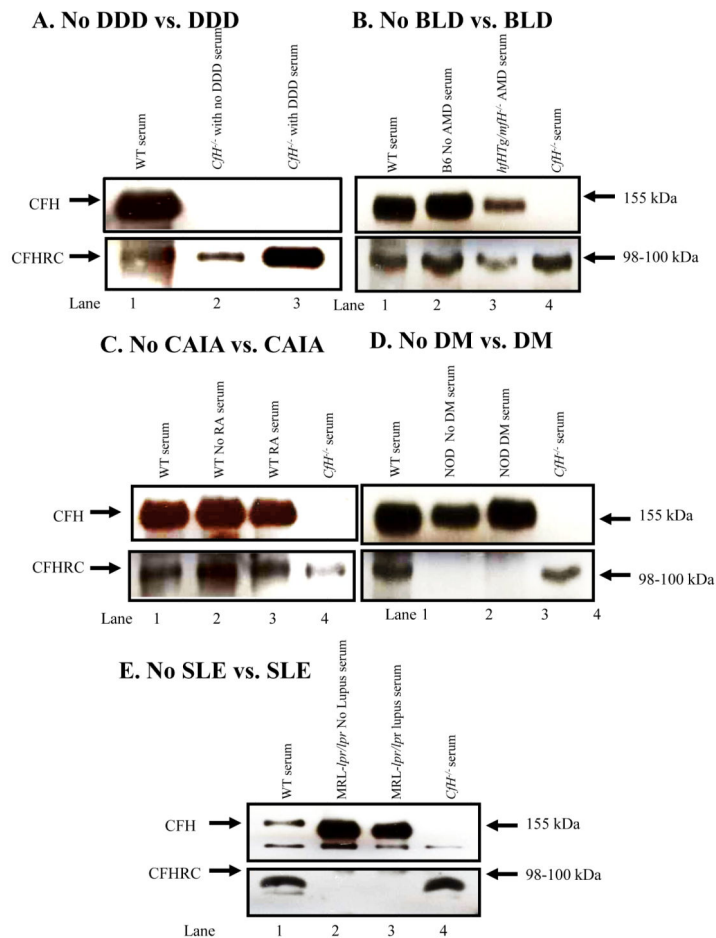


FIGURE 5.

mRNA expression levels of CFH, CFHR-B and CFHR-C analysis of various organs in mice with and without SLE. At 23 weeks of age vs. 9 weeks MRL-*lpr/lpr* showed clinical signs of lupus. Total RNA was extracted from brain, liver, lung, heart, spleen, kidney, knee joint and skin from MRL-*lpr/lpr* with and without lupus. All RNA samples were analyzed in duplicate by qRT-PCR. The mRNA for CFHR-C was absent not only in the liver of mice with and without lupus, but it was absent universally in brain, lung, heart, spleen, kidney, knee joint and skin. **A.** fH mRNA in various organs of MRL-*lpr/lpr* mice with no lupus. **B.** CFHR-B mRNA in various organs of MRL-*lpr/lpr* mice with no lupus. The mRNA was present in all organs, but in skin it was very low. **b.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in BR, LG, HT, SPL, KID, KJ and SK. **C.** Undetectable levels of CFHR-C mRNA in all organs of MRL-*lpr/lpr* mice with no lupus. **D.** CFH mRNA in various organs of MRL-*lpr/lpr* mice with lupus. It was detectable in all organs except in skin it was low. **E.** CFHRB mRNA in various organs of MRL-*lpr/lpr* mice with lupus. **e.** A small histogram embedded in the main histogram shows detectable mRNA levels of CFHR-B in BR, LG, HT, SPL, KID, KJ and SK. **F.** Undetectable levels of CFHR-C mRNA in various organs of FH mRNA in various organs of MRL-*lpr/lpr* mice with lupus. All data presented in these graphs, as Mean \pm SEM, were from MRL-*lpr/lpr* mice with no lupus (n = 5) and MRL-*lpr/lpr* mice with lupus (n = 5). These experiments were repeated three times. BR = brain, LG = lung, HT = heart, SPL = spleen, KID = kidney, KJ = knee joint and SK = Skin. *p-value < 0.05 compared to liver.

**FIGURE 6.**

Analysis of the mRNA expression levels of CFH, CFHR-B and CFHR-C in various organs of NZB/NZW mice, with and without SLE, and in LG/J mice without SLE. At ~54 weeks of age NZB/NZW showed clinical signs of lupus. Total RNA was extracted from liver of NZB/NZW with (54 weeks) and without lupus (9 weeks). Liver was also obtained from LG/J mice without lupus (age 9 weeks). All RNA samples were analyzed in duplicate by qRT-PCR. The mRNA for CFH and CFHR-B were present in liver of NZB/NZW and LG/J mice with and without lupus. The mRNA for CFHR-C was totally absent in liver of both NZB/NZW and LG/J mice regardless of disease.

**FIGURE 7.**

Western blot analyses showing the presence of CFHR-C in mouse serum with and without DDD, BLD and CAIA and also showing the universal absence of mice with and without DM and SLE. Sera from WT C57BL/6 and $fH^{-/-}$ mice were used as a positive and a negative control for CFH and CFHR-C, respectively. Mouse serum was analyzed by using 8% Trisglycine SDS-PAGE under reducing conditions. Immuno-blotting was done using a primary anti-CFH and a secondary HRP-conjugated antibody. **A.** Serum was analyzed from WT ($fH^{+/+}$) and $fH^{-/-}$ mice with no DDD and with DDD. *Top panel* (lanes 1, 2 & 3): Serum from WT ($fH^{+/+}$) mice, 6-week-old $fH^{-/-}$ mice with no DDD and 40-week-old $fH^{-/-}$ mice with DDD. No CFH is present in $fH^{-/-}$ mice with DDD, as expected. *Bottom panel* (lanes 1, 2 & 3): Serum from WT ($fH^{+/+}$) mice with no DDD and $fH^{-/-}$ mice with DDD. A band of ~98-100 kDa of CFHR-C was present in serum from WT, and in $fH^{-/-}$ with no DDD and with DDD, respectively. **B.** Sera from B6 mice with no BLD and from $CfhTg/mCfh^{-/-}$ chimeric mice with BLD were analyzed. *Top panel* (lanes 1, 2 & 3): A band of CFH was present in serum from WT, with no BLD and with AMD mouse. *Bottom panel* (Lanes 1, 2, 3 & 4): CFHR-C was present in serum from WT, B6, $CfhTg/mCfh^{-/-}$ and $fH^{-/-}$ mouse. **C.** Sera from WT C57BL/6 mice with no CAIA, at day 0, and WT C57BL/6 mice with CAIA, at day 10, were analyzed. *Top panel* (lanes 1, 2 & 3): A band of CFH was present in the sera from WT, with no CAIA and with CAIA mice. Lane 4. Absence of CFH

in serum from $fH^{-/-}$ mice, as expected. *Bottom panel* (Lanes 1, 4): A band of CFHR-C was present in sera from WT, with no arthritis, with arthritis and $fH^{-/-}$ mice. **D.** Sera from NOD mice with no DM, and NOD mice with DM were analyzed. *Top panel* (lanes 1, 2 & 3): A band of fH ~155 kDa was present in serum from WT, no DM and DM mice. Lane 4. Absence of CFH in serum from $fH^{-/-}$ mouse as a negative control. *Bottom panel* (Lanes 1, 4): A band of ~98-100 kDa of CFHR-C was present in serum from WT and $fH^{-/-}$ mice. Lanes 2, 3. CFHR-C was absent in serum from mice with no DM or with DM. **E.** Sera from MRL-*lpr/lpr* without disease and MRL-*lpr/lpr* with disease mice were analyzed. *Top panel* (lanes 1, 2 & 3): A band of CFH was present in sera from WT, MRL-*lpr/lpr* (12 Wks) and MRL-*lpr/lpr* (23 Wks) mouse. Lane 4. Absence of CFH in the sera from $fH^{-/-}$ mice as expected. *Bottom panel* (lanes 1): A band of CFHR-C was present in sera from WT mouse. Occasionally, twin bands were also seen at ~96-100 kDa and these may be variants of CFHR-C. Lanes 2 & 3. No CFHR-C protein was present in sera from MRL-*lpr/lpr* mice with and without lupus, respectively. Lane 4. CFHR-C protein was present in sera from $fH^{-/-}$ mice even in the absence of CFH. Western blots using each serum were repeated at least three times and results were highly reproducible.

Table 1

Primers and probes for quantitative RT-PCR used to determine the levels of mouse CFH, CFHR-B and CFHR-C mRNAs

Gene name: <i>CFH</i> *	
Forward Primer (5'-3')	GTGGGCATCCCGGAGAC
Reverse Primer (5'-3')	CACCAAACCTCAAATTGAGATCCAA
Probe (5'-3')	CACCCTTTGGGTCCTTTAGGCTGGC
Product size	69 bp
Gene name: <i>CFHR-B</i>	
Forward Primer (5'-3')	AAAATGGTCAAGACACAATGACATG
Reverse Primer (5'-3')	TTGTTGAGTTGATACGGATGCAT
Probe (5'-3')	AGAATGGCTGGTCCC
Product size	78 bp
Gene name: <i>CFHR-C</i>	
Forward Primer (5'-3')	TCCCAGCCCCCTACAATAGA
Reverse Primer (5'-3')	TCATGACTGCTGAACTCAATGGA
Probe (5'-3')	ACCCAGAACATCTGAAAA
Product size	89 bp

* primers for mouse CFH have been designed using exon #3 except for *cfhTg/mCfh*^{-/-} mice. The designs for CFHR-B and CFHR-B primers and probes have been explained in the Methods section. Primers and probes were purchased from Life Technologies, Grand Island, NY