Blood Cells, Molecules and Diseases 79 (2019) 102349

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Elevated surface-bound complement FH alters the function of platelets and monocytes in *FHR1/3* null healthy individuals

Angika Bhasym, Teena Bhakuni, Prasenjit Guchhait*

Disease Biology Laboratory, Regional Centre for Biotechnology, National Capital Region Biotech Science Cluster, Faridabad, India

ARTICLE INFO

Editor: Mohandas Narla

Keywords:
Complement factor F (FH)
FHR1/3
Platelet
Monocyte

ABSTRACT

Complement factor H (FH) and FH-related proteins (FHRs), structurally similar proteins are involved in the regulation of complement activation. Homozygous deletion of FHR 1 and 3 proteins *(FHR1/3−/−)* is known as a risk factor for disorders such as aHUS and SLE, characterised by thrombo-inflammatory complications. Interestingly, *FHR1/3−/−* genotype also exists as polymorphism in healthy population of various ethnicities around the world including 8–10% Indians. In an effort to understand the functional role of this polymorphism, we describe in this study an elevated surface-bound FH on platelets and monocytes, but not other blood cells in *FHR1/3 −/−* healthy individuals. The *FHR1/3−/−* platelets displayed diminish ability to form aggregates in response to agonists *in vitro*. The *FHR1/3−/−* monocytes displayed elevated secretion of TNFα, IL1β, IL6 and IL10 in response to TLR ligands. However, exogenous FH limits platelet aggregates formation as well as cytokine secretion in monocytes. Therefore, observations together suggest a differential regulation of platelets and monocytes by FH-FHR1/3 axis in healthy individuals. While these findings will need more detailed investigation, it is clear that the connection between FH-FHR axis and thrombo-inflammatory complications is likely to be complex in diseases including aHUS and SLE, and provide interesting new directions for future study.

1. Introduction

Complement factor H (FH) and complement factor H related (FHRs) proteins 1–5 (FHR1–5) are a group of structurally similar proteins. The *FHR1–5* genes are located on chromosome1q32 within the region of recombinant for complement activation (RCA), downstream to *FH*. The highly repetitive sequences of both *FH* and *FHR* genes is the reason for multiple genomic variations of these factors [1,2]. Among all these, homozygous deletion of *FHR1* and *FHR3* genes (*FHR1/3−/−*) is common and described as the risk factor for a broad range of immune-inflammatory disorders including atypical hemolytic syndrome (aHUS), systemic lupus erythematosus (SLE), C3 glomerulopathy, dense deposit disease and C3 glomerulonephritis [3–6]. Besides, studies including our own work described the presence of *FHR1/3−/−* polymorphism in healthy individuals from various ethnic populations including 8–10% Indians [7–10].

Complement factor-H (FH) plays significant role to maintain the level of another complement factor C3b, the central activator of complement pathways, on cell-surface and protect the cells from complement-mediated damage [11,12]. The C-terminal of FH binds to C3b while N-terminal hydrolyze C3b into inactive form. Due to high structural similarity, the FHRs compete with FH for binding to C3b but are unable to regulate its activity on the cell surface [2,12,13]. Therefore, imbalance in the concentration of FH and FHRs proteins may have role in altering the cellular activity [13]. Studies including our own work have described the role of FHR and FH in the regulation of immune cell functions and the pathogenesis of inflammation in diseases such as aHUS and SLE [8,9]. Likewise, other studies also have suggested the impairment in activation of complement factor C3 on cell surface and in turn alteration in the phenotype and function of various cell types including platelets and leukocytes in diseases such as aHUS and SLE [12,14,15]. Besides, we also have described the association of complement factors FHR and FH in the regulation of leukocytes functions in healthy individuals. We have shown the association of *FHR1/3* homozygous deletion polymorphism with the alteration in inflammatory response of the monocytes from peripheral blood of healthy individuals [10].

With the above observations, we investigated further the association of FHR1 and FHR3 (FHR1/3) proteins, more specifically the interaction of FH-FHR1/3 in the regulation of other blood cells in healthy individuals with homozygous *FHR1/3* deletion *(FHR1/3−/−)*. Like in monocytes but unlike other blood cells, platelets or thrombocytes of *FHR1/3−/−* healthy individuals displayed a higher surface-bound FH and lower ability to platelet-platelet aggregate formation compared to

* Corresponding author.
E-mail address: prasenjit@rcb.res.in (P. Guchhait).

https://doi.org/10.1016/j.bcmd.2019.102349
Received 9 June 2019; Received in revised form 14 July 2019; Accepted 14 July 2019
Available online 22 July 2019

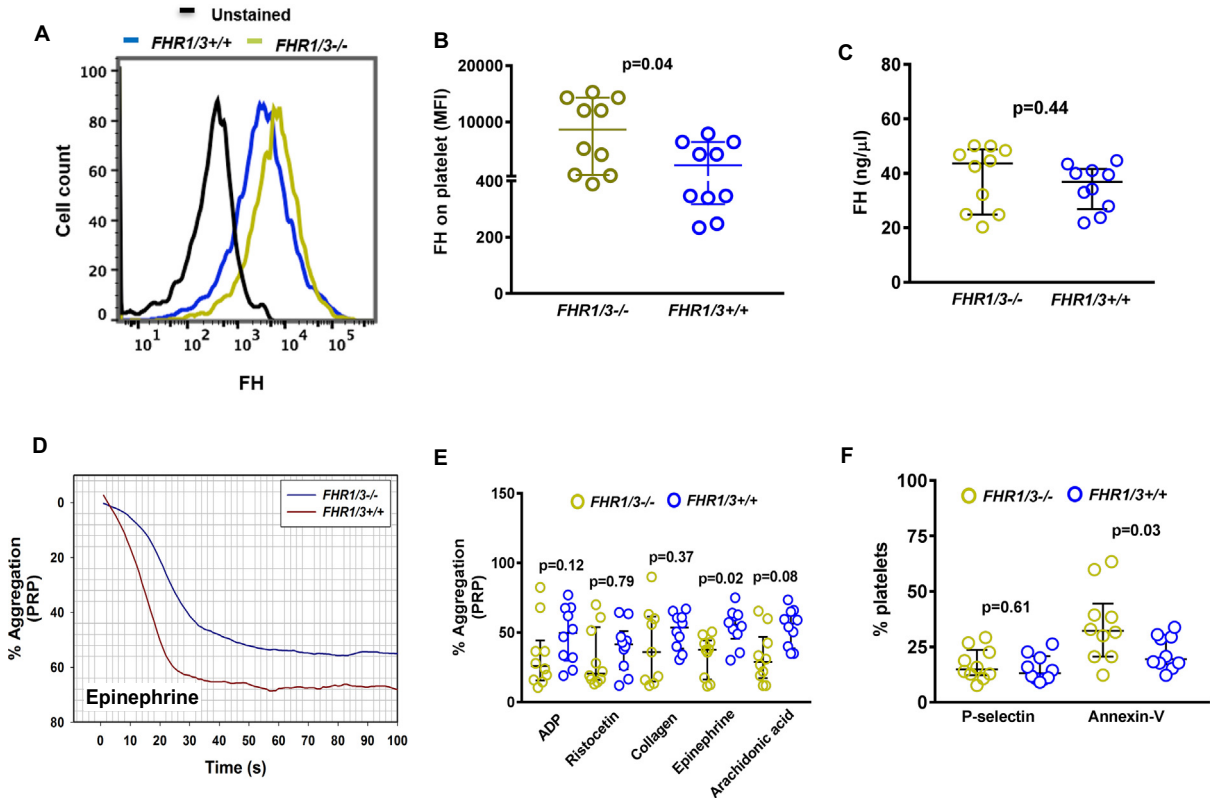

Fig. 1. Phenotype and function of platelets in *FHR1/3−/−* and *FHR1/3+/+* individual. Platelet rich plasma (PRP) was separated from whole blood of *FHR1/3−/−* and *FHR1/3+/+* individuals and analyzed for surface level of factor H (FH) on platelets using flow cytometry (gating strategy is mentioned in suppl. Fig. S3), (A) histogram (cell counts) of one individual each, and (B) mean (n = 10 each). (C) Plasma of above individuals was used for quantification of FH using ELISA assay. The PRP was used for platelet aggregation using platelet agonists such as ADP (20 µM), ristocetin (1.25 mg/ml), collagen (10 µg/ml), epinephrine (0.1 µM) or arachidonic acid (500 µg/ml); (D) histogram of one individual each from both groups with agonist epinephrine and (E) mean from all the mentioned platelet agonists. The activation state of platelets of both *FHR1/3−/−* and *FHR1/3+/+* individuals was analyzed by assessing surface level of (F) P-selectin and phosphatidylserine (PS) using flow cytometry. Each dot represents individual data. Data shown as mean and p value was calculated between *FHR1/3−/−* and *FHR1/3+/+* groups using Mann-Whitney *U* test.

FHR1/3+/+ individuals. On the other hand, monocytes of same *FHR1/3−/−* individuals exhibited elevated surface-bound FH but increased secretion of inflammatory cytokines. Thus, this study for the first time describes the mechanism underlying the interaction between two structurally-homologous complement proteins such as FH and FHR in the regulation of functions of platelets and monocytes, two major cell types of thrombo-inflammatory pathway.

2. Materials and methods

2.1. Antibodies and reagents

Fluorescence-labeled monoclonal anti-human CD62P (AC1.2) FITC was purchased from BD Bioscience (San Diego, CA), Annexin-V PE from Biolegend (San Diego, CA) and anti-FH (OX-24, catalog no. 118620) FITC antibody from abcam (Cambridge, USA) and anti-C3b (3E7/C3b) PE from BioLegend (San Diego, USA). Platelet agonists including epinephrine, ADP, arachidonic acid and ristocetin were purchased from BIO/DATA Corporation (Horsham, USA). Human collagen and factor-H protein were purchased from Sigma-Aldrich (St. Louis, USA), Lipofectamine-based transfection reagent, GeneIn was purchased from amsbio (Milton Park, UK) and most of the other laboratory reagent was purchased from Sigma (St. Louis, USA).

2.2. Human subjects

The ethics approval was obtained from the Institute Human Ethics Committee of Regional Centre for Biotechnology (RCB), Faridabad for the collection of whole blood from healthy adults. Informed consent was provided according to the recommendations of the declaration of Helsinki. Initially we recruited 100 healthy individuals from RCB and collected 2 ml of peripheral blood for *FHR1/3−/−* or *FHR1/3+/+* genotyping. We could get ten *FHR1/3−/−* individuals from the above pool. We recruited ten each *FHR1/3−/−* or *FHR1/3+/+* individuals one more time and collected 7–10 ml of blood in acid citrate-dextrose (ACD) anticoagulant (BD Bioscience, San Diego, CA) for platelet related assays and other experiments.

2.3. Genotyping of CHR1/3

Genomic DNA was isolated from PBMCs (FlexiGene DNA kit, Qiagen, Germany). Real-time quantitative PCR (qPCR) was performed to detect homozygous deficiency of FHR1 and FHR3 genes in all samples as described in our recent publication [9,10].

2.4. Platelet-rich plasma (PRP) and washed platelets preparation

Platelet-rich plasma (PRP) was obtained from whole blood of healthy donors. Whole blood collected in ACD anti-coagulant and centrifuged at 500 rpm for 15 min at break free condition at room temperature (RT). Platelet-rich plasma (PRP) was separated from top layer carefully. PRP was centrifuged at 1000*g* for 10 min without break at RT. To avoid platelet activation 100 µl of ACD was added in 5 ml (1:50) of PRP before centrifugation. Platelets were resuspended in calcium-free Tyrode buffer (pH 6.5) and filtered through Sepharose 2B column (Sigma-Aldrich, St. Louis, USA) using same buffer of pH 7.2

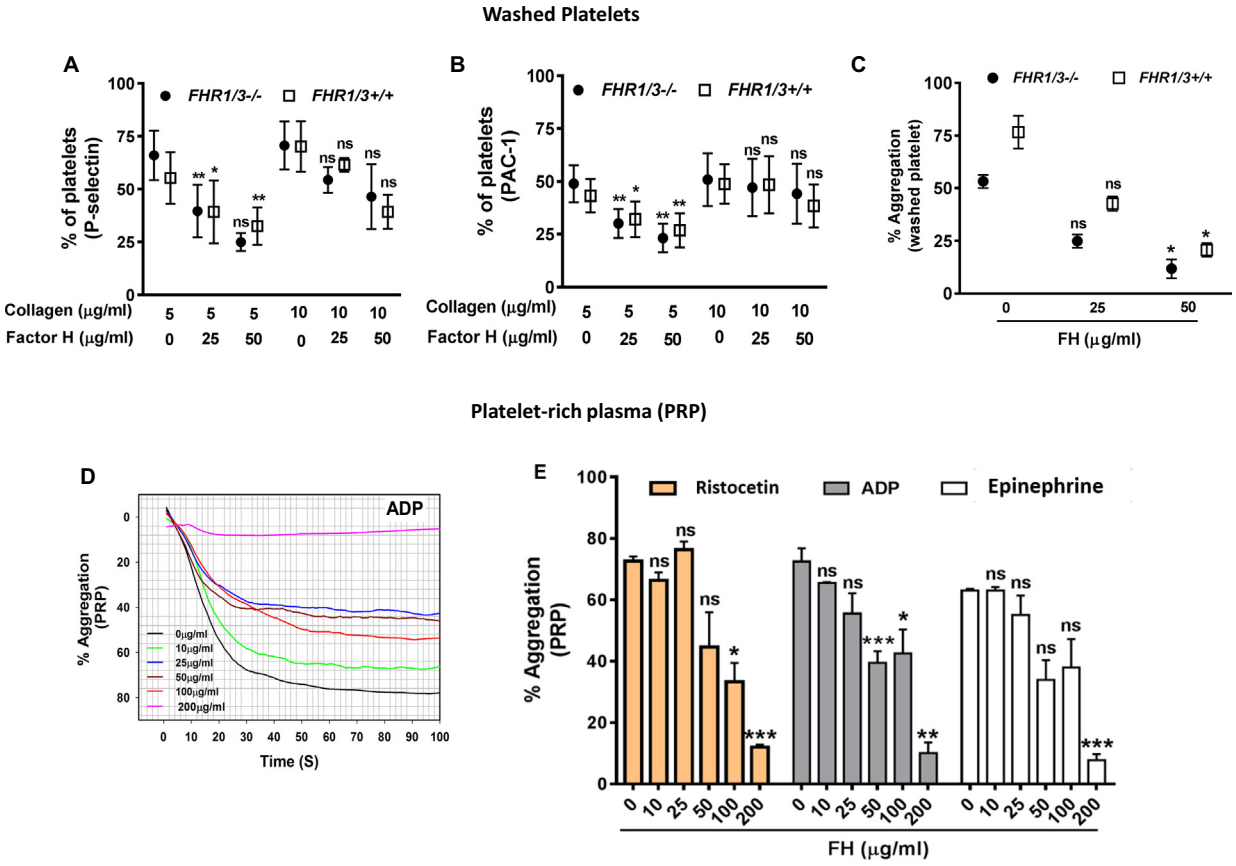

Fig. 2. Platelet activation and aggregation in presence of purified FH. Washed-platelets isolated from *FHR1/3−/−* and *FHR1/3+/+* individuals were incubated with various concentrations of FH for 30 min at 37 °C in presence of platelet agonist such as collagen, and platelet activation markers such as (A) P-selectin and (B) PAC-1 was measured using flow cytometry. (C) Aggregation of washed platelets in presence of various concentrations of FH and agonist such as ristocetin 0.5 mg/ml and purified human fibrinogen 0.5 mg/ml. Each dot in (A-C) represents data pooled from 3-independent experiments with different donors of *FHR1/3−/−* (n = 3) and *FHR1/3+/+* (n = 3). Data shown as mean ± SEM and p value calculated by paired *t*-test. Platelet aggregates were analyzed using PRP; (D) representative diagram of platelet aggregation in presence of various concentrations of FH and agonist ADP; and (E) mean of similar experiments of platelet aggregation in presence of FH and agonist such as ristocetin (1.25 mg/ml), ADP (20 µM) and epinephrine (0.1 µM). Data shown as mean ± SEM from 3 independent experiments and p value is calculated by paired *t*-test, * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ and ns = non-significant represent comparison of various doses of FH with no-FH treatment among respective genotypes.

[16].

2.5. Platelet aggregation assay

Platelet aggregation assay was performed using PRP of *FHR1/3−/−* or *FHR1/3+/+* individuals in presence of agonists such as ADP (20 µM), ristocetin (1.25 mg/ml), collagen (10 µg/ml), epinephrine (0.1 µM) or arachidonic acid (500 µg/ml). Washed platelets were prepared from PRP from healthy individuals from *FHR1/3−/−* and *FHR1/3+/+* genotype each, and incubated with human plasma-purified FH (Sigma-Aldrich, St. Louis, USA), concentrations ranging from 0 to 50 µg/ml for 20 min at 37 °C before aggregation assay using PAP-8 Platelet Aggregation System from BIO/DATA Corporation (Horsham, USA), as mentioned [17].

2.6. Analysis of surface level of FH on platelets and monocytes

Surface bound factor H (FH) was assessed in platelets from *FHR1/3−/−* or *FHR1/3+/+* individuals. Platelets were incubated with anti-FH monoclonal antibody (abcam, Cambridge, USA) at 1 µg/ml concentration for 30 min at RT in 200 µl of Tyrode buffer, and analyzed using flow cytometry. Similarly, the PBMCs were labeled with anti-FH antibody and detected using flow cytometry as mentioned in our previous work [10].

2.7. Analysis of platelet activation

The activation status of platelets was measured in *FHR1/3−/−* or *FHR1/3+/+* individuals. 10 µl PRP was incubated with anti-P selectin FITC (CD62P; BD Bioscience) or annexin-V PE (Biolegend, San Diego, CA) in 200 µl of Tyrode buffer for 30 min and analyzed using flow cytometry. Washed platelets, isolated from different *FHR1/3−/−* or *FHR1/3+/+* individuals (n = 3 for each genotype) were incubated with FH protein (concentration ranges from 0 to 50 µg/ml) for 20 min at 37 °C with mild rotation, and further incubated with agonist collagen (5 or 10 µg/ml) and analyzed for surface expression of P-selectin and phosphatidylserine (PS) using flow cytometry.

2.8. Quantification of plasma FH using ELISA

Plasma level of FH in *FHR1/3−/−* or *FHR1/3+/+* individuals was analyzed using human complement FH ELISA Kit (abcam, Cambridge, USA, catalog no. 137975). 100 µl of 1:10 diluted plasma was added into an ELISA plate pre-coated with anti-FH monoclonal antibody and FH level was detected according to the manufactures protocol.

2.9. FH deletion in U937 cells

Complement FH was deleted in monocytic U937 cells by using CRISPR/cas9 technique. FH gene sequence were inserted in

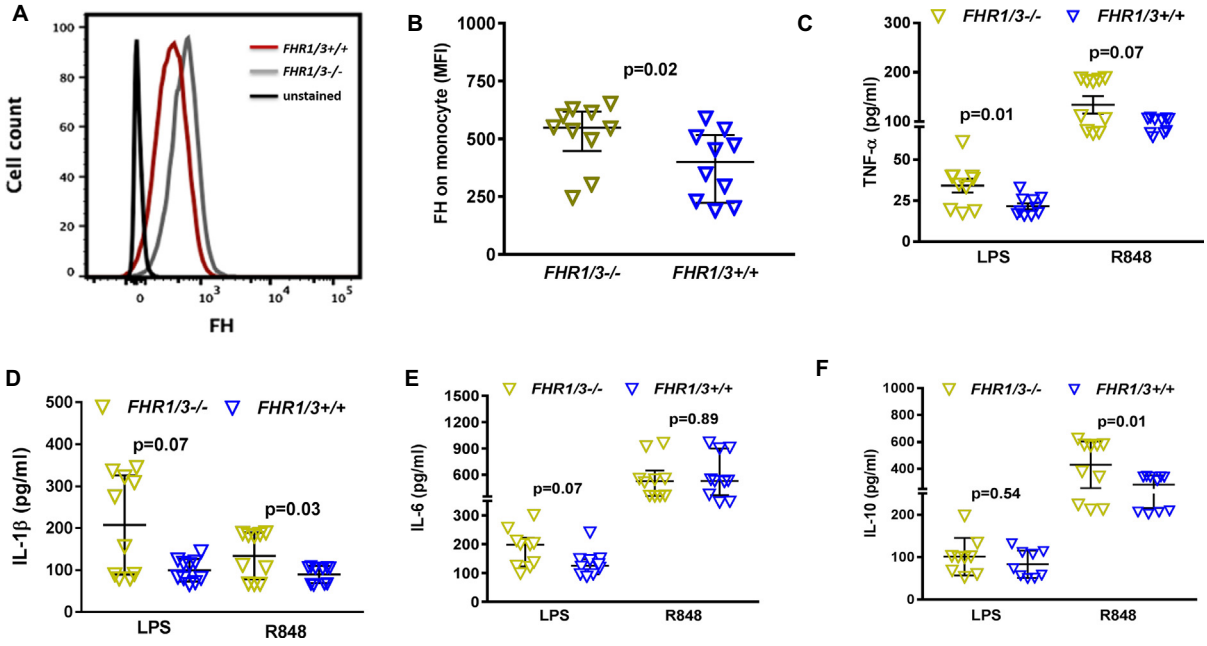

Fig. 3. Phenotype and function of monocytes in *FHR1/3−/−* and *FHR1/3+/+* individual. PBMCs isolated from same *FHR1/3−/−* (n = 10) and *FHR1/3+/+* (n = 10) individuals mentioned in Fig. 1 and were analyzed for surface level of FH on monocyte by using flow cytometry (gating strategy is mentioned in suppl. Fig. S4). (A) Histogram of single experiment of an individual and (B) mean of above experiment. Data shown as mean ± SEM and p-value calculated by Mann-Whitney U test. Each dot represents individual value. Monocytes were isolated from PBMCs using CD14 conjugated magnetic beads-based separation and stimulated with 50 ng/ml LPS or 10 μg/ml R848 for 24 h and different cytokines such as (C) TNF-α, (D) IL-1β, (E) IL-6 and (F) IL-10 were analyzed by using cytometric beads array (CBA). Data analyzed as mentioned above in Fig. 1.

pSpCas9(BB)-2A-Puro plasmid backbone and cloned in *E. coli* DH5α. Plasmid purification was done by using midi kit (Qiagen, Hilden, Germany). Lipofectamine based transfection was performed using GeneIn Transfection Reagent (amsbio, Milton Park, UK) according to manufacturer protocol.

2.10. Cell culture and cytokine analysis

CD14+ monocytes, separated by magnetic bead from PBMCs of *FHR1/3−/−* or *FHR1/3+/+* individuals, and seeded in 24-wells plate containing RPMI media supplemented with 10% FBS. Cells were stimulated with 50 ng/ml of LPS or 10 μg/ml of R848 for 24 h, and cytokines such as TNF-α, IL-1β, IL-6 or IL-10 were analyzed in culture supernatant using flow cytometry-based beads array (CBA; BD Bioscience).

2.11. Statistical analysis

Data from at least 3 experiments are presented as mean ± standard error (SEM). Statistical analysis was performed using Mann-Whitney *U* test for individual's studies and paired *t*-test for *ex vivo* or *in vitro* experiments. Graph Pad Prism 7.0 or Sigma plot software was used for data analysis and p values < 0.05 were considered to be statistically significant.

3. Results

3.1. Platelets with elevated FH on surface from FHR1/3−/− healthy individuals exhibit less aggregation

We assessed the level of surface-bound FH on platelets of healthy individuals with *FHR1/3−/−* and *FHR1/3+/+* genotypes. *FHR1/3−/−* platelets exhibited increased level of FH on surface (Fig. 1A–B) but no-difference in plasma level of FH (Fig. 1C) compared to +/+ individuals. No significant difference in the surface level of complement factor C3b in platelets was observed between genotypes (suppl. Fig. S1A). The *FHR1/3−/−* platelets displayed significantly less aggregation in presence of agonist epinephrine (Fig. 1D–E). Also the *FHR1/3−/−* platelets showed a decreased trend in aggregation in presence of other agonists such as arachidonic acid, ADP, collagen and ristocetin when compared with *FHR1/3+/+* platelets (Fig. 1E). Although we measured the higher annexin-V binding (representing PS exposure) on platelet surface from *FHR1/3−/−* individuals but no such difference was observed for other platelet-surface activation marker such as P-selectin in these individuals compared to *FHR1/3+/+* individuals (Fig. 1F).

3.2. Exogenous FH diminishes further platelet aggregation

Since we observed an elevated surface-bound FH and decreased aggregate formation in platelets of *FHR1/3−/−* healthy individuals, we have investigated the effects of exogenous FH on platelet function. We prepared washed-platelet from both *FHR1/3−/−* and *FHR1/3+/+* genotypes and incubated with various concentrations (0–50 μg/ml) of purified human FH, and measured platelet activation in presence of agonists such as collagen. The activation markers such as P-selectin expression on platelets (Fig. 2A) and PAC-1 binding to platelets (Fig. 2B) were inhibited by FH in a concentration-dependent manner. Similarly, the addition of FH inhibited the aggregation (in response to agonist ristocetin) of washed-platelets in a concentration-dependent manner independent of *FHR1/3−/−* and *FHR1/3+/+* genotypes (Fig. 2C). Also, we analyzed platelet aggregation using platelet-rich plasma (PRP) of *FHR1/3+/+* individuals to mimic the *ex vivo* platelet aggregation in the presence of various concentration of purified human FH (0–200 μg/ml) and observed concentration-dependent inhibition of platelet aggregation by FH with maximum inhibition at 200 μg/ml (Fig. 2D–E).

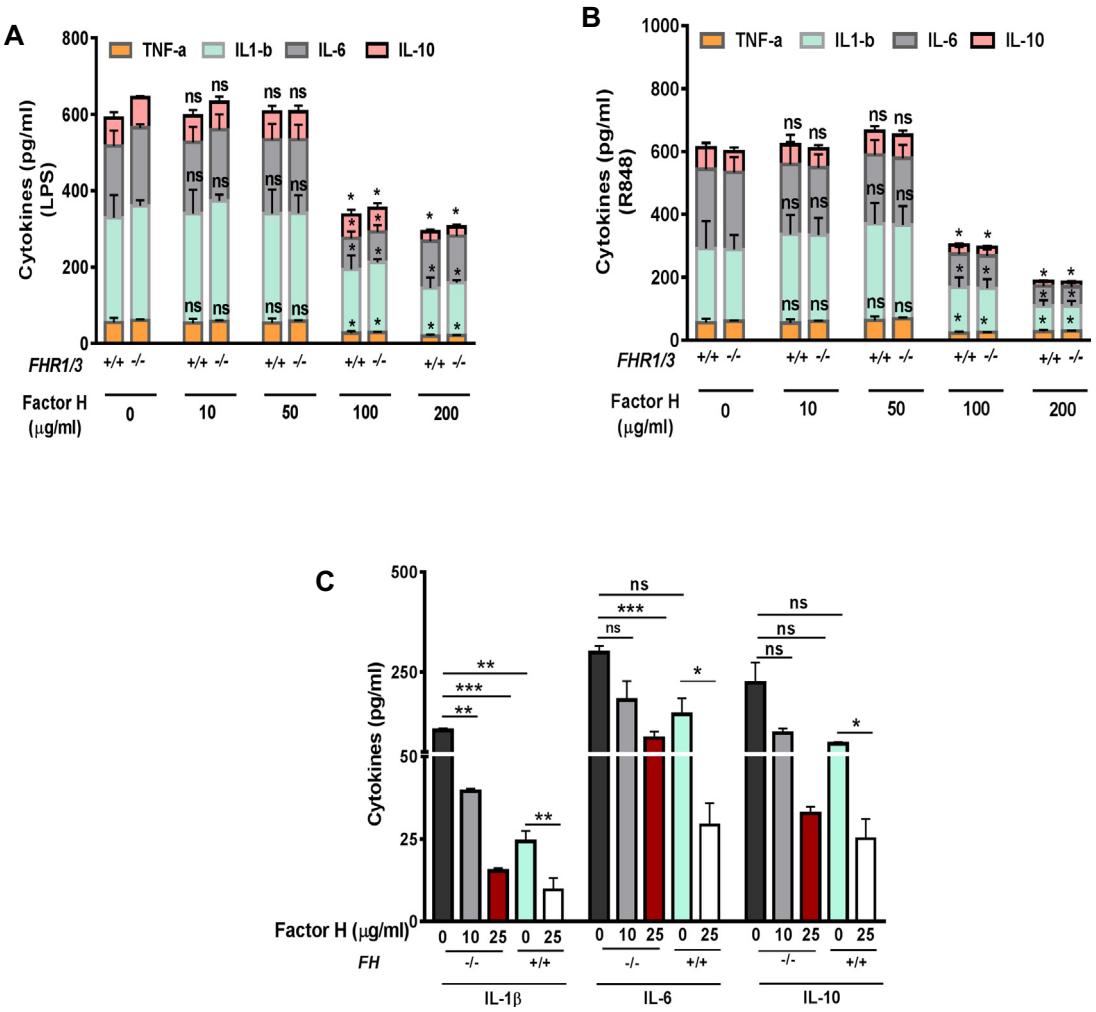

Fig. 4. Function of FHR1/3−/− monocytes and FH−/− U937 cells in presence of purified FH. Monocytes from *FHR1/3*−/− or *FHR1/3*+/+ individuals were incubated with different doses of purified human FH at 37 °C and further stimulated with 50 ng/ml of LPS or 10 μg/ml of R848 for 24 h. Cytokines were measured from supernatant of (A) LPS and (B) R848 -stimulated cells using CBA assay. Data shown as mean ± SEM of 3 independent experiments and p-value calculated using paired t-test, *$P < 0.05$ and ns = non-significant represents comparison of various doses of FH with no-FH treatment among respective genotypes. (C) *FH*−/− monocytic U937 cells were treated with purified FH and stimulated with 10 μg/ml of R848 for 24 h and cytokines were measured as mentioned above. P value was calculated using paired t-test * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ and ns = non-significant. *FH*−/− U937 cells were generated using CRISPR-cas9 technology, mentioned in suppl. Fig. S4.

3.3. Monocytes with elevated FH on surface from FHR1/3−/− individuals display increased secretion of cytokines

The monocytes (isolated from PBMCs using CD14+ beads) of the same *FHR1/3*−/− healthy individuals (mentioned in above Figs. 1 and 2) exhibited increased level of surface-bound FH (Fig. 3A–B), similar to our previous observation [13]. The monocytes from *FHR1/3*−/− individuals when challenged with TLR ligands such as LPS (ligand for cell-surface TLR4) and R848 (ligand for endosomal TLR7/8), exhibited elevated secretion of TNF-α, IL-1β, IL-6 and IL-10 (Fig. 3C–F), suggesting the differential association of *FHR1/3*−/− genotype with platelets and monocytes in these heathy individuals.

3.4. Exogenous FH decreases the cytokine secretion by monocytes, however knockdown of endogenous FH increases their cytokine response

To assess the effects of FH, the various concentrations of purified human FH (0–200 μg/ml) were added to monocytes isolated from *FHR1/3*+/+ individuals and stimulated with TLR ligands such as LPS and R848. Data show a significant inhibition of cytokine secretion (TNF-α, IL-1β, IL-6 and IL-10) by monocyte in a concentration-dependent manner by FH (Fig. 4A–B). Further, to assess the effect of endogenous FH, we deleted *FH* (−/−) in monocytic U937 cells using CRISPR/Cas9 technique (mentioned in suppl. Fig. S2) and have stimulated these cells with R848. Data show the significant increase in secretion of IL-1β, IL-6 and IL-10 by the *FH*−/− U937 cells compared to control counterparts (Fig. 4C). Interestingly, the addition of exogenous FH decreased the secretion of above cytokines in U937 cells independent of *FH* genotype (Fig. 4C).

4. Discussion

The complement factor H-related protein 1 and 3 homozygous deletion (*FHR1/3*−/−) and association of this polymorphism with immune-inflammatory disorders including aHUS has been described by us as well as others [3,4]. There is evidence that FHR proteins form heterodimers with a predominant participation of the FHR1 polypeptide, and that these hetero-dimers may function as inhibitors of FH particularly at cell surfaces by competitively modulating FH binding to complement factor C3b [10,18,19]. Since both FH and FHR1 bound to cell surfaces is known to be a critical regulator of cell-surface activation of complement factors including C3b [10,19], therefore, it is possible that in the

absence of FHR hetero-dimers in the *FHR1/3−/−* genotype, elevated amount of FH may easily bind to cell surfaces and such increased binding may contribute to FH-specific regulation of complement activation as well as other cellular functions.

In an effort to study the FH-FHR1/3 axis, we observed the elevated surface-bound FH in platelets and monocytes, but not in other blood cells in *FHR1/3−/−* healthy individuals. The platelets from *FHR1/3−/−* individuals displayed less aggregation *in vitro* in response to agonists such as epinephrine, arachidonic acid and collagen. The higher binding of FH on platelet surface correlated with the inhibition of activation as well as aggregation of platelets in *FHR1/3−/−* individuals. The inhibitory effect of FH on platelet was further confirmed by our *in vitro* study showing the significant inhibition of platelet activation and aggregation by exogenous FH in a concentration-dependent manner. Although several other studies have reported the anti-thrombotic effect of exogenous FH on platelet functions [20,21], but our study describes the anti-platelet effects of both surface-bound as well as soluble FH, highlighting further the non-canonical role of FH on platelet aggregation rather altering the activation of complement factor C3b on platelet surface.

On the other hand, the monocytes from same above *FHR1/3−/−* individuals, having higher level of surface-bound FH, secreted elevated levels of cytokines such as TNF-α, IL-1b, IL-6 and IL-10 in response to TLR-ligands such as LPS and R848 than the counterparts from *FHR1/3+/+* individuals *in vitro*. Similar to our recent observations where *FHR1/3−/−* monocytes appeared to respond by making higher levels of inflammatory cytokines; in other words, FHR1/3 limits inflammatory responses of these cells [10]. Interestingly, the blood cells other than platelets and monocytes did not displayed any significant difference in surface level of FH between *FHR1/3−/−* and *FHR1/3+/+* individuals.

Further to study the role of FH, we have generated *FH−/−* monocytic U937 cells. These cells are known to synthesize significant FH, which are not secreted but remain bound to cell-surface to regulate complement activation [22]. Our data show that *FH−/−* U937 cells secreted elevated TNF-α, IL-1b, IL-6 and IL-10 in response to TLR ligands, a similar mechanism observed for *FHR1/3−/−* monocytes. Upon treatment with exogenous FH these *FH−/−* U937 cells secreted significantly less cytokines, suggesting further crucial role of FH in mediating non-canonical functions beyond its role in the modulation of complement activation. The FH (endogenous, exogenous or surface-bound forms) displayed differential regulatory role on inflammatory response of monocytes depending on the context, such as the nature of additional stimuli like exposures to TLR ligands. However this speculation remains to be investigated.

Therefore, our data together suggest that the *FHR1/3−/−* genotype is not sufficient in itself to give rise to a disease condition, as evidenced by the wide prevalence of this genotype in healthy populations of various ethnic origins. However, it is likely to modulate function of specific cell types such as thrombocytes and monocytes in both FH-dependent/independent pathways. While these findings will need more detailed investigation, it is clear that the connection between the FHR1/3-FH axis and thrombo-inflammatory complications in diseases such as aHUS and SLE is likely to be complex and provide interesting new directions for future investigations.

Declaration of Competing Interest

Authors have no financial or other interest to declare.

Acknowledgements

Authors sincerely acknowledge Dr. Amit Pandey of THSTI, Faridabad, India for helping us to generate complement FH knockdown cell line using CRISPR/cas9 technique. This study was supported financially by grants BT/PR8591 and BT/PR22985 from the Department of Biotechnology (DBT), Government of India (GoI) and from the Regional Centre for Biotechnology grants-in-aid from the DBT, GoI to P. G.

Authorship contributions

A. B. and P. G. designed the experiments. A. B. and T. B. performed all experiments. A. B. and P. G. analyzed all data and prepared the manuscript. P. G. was responsible for oversight of the project and preparation of the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2019.102349.

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