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ORIGINAL ARTICLE

Altered Peripheral Blood Leucocyte Phenotype and Responses in Healthy Individuals with Homozygous Deletion of *FHR1* and *FHR3* Genes

Angika Bhasym[1,2] · Bahadur Singh Gurjar[3] · Savit Prabhu[4] · Mamta Puraswani[5] · Priyanka Khandelwal[5] · Himanshi Saini[5] · Savita Saini[5] · Priyadarshini Chatterjee[1] · Vineeta Bal[3,4,6] · Anna George[3] · Poonam Coshic[7] · Gopal Patidar[7] · Pankaj Hari[5] · Aditi Sinha[5] · Arvind Bagga[5] · Satyajit Rath[3,4] · Prasenjit Guchhait[1]

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Abstract

A homozygous 83-kb deletion encompassing the genes for complement factor-H-related proteins 1 and 3 (*FHR 1*, *FHR3*) is known as a risk factor for some immune inflammatory disorders. However, the functional relevance of this *FHR1/3* deletion is relatively unexplored. Globally, healthy populations of all ethnic groups tested show an 8–10% prevalence of homozygosity for this deletion polymorphism. We have begun to compare the peripheral leucocyte phenotype and functionality between *FHR1/3*−/− and *FHR1/3*+/+ healthy adult individuals. We report that the two groups show significant differences in their peripheral blood innate leucocyte subset composition, although the adaptive immune subsets are similar between them. Specifically, *FHR1/3*−/− individuals show higher frequencies of patrolling monocytes and lower frequencies of classical monocytes than *FHR1/3*+/+ individuals. Similarly, *FHR1/3*−/− individuals show higher frequencies of plasmacytoid dendritic cells (pDCs) and lower frequencies of myeloid DCs (mDCs) than *FHR1/3*+/+ individuals. Notably, classical monocytes specifically showed cell-surface-associated factor H (FH), and cells from the *FHR1/3*−/− group had somewhat higher surface-associated FH levels than those from *FHR1/3*+/+ individuals. *FHR1/3*−/− monocytes also showed elevated secretion of TNF-α, IL-1β, and IL-10 in response to TLR7/8 or TLR4 ligands. Similarly, FHR1/3−/− mDCs and pDCs showed modest but evident hyper-responsiveness to TLR ligands. Our findings, that the *FHR1/3*−/− genotype is associated with significant alterations of both the relative prominence and the functioning of monocyte and DC subsets, may be relevant in understanding the mechanism underlying the association of the genotype with immune inflammatory disorders.

Keywords *FHR1/3* null genotype · complement factor H · monocytes · dendritic cells

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✉ Satyajit Rath
satyajit@nii.ac.in

✉ Prasenjit Guchhait
prasenjit@rcb.res.in

[1] Regional Centre for Biotechnology, Faridabad, India

[2] Department of Biotechnology, Manipal Academy of Higher Education, Manipal, India

[3] National Institute of Immunology, New Delhi, India

[4] Translational Health Science and Technology Institute, Faridabad, India

[5] Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India

[6] Indian Institute of Science Education and Research, Pune, India

[7] Department of Transfusion Medicine, All India Institute of Medical Sciences, New Delhi, India

Introduction

Complement factor H (FH)-related proteins (FHRs) are a group of five highly related FH family proteins, FHR1–5. The genes for these proteins are located on human chromosome 1 downstream from the gene for FH [1, 2]. FH as well as FHR proteins contain multiple short consensus repeat (SCR) sequences. The conserved C-terminal of FH and FHR binds to cell surfaces, but unlike FH, FHRs do not appear to have the N-terminal region crucial for C3b regulation [3–5]. This suggests that FHRs can interact with FH ligands on cell surface with limited complement regulatory activity, thereby modulating FH-mediated complement regulatory activity on cell surface [6, 7]. FHRs have been reported to act as either promoters or as regulators of complement activation [3, 8–10].

However, unlike FH, the role of FHRs in actual complement regulation is poorly understood [5, 11–13]. It is suggested that FHR1 binds to C3b component of C5 convertase and inhibits the cleavage of C5 protein [10]. The N-terminal domain (SCR1–2) of FHR1 can also inhibit the assembly of the membrane attack complex of complement [8]. FHR1 lacks the co-factor activity for complement factor I, and hence, it is unable to cleave C3b into its inactivated form [9, 10]. Like FHR1, FHR3 also competes with FH for binding to C3b; however, the specific role of FHR3 in the complement regulation is still unclear [14].

Part of the function complexity of the FHR1/3−/− genotype is related to the diversity of functional significance of FHRs. There is evidence that the FHRs can promote C3 activation [15]. This is thought to be particularly relevant when the FHRs may compete with the FH for cell-surface binding, thereby preventing the negative regulatory action of FH. Such competition between FHR and FH has been suggested to ameliorate the complement evasion mediated by the malarial parasite [16].

FHRs also share unique and highly conserved N-terminal sequences with ~ 85% sequence similarity with each other. The FHRs appear to form homo-dimers as well as hetero-dimers, predominantly with FHR1 [3]. These dimers show enhanced functional abilities, suggesting that they may be the major physiological functioning units.

The FH/FHR gene cluster contains many large genomic repeats with high degrees of sequence similarity among them, making this region prone to genomic rearrangements through non-homologous recombination [17]. Variations in FHR gene loci are associated with several diseases including atypical hemolytic syndrome (aHUS), systemic lupus erythematosus (SLE), C3 glomerulopathy (C3G), dense deposit disease (DDD), and C3 glomerulonephritis (C3GN), which are commonly characterized by deregulated complement activation and inflammation [18–22]. Many genomic re-arrangements within FH and FHR genes including FH:FHR1 or FH:FHR3 hybrid gene formation, *FHR1* or *FHR5* gene duplication and *FHR1* and *FHR3* homozygous deletion have been observed to be associated with these diseases [20, 21]. Unlike most of these genomic variations which are rare, the deletion of *FHR1* and *FHR3* is a common polymorphism mediated by a ~ 83-kb deletion.

This homozygous *FHR1/3−/−* genotype is associated as a risk factor for aHUS, SLE, and C3G and as a protective factor for age-related macular degeneration (AMD) and IgA nephropathy (IgAN) [23, 24]. It is not clear how these apparently paradoxical findings of susceptibility association in some complement-mediated diseases and protection association in other diseases are related with the positive- or negative-regulatory roles the FHRs appear to play in different situations in complement biology.

While the association of the *FHR1/3−/−* genotype with aHUS is strongly associated with an autoantibody response to FH, our recent findings suggest that report described that there is also an anti-FH autoantibody-independent association [25, 26]. While this *FHR1/3−/−* genotype is thus associated, positively or negatively, with a wide range of immuno-inflammatory disorders, the mechanistic connections between this genotype and the regulation of immune inflammation are unclear as yet.

It is noteworthy that, despite these disease associations, the *FHR1/3−/−* genotype is common and ubiquitous in healthy humans of different ethnicities including Africans, indicating the ancient origin of this variation [27]. Variation in the frequencies of *FHR1/3−/−* genotype has been observed in different ethnic groups. The highest frequency of this variation (~ 54%) is reported in African communities, while frequencies of 15–25% are reported from Europe, and the genotype is almost absent in many South American communities [28]. We have observed an ~ 8–10% frequency in India among healthy adults [26]. Thus, while the associations of this genotype with immune-inflammatory diseases suggest an immune-regulatory role; major immune perturbations associated with the *FHR1/3−/−* genotype would be unlikely.

On this background, we have begun to examine this issue by initiating a comparison of a number of immune parameters between healthy Indian adults carrying the *FHR1/3+/+* or the *FHR1/3−/−* genotypes. Our results suggest that healthy *FHR1/3 −/−* individuals show subtle but clear innate immune alterations and provide directions of further exploration of the mechanistic basis of the association of this common polymorphism with immune-inflammatory disease.

Materials and Methods

Antibodies and Reagents

Monoclonal fluorescent conjugated antibodies to CD45 (2D1), CD16 (3G8), CD14 (M5E2), CD11c (B-ly6), CD25

(M-A251), CD3 (OKT-3), CD4 (RPA-T4), CD8 (RPA-T8), TCR gamma/delta (γδ) (B1), CD45RA (HI100), CCR7 (3D12), CD19 (HIB19), CD20 (2H7), CD27 (M-T271), lineage (LIN: CD3, CD14, CD16, CD19, CD20, CD56), HLA-DR (L243), CD123 (7G3), CCR5 (HEK/1/85a), CCR6 (11A9), CXCR5 (RF8B2) (from BD Biosciences, San Jose, USA), CD43 (10G7), CD10 (HI10a), CD38 (HB-7), CD45RO (UCHL1) (from BioLegend, San Diego, USA) Helios (22F6), Foxp3 (150D/E4) (from eBioscience, San Diego, USA), CRTH2 (301108) (from R&D Systems, Minneapolis, USA), FH (OX-24), C3b (3E7/C3b) (from Abcam, Cambridge, USA, and BioLegend, San Diego, USA), and intracellular cytokines: IFN-g (4S.B3), IL-10 (JES3-9D7), IL-4 (MP4-25D2), and IL-17 (BL168) (from BioLegend, San Diego, USA) were used. Cytokine bead array (CBA) kit (from BD bioscience, San Jose, CA) was used to analyze cytokines in culture supernatant. CpGA and resiquimod (R848) (InvivoGen, San Diego, CA, USA) were used as appropriate.

Human Subjects

Samples were collected from 356 healthy males ranging in age from 20 to 50 years (Supplementary data; Table S1), with no history of any chronic illness or acute illness in the previous 2 weeks, from the blood bank of the Department of Transfusion Medicine, AIIMS, New Delhi, using leukapheresis (Leukapaks) to separate leucocytes from whole blood. The limitation of our analysis to males was a consequence of the use of blood bank donor PBMCs, since the overwhelming majority of donors were males. Further, since only leucocytes were available as sample post-leukapheresis, the sera could not tested for anti-FH autoantibodies.

PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). One aliquot of the cells was used for DNA isolation, and the remaining cells were stored in liquid nitrogen in cryomedia (20% DMSO in heat-inactivated FBS) at a density of 15–20 million cells/ml in liquid nitrogen until use.

Genotyping

Genomic DNA was isolated from PBMCs (FlexiGene DNA kit, Qiagen, Germany). Real-time quantitative PCR (qPCR) was performed to detect homozygous sufficiency or deficiency of *FHR1* and *FHR3* genes in all samples as described previously [25]. Genomic DNA (125 ng) and 0.2-μM reverse and forward primers were used for the qPCR. All reactions were performed in triplicates. The CT values obtained for *FHR1* and *FHR3* gene copy numbers were normalized to the endogenous control gene *BETA-ACTIN* and quantified relative to the copy number of control samples using the ΔΔCT method. A total of 33 individuals showing the *FHR1/3−/−* genotype and 33 individuals showing the *FHR1/3+/+* genotypes were selected for further analysis (Supplementary data; Fig. S1).

Immuno-phenotyping

PBMCs were stained using various fluorochrome-labeled antibody cocktails. The cocktails were the following: (A) CD45, CD16, CD14, CD11c; (B) CD45, CD3, CD4, CD25, CD8, CD45RA, CCR7, TCRγδ, CD25; (C) CD45, CD19, CD20, CD10, CD43, CD38 CD27; (D) CD45, LIN, HLA-DR, CD11c, CD123; (E) CCR5, CRTH2, CCR6, CXCR5, CD4, CD25, CD45RO; and (F) helios, Foxp3, CD4, CD25, CD45RO. Anti-FH and anti-C3b monoclonal antibodies were used for cell-surface-bound FH and C3b detection. Staining was performed on ice for 30 min in 96-well U-bottom plates containing 3 million cells in each well, followed by washing in 1× PBS. Cells were fixed in 2% paraformaldehyde and subjected to flow cytometric analysis (FACS Verse, BD Biosciences, San Jose, USA). Data were analyzed using FlowJo software (TreeStar, Ashland, USA).

Functional Assays

Monocyte Stimulation and Cytokine Assays

Monocytes were isolated from PBMCs using anti-CD14 magnetic bead-based purification (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were plated in 24-well plates (5×10^6 in 1 ml) in RPMI1640 medium supplemented with 10% heat-inactivated FCS and stimulated with 50 ng/ml lipopolysaccharide (LPS) (from *Escherichia coli*, serotype O55:B5; Sigma-Aldrich) or 10 μg/ml R848 (InvivoGen, San Diego, USA) for 24 h. Cytokine levels (TNF-alpha, IL-1β, IL-6, and IL-10) were measured in the culture supernatants using cytokine bead arrays (CBAs; BD Biosciences, San Jose, USA) and analyzed using FCAP array software (BD Biosciences, San Jose, USA).

DC Stimulation and Cytokine Assays

Myeloid DCs (mDCs; LIN−HLADR+CD11c+) and plasmacytoid DCs (pDCs; LIN−HLADR+CD123+) were electronically sorted on a flow cytometer (FACS Influx; BD Bioscience, San Jose, USA). These mDCs or pDCs were seeded (4×10^4 mDCs or 2.5×10^4 pDCs) in 96-well U-bottomed plates containing 150 μl of RPMI1640+FCS. While mDCs were stimulated with 50 ng/ml of LPS or 10 μg/ml of R848, pDCs were stimulated with 5 μg/ml of CpGA. After 24 h,

levels of TNF-alpha, IL-1β, IL-10, and IFN-α were measured in culture supernatants using CBA cytokine assays and analyzed by FCAP array software.

CD4 T Cell Cytokine Assays

PBMCs (5×10^6/ml) were seeded in 24-well plates containing 1 ml RPMI 1640+FCS and stimulated with 200 ng/ml PMA and 1 μg/ml ionomycin for 4 h in the presence of 3 μM monensin. Cells were fixed and permeabilized by using fixation and permeabilization buffer set (eBioscience, San Diego, USA) according to manufacturer's protocol and intracellular cytokines: IFN-γ, IL-4, IL-17, and IL-10 that were analyzed in CD4 T cells on flow cytometry.

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney *U* test or the unpaired *t* test as indicated. GraphPad Prism 7.0 software was used for data analysis, and *p* values < 0.05 were considered to be statistically significant.

Results

Differences in Circulating Leucocyte Subset Frequencies between *FHR1/3−/−* and *FHR1/3+/+* Genotypes

A number of peripheral blood leucocyte subsets (~30) as previously described [29, 30] (Supplementary data; Table S2 and Fig. S2) from *FHR1/3−/−* and *FHR1/3+/+* individuals were estimated and compared. None of the major leucocyte subsets such as CD4 and CD8 T cells as well as the CD4 to CD8 ratio, γ/δ T cells, B cells, monocytes, or dendritic cells (DCs) showed any differences between the two groups, *FHR1/3−/−* and *FHR1/3+/+* (Fig. 1a). However, significant differences were observed within the monocytic and DC compartments. The frequencies of patrolling monocytes (PMs) were higher in the *FHR1/3−/−* group than in the *FHR1/3+/+* group (Fig.1b), although the frequencies of classical monocytes (CMs) were similar. Similarly, the frequencies of plasmacytoid DCs (pDCs) were higher in the *FHR1/3−/−* group than in the *FHR1/3+/+* group (Fig. 1c), although the frequencies of myeloid DCs (mDCs) were similar. As a result, the mDC:pDC ratios were significantly different between the two groups (Fig. 1c).

The B cell compartment subsets showed little difference by and large between the two genotypes. While

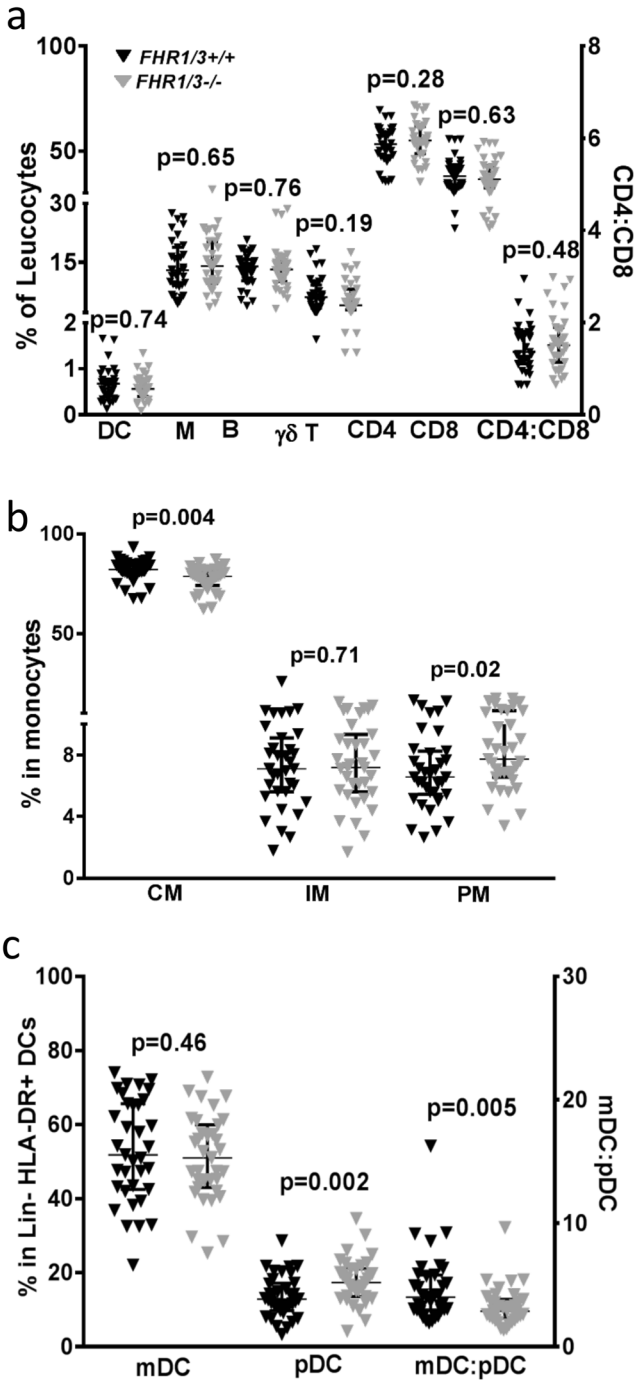

Fig. 1 Innate immune leucocyte profiles of *FHR1/3+/+ and FHR1/3−/−* healthy individuals. Frequencies of major leucocyte subsets and CD4:CD8 T cell ratios (right axis) (**a**), monocyte subsets: classical monocytes (CM), inflammatory monocytes (IM) and patrolling monocytes (PM) (**b**), and dendritic cell subsets (**c**) in PBMCs of *FHR1/3+/+* (black; $n = 33$) and *FHR1/3−/−* (gray; $n = 33$) healthy adults are shown as individual data points and median+/− interquartile range (IQR). The Mann–Whitney *U* test was used to estimate *p* values.

naive, immature memory and B1 B cell subset frequencies were quite similar between the two groups, *FHR1/3−/−* individuals showed a somewhat higher frequency of circulating plasmablasts (Fig. 2a), possibly indicating

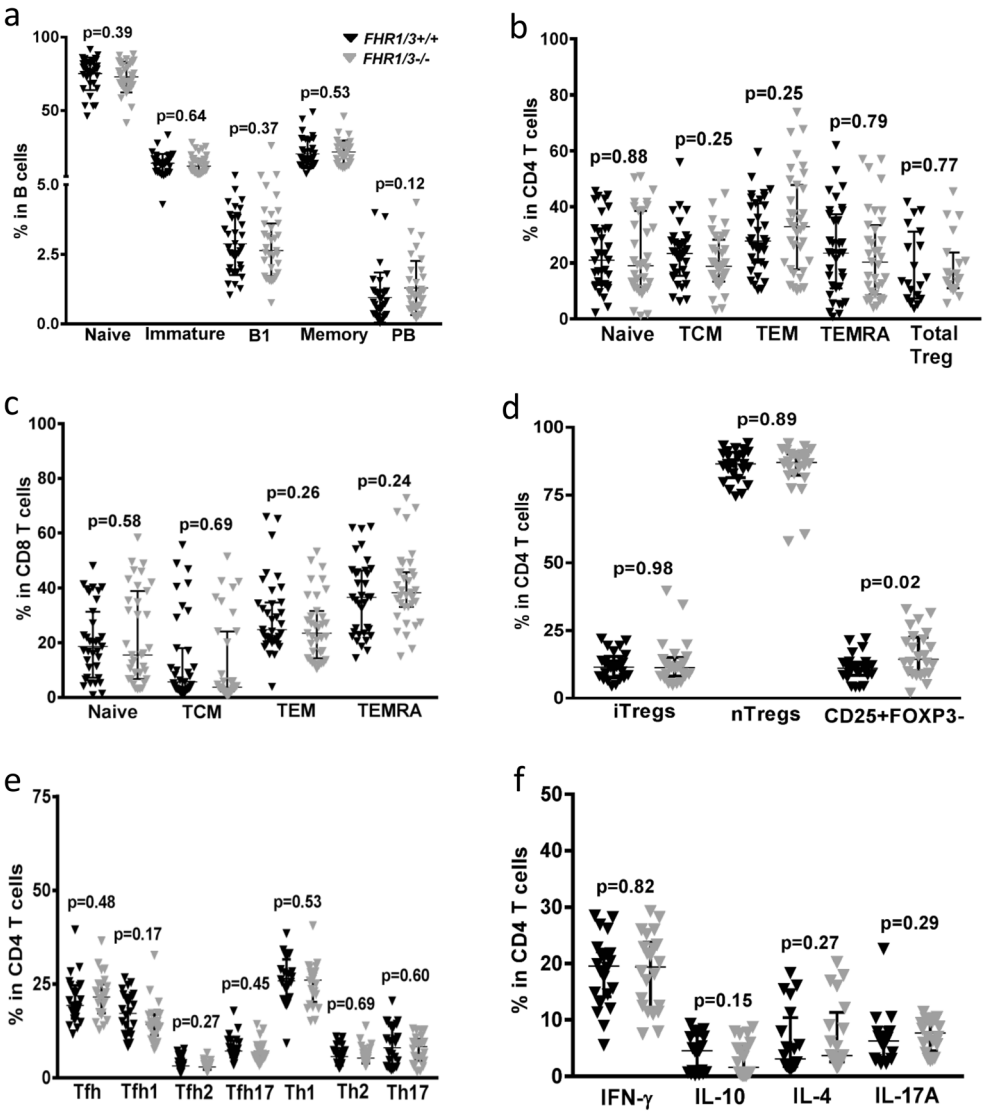

Fig. 2 Adaptive immune leucocyte profiles of *FHR1/3+/+* and *FHR1/3−/−* healthy individuals. Frequencies of B cell subsets (**a**), CD4 T cell subsets (**b**), CD8 T cell subsets (**c**), Treg cell subsets (**d**), and CD4 Tem cell subsets (**e**) in PBMCs of *FHR1/3+/+* (black; $n = 33$) and *FHR1/3−/−* (gray; $n = 33$) healthy adults are shown as individual data points and median+/− interquartile range (IQR). **f** PBMCs were stimulated in vitro with PMA + ionomycin followed by intracellular cytokine analysis as described, and cytokine-expressing subsets of *FHR1/3+/+* (black; $n = 25$) and *FHR1/3−/−* (gray; $n = 25$) healthy adults are shown as individual data points and median+/− interquartile range (IQR). The Mann–Whitney *U* test was used to estimate *p* values.

a tendency to greater immune activation, although this did not reach statistical significance.

Given this, as well as the association of the *FHR1/3−/−* genotype with a range of immune inflammatory syndromes, we also examined the T cell compartment in some detail. The major subsets of both CD4 and CD8 T cells, namely, naive, central memory (Tcm), effector memory (Tem), and the recently activated subset (effector memory CD45RA+; TEMRA) showed no differences between the two genotypes (Figs. 2b–c). We also examined the CD4 regulatory T (Treg) cell compartment. Total CD4 Treg cells showed no differences between the two genotypes (Fig. 2b). Neither the thymic "natural" Treg cells (Foxp3+Helios+; nTregs) nor the peripherally induced Tregs (Foxp3+Helios−; iTregs) showed any differences either (Fig. 2d). It was noticeable, however, that the population of CD25+ but FoxP3− CD4 T cells were higher in the *FHR1/3−/−* genotype group (Fig. 2d). When we examined the phenotype of these cells further, they were CD45RO+, indicating that they could be activated CD4 T cells, although no other group of effector, memory, or recently activated CD4 T cells showed any differences between the two genotypes.

Given the trends to differences in plasmablasts and in possibly activated CD4 T cells, we examined the functional subsets of the CD4 Tem compartment further. None of the subsets tested, T helper (Th) Th1, Th2, and Th17, in either follicular helper T cell (Tfh) or non-Tfh CD4 T cells, showed any differences between the two groups (Fig. 2e). We confirmed this by activating T cells from PBMCs from the two groups and estimating the frequencies of cytokine-expressing CD4 T cells; we found no differences between the two groups (Fig. 2f).

Higher Levels of Cell-Surface FH on *FHR1/3−/−* Classical Monocytes

The FHRs are reported to compete with FH for cell-surface binding, thereby regulating the dynamic balance of complement-related self-non-self discrimination [31]. We therefore investigated whether the absence of FHR1 and FHR3 proteins altered the levels of FH found on leukocyte surfaces. Two notable findings emerged. Firstly, FH was indeed clearly detectable ex vivo on leucocyte surfaces. However, all leucocyte subsets did not show equal levels of FH on them. Instead, FH was detectable mainly on monocytes and not on CD14-negative non-monocytic cells such as B cells and T cells (Fig. 3a). Among the monocyte subsets, the highest levels of FH were specifically detectable on classical monocytes compared to the patrolling monocytes (Fig. 3b). Secondly, FH levels on monocytes ex vivo were higher on average on cells from *FHR1/3 −/−* than *FHR1/3+/+* individuals (Fig. 3b). It was notable that DCs did not show appreciable levels of cell-surface FH in comparison to monocytes, and unlike monocytes, the levels of FH on DCs did not vary between *FHR1/3 −/−* and *FHR1/3+/+* individuals (Supplementary data; Fig. S3).

Since FH regulates C3 activity, we tested if these differences in cell-surface FH levels were correlated with differences in C3b levels. Like FH, C3b was detectable mainly on monocytes (Fig. 3c) ex vivo, and most prominently on inflammatory monocytes (IM) (Fig. 3d). However, unlike FH, C3b levels on monocytes from *FHR1/3−/−* and *FHR1/3+/+* individuals were comparable (Fig. 3d).

Differences in TLR-Responsiveness of *FHR1/3−/−* Versus *FHR1/3+/+* Monocytes and DCs

Clearly, both the monocyte and the DC compartment landscapes were different between the *FHR1/3−/−* and *FHR1/3+/+* groups, but cell-surface FH levels and differences therein were detectable only on monocytes and not in DCs. We therefore tested functional responses of both monocytes and DCs from the two groups.

Monocytes were isolated using CD14-based positive selection, thereby enriching the purified population for classical monocytes. Isolated monocytes were exposed to two TLR ligands: LPS as a ligand for cell-surface TLR4 and R848 as a ligand for endosomal TLR7/8. Curiously, *FHR1/3−/−* monocytes responded to LPS with higher levels of IL-1ß, but not of TNF-alpha, IL-6, or IL-10

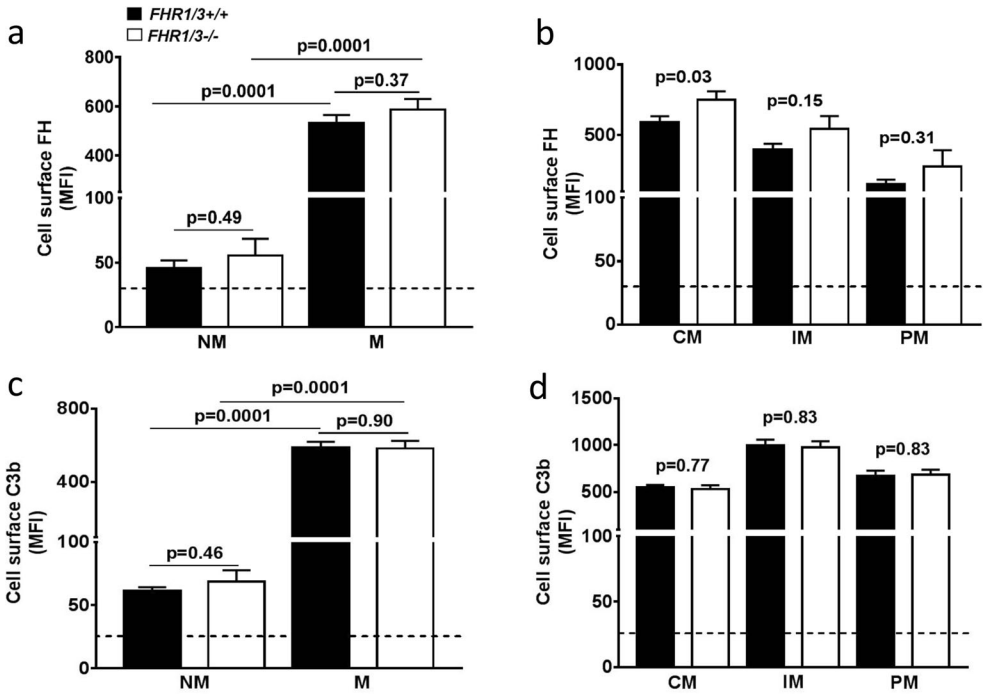

Fig. 3 Cell-surface-bound FH and C3b levels in leukocyte subsets. PBMCs of *FHR1/3+/+* (black bars; $n = 33$) and *FHR1/3−/−* (white bars; $n = 33$) healthy adults were stained for cell-surface-bound FH (panels **a** and **b**) or C3b (panels **c** and **d**) and for monocyte subsets. Data are shown as the mean fluorescence intensity (MFI) values (mean ± SEM). Dotted lines represent background isotype control MFI values. The *p* values were calculated using Student's *t* test. Non-monocytic cells (NM), total monocytes (M), classical monocytes (CM), IM, and patrolling monocytes (PM) are shown. Representative histograms are shown in supplementary data (Supplementary data; Figs. S4 and S5).

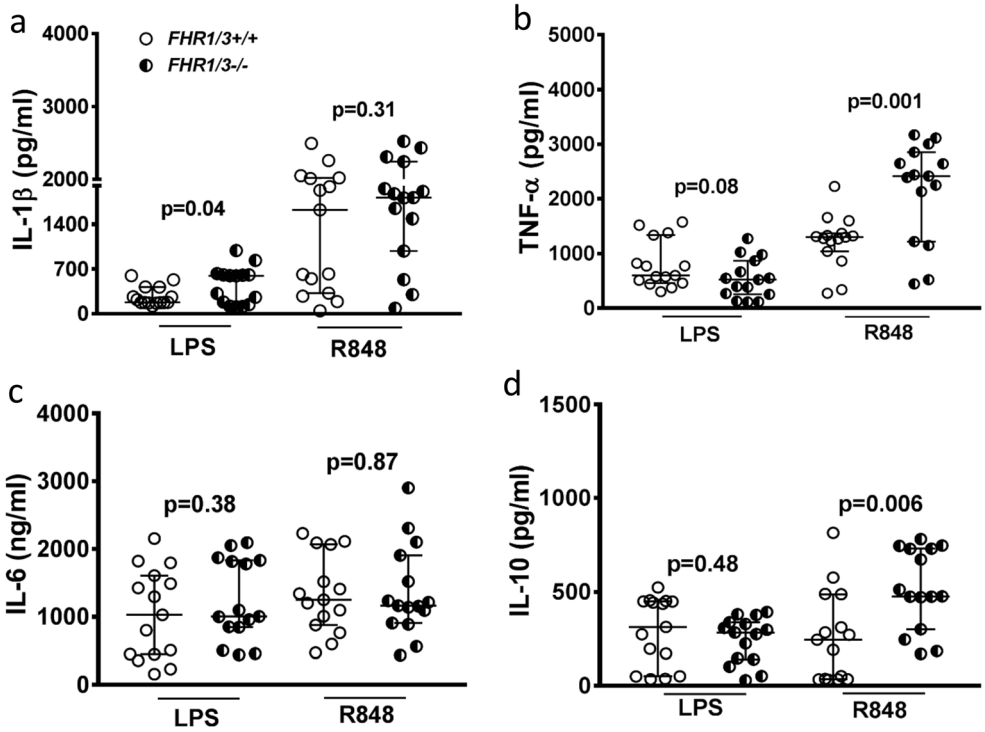

Fig. 4 Cytokine responses to TLR ligands of monocytes from *FHR1/3+/+* and *FHR1/3−/−* healthy individuals. CD14+ monocytes were isolated from PBMCs of *FHR1/3+/+* (white dots, $n = 15$) or *FHR1/3−/−* (black dots, $n = 15$) healthy individuals and stimulated with LPS or R848 as described for 24 h. Culture supernatants was analyzed for IL1β (**a**), TNFα (**b**), IL6 (**c**), and IL10 (**d**). Data are also shown as median+/−IQR. The *p* values shown were calculated by the Mann–Whitney *U* test.

(Fig. 4a–d). However, in response to R848, *FHR1/3−/−* monocytes responded with higher levels of TNFα and IL-10, but not of IL-6 or IL-1ß (Fig. 4a–d).

In the DC compartment, we purified mDCs and pDCS and examined their functional responses. The mDCs were stimulated with TLR4 and TLR7/8 ligands as above. *FHR1/3−/−* mDCs responded to LPS with higher levels of IL-10 but not of TNF-alpha or IL-1ß, while they response to R848 with higher levels of TNF-alpha and IL-1ß but not of IL-10 (Fig. 5a–c). Purified pDCs were tested with the TLR9 ligand CpGA for their specific ability to show type 1 interferon induction.

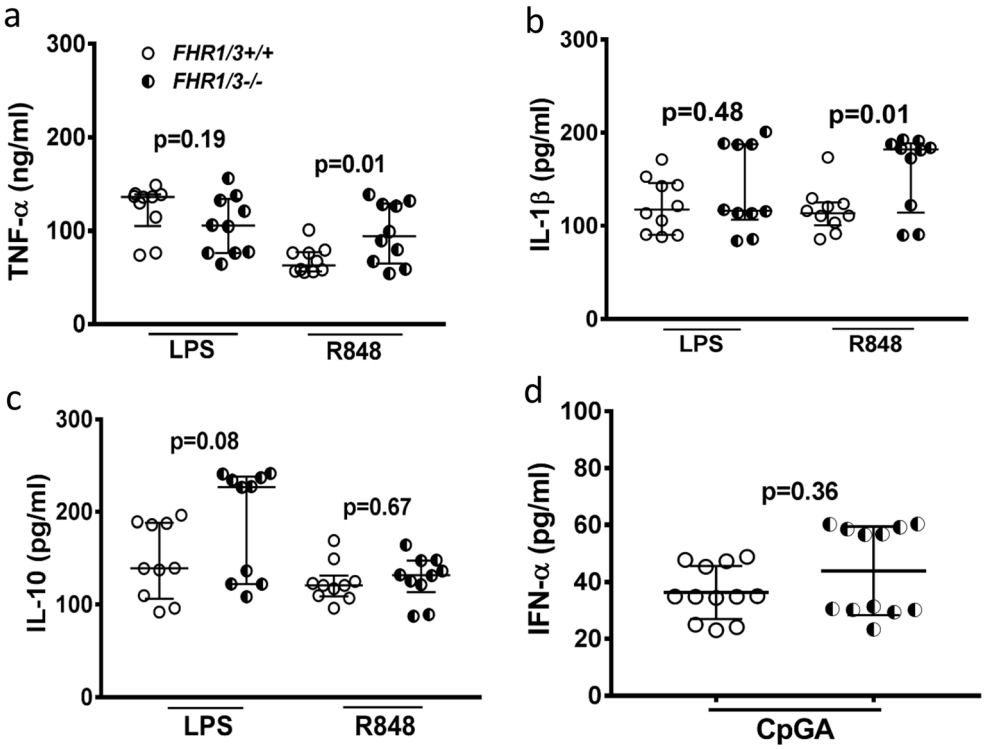

Fig. 5 Cytokine responses to TLR ligands of DCs from *FHR1/3+/+* and *FHR1/3−/−* healthy individuals. DC subsets, mDCs (**a**–**c**), and pDCs (**d**) were isolated from PBMCs of *FHR1/3+/+* (white dots) or *FHR1/3−/−* (black dots) healthy individuals ($n = 8$–10). The mDCs were stimulated with LPS or R848 as described for 24 h, and culture supernatants analyzed for TNFα (**a**), IL1β (**b**), and IL10 (**c**). The pDCs were stimulated with CpGA as described for 24 h, and culture supernatants analyzed for IFNα (**d**). Data are also shown as median+/−IQR. The *p* values shown were calculated by the Mann–Whitney *U* test.

However, pDCs of the two genotypes did not show any differences in this property (Fig. 5d).

Discussion

The *FHR1/3−/−* genotype is associated in various ways with a number of immune-inflammatory diseases such as SLE, IgA nephropathy, and age-related macular degeneration. Our recent data also suggest that, even in its association with pediatric aHUS, it may contribute both an anti-FH autoantibody-dependent and an independent pathway [25]. Clearly, this genotype is not sufficient in itself to give rise to autoimmune disease, as evidenced by the wide prevalence of this genotype in healthy populations. Therefore, it is likely to modulate immunity in both FH-specific and more general ways. Modulation of complement pathways has been increasingly shown to be associated with a number of alterations in adaptive immune responses. On this background, we hypothesized that the peripheral leukocyte subset phenotype in the *FHR1/3−/−* genotype may show evidence of tendencies to altered immune/inflammatory states even in apparently healthy individuals.

When we compared the peripheral leukocyte subset phenotypes in healthy asymptomatic adults with and without the *FHR1/3−/−* genotype, it was evident that individuals with the *FHR1-FHR3* deletion genotype showed some interesting alterations. They showed an altered balance of the pDC:mDC ratio with greater predominance of plasmacytoid DCs, a finding of interest given the role that these DC subsets play in inducing and maintaining immune tolerance [31–34]. They also showed a relative prominence of patrolling over classical monocytes, a finding of interest because of the potential differing roles of these subsets in maintaining immune surveillance as well as inflammatory responses under several disease conditions such as sepsis [35], atherosclerosis [36], rheumatoid arthritis [37], and SLE [31–33, 38]. There is also accumulating evidence suggesting that increased prominence of non-classical monocytes and of pDCs is associated with exacerbated inflammatory responses in immune inflammatory disorders [31, 36, 39–41]. Complement-mediated modulation of adaptive immune responses has been shown to be mediated via plasmacytoid DCs [42]. Further, patrolling monocytes have recently been shown to prevent complement-mediated vascular injury in sickle cell disease [43]. Our data add further complexity to the uncertainty over the differing situations of complement activation in which the FHRs play positive or negative roles [15], and to the paradoxical association of the FHR1/3−/− genotype with either lower or higher risk for differing immune inflammatory diseases [23, 24, 28].

It is however noteworthy that, despite these alterations, *FHR1/3−/−* individuals show no substantial trend to alterations in their adaptive leucocyte subsets, with the exception of a significantly higher prominence of CD25+FoxP3− CD4 T cells and a tendency to higher plasmablast frequencies that did not reach statistical significance. While these findings will need more detailed investigation, it is clear that additional "hits" are likely to be required before the *FHR1/3−/−* genotype can manifest as full-fledged autoimmune/inflammatory disease. In this context, it must be noted that, while many of these diseases show gender-specific differences in susceptibility, the present study does not provide any insights into gender-related differences in the phenotype of the FHR1/3−/− genotype since we have used only male samples as a consequence of blood bank donorship. Similarly, our assumption that as healthy adults these donors were not likely to harbor anti-FH autoantibodies is well supported by literature and our reported prior experience [18, 25, 26]. We were unable to test these sera for anti-FH autoantibodies since only leucocytes were available as samples post-leukapheresis.

While the *FHR1/3−/−* genotype is associated anti-FH autoantibodies in aHUS, it is clearly not sufficient to induce this autoimmune response, raising questions about the identity of additional factor/s required. Further, it remains a puzzle of how the *FHR1/3−/−* genotype is connected specifically to anti-FH autoantibodies but not other autoantibodies [44, 45]. There is now evidence that FHR proteins are likely to be hetero-dimers 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 [3]. Thus, it was now possible to hypothesize that, in the absence of FHR hetero-dimers in the *FHR1/3−/−* genotype, more FH may easily bind to cell surfaces, and such increased binding to antigen-presenting cell (APC) surfaces may contribute to FH-specific loss of immune tolerance as well as to other effects on complement regulation. We tested this possibility in cells of healthy adults bearing the *FHR1/3−/−* genotype. Our data show that, remarkably, among peripheral leukocytes ex vivo, only classical monocytes show significant levels of FH bound on the cell surface. Further, we find that the *FHR1–FHR3* deletion is associated with higher levels of FH on classical monocytes ex vivo.

Since FH bound to cell surfaces is known to be a critical regulator of cell-surface complement activation [3], it was possible that, with lower levels of cell-surface-bound FH in *FHR1/3+/+* individuals, classical monocytes would show higher levels of active complement components [13, 15] vice versa; with higher levels of FH on cell surface in *FHR1/3−/−* individuals,

monocytes would show less complement activity. However, this turned out not to be the case, at least with respect to the levels of detectable cell-surface C3b. Both *FHR1/3+/+* and *FHR1/3−/−* individuals exhibited similar levels of cell-surface C3b on monocytes. However, there is increasing evidence that FH-binding to monocyte/macrophage surfaces has functionally meaningful consequences. While such binding on monocytes leads to their differentiation into functionally different dendritic cells [46], FH binding to macrophages enhances their inflammatory responses to *Candida albicans* [47].

On this background, it is notable that, when we stimulated purified monocytes from *FHR1/3−/−* and *FHR1/3+/+* genotypes with TLR ligands, FHR1/3−/− monocytes appeared to respond by making higher levels of inflammatory cytokines; in other words, FHR1/3 limits inflammatory responses of these cells. Whether and how cell-surface FH modulates TLR-mediated monocyte responses, and what roles if any these modulations play in the immuno-inflammatory morbidities associated with the *FHR1/3−/−* genotype, remains to be investigated. Thus, while our data do not immediately provide an explanation of the biology of the FHR1/3−/− genotype, they demonstrate the complexity of the functional consequences of the genotype in vivo; they indicate that the connection between the *FHR1/3−/−* genotype and immuno-inflammatory diseases is likely to be complex and provide interesting new directions for future investigations.

Author Contributions AB performed experiments, analyzed the data, and helped in writing the manuscript. BSG, SP, TMS, and MP developed critical tools and analyzed data. AB and SS developed critical methodologies and helped collect the data. AB, PK, HS, PoC, and GP conceptualized the approach, did the critical clinical components of the work, supervised the data collection, and analyzed the data. AKV, PrC, VB, AG, ArS, AB, AmS, PH, and AdS provided crucial conceptual inputs. AB, PG, and SR conceptualized the approach, designed the experiments, analyzed the data, and wrote the manuscript. All authors read, edited, and approved the final manuscript.

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Compliance with Ethical Standards

All procedures followed were in accordance with the ethical standards of the ethics committee for human research, and the study was approved by the Institutional Ethics Committee for Human Research of Regional Centre for Biotechnology (RCB, Reference No. RCB-IEC-H-8), Faridabad, India, and the Institutional Human Ethics Committee of the All India Institute of Medical Sciences (AIIMS, Reference No. IEC-/05. 02. 20, RP44/2016), New Delhi, India.

Conflict of Interest SR is a non-executive director of Ahammune Biosciences Private Limited, Pune, India, and a member of the scientific advisory boards of Curadev Pharma Private Limited, NOIDA, India, and Mynvax Private Limited, Bangalore, India. Other authors have no financial or other interest to declare.

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