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Reduced red blood cell surface level of Factor H as a mechanism underlying paroxysmal nocturnal hemoglobinuria

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Abstract

The absence of the cell-surface complement inhibitors CD55 and CD59 is considered the mechanism underlying the complement-mediated destruction of affected red blood cells (RBCs) in paroxysmal nocturnal hemoglobinuria (PNH) patients, but Factor H (FH), a fluid-phase complement inhibitor, has also been proposed to be involved. However, the status of FH on the PNH patient RBC surface is unclear and its precise role in PNH pathogenesis remains to be further defined. In this study, we identified significantly lower levels of surface-bound FH on the affected $CD59^-$ RBCs than on the unaffected $CD59^+$ RBCs. Although this reduction in surface-bound FH on PNH RBCs was accompanied by decreased surface sialic acid levels, the enzymatic removal of sialic acids from these RBCs did not significantly affect the levels of surface-bound FH. We further observed higher surface levels of FH on the C3b/iC3b/^{high} RBCs than on C3b/iC3b^{low} RBCs within the affected PNH RBCs of patients treated with eculizumab. Finally, we determined that enhanced surface levels of FH on CD55/CD59-deficient RBCs from mice and PNH patients protected against complement-mediated hemolysis. Taken together, our results suggest that a reduced surface level of FH is another important mechanism underlying the pathogenesis of PNH.

Introduction

Complement is a key component of innate immunity that primarily acts to fight infection and clear apoptotic cells(1). Upon activation, the complement cascade culminates in the formation of membrane attack complexes (MAC, C5b-9) on the surfaces of invading pathogens, which cause damage and even lysis of the intruders. However, activated complement cannot distinguish autologous cells from invading pathogens. Accordingly, cells

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Authorship

L.Z. and J.C. did the experiments, analyzed the data and edited the manuscript; C.K. collected and organized the patient samples, B.C and J.M. discussed the results and edited the manuscript; F.L. designed the experiments, interpret the results and wrote the manuscript.

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in host tissues must express complement inhibitors on their surfaces to protect against bystander complement attacks(2).

To date, four common cell surface complement inhibitors have been identified: CD35(3), CD46(4), CD55(5) and CD59(6). These inhibitors are expressed concurrently in different combinations on the surfaces of all host cells, where they control activation at different steps of the complement cascade and thus protect autologous cells from a misdirected complement attack. CD35 and CD46 are classical transmembrane proteins expressed on the cell surface, whereas CD55 and CD59 lack transmembrane domains but attach to the cell membrane via C-terminal glycosylphosphatidylinositol (GPI) anchors that are added during post-translational modifications(7). In addition to the cell surface complement regulators, inhibitors of complement activation also exist in the fluid phase. Among them, factor H (FH) is a potent soluble complement inhibitor that works by accelerating the decay of the C3/C5 convertases and by serving as a co-factor for factor I to inactivate C3b. Moreover, FH can also attach to the cell surface via different mechanisms, where it could function as another cell surface complement inhibitor to provide further protection to self-cells(8). Despite these defensive mechanisms, however, self-tissue injury and disease still occur when activated complement overwhelms the combined protective effects of these complement regulators.

In paroxysmal nocturnal hemoglobinuria (PNH), under-controlled activation of the terminal complement activation pathway in the absence of GPI-anchored CD55 and CD59 is considered responsible for hemolysis(9). While these fundamental pathologic mechanisms have been intensely investigated, several clinical observations in PNH patients have not been well explained. For instance, there is a great variability of clinical courses irrespective of the size of the PNH clone and the fraction of GPI-deficient RBCs(10). PNH patients may have hemolysis that progresses at various ferocity or their course is characterized with period hemolytic crises(11). The causes for the latter are not well explored: why patients may have a residual seemingly complement-resistant fraction of PNH RBCs? Finally, the variable susceptibility of some patients to C3-mediated medullary destruction of GPI-deficient RBCs evolving upon C5 blockade remains not completely understood, despite the discovery of the role of CR1 polymorphisms(12, 13).

According to the currently accepted view, PNH is caused by a lack of CD55 and CD59 on the affected RBCs due to the GPI-anchor pathway deficiency(14). However, a few studies have also suggested that FH plays an important RBC-protective role (15, 16). While its complement-inhibitor function is described, previous studies have not explored the status of cell surface-bound FH on RBCs from patients with PNH, and the role of this factor in PNH pathogenesis remains to be defined rigorously. Clarification of this function of FH in PNH may provide answers to these and other questions which persist in particular after the introduction of the development of other targeted therapies. In this project, we examined the levels of cell surface-bound FH on RBCs from PNH patients using flow cytometry, immunofluorescence staining, ELISA and Western blotting. We also studied the significance and mechanistic role of surface-bound FH in complement-mediated hemolysis, using RBCs from PNH patients and from CD55/CD59 double-knockout (KO) mice. Our data demonstrate a previously unclarified role for cell surface-bound FH in the pathogenesis of PNH.

Materials and Methods

All Method details are in the Supplemental Materials.

PNH patient samples, mice and normal human serum

Blood samples from diagnosed PNH patients (Table 1) were collected with consent following a Cleveland Clinic IRB approved protocol. WT mice and CD55/CD59 double knockout mice(17) were maintained under pathogen-free conditions at Cleveland Clinic. All the procedures involving the animals were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic. Pooled normal human serum (NHS) was purchased from Innovative Research Inc (Novi, MI).

CFH detection on PNH RBCs by flow cytometry, confocal microscopy, ELISA and Western blot

CFH on RBCs from PNH patients was assessed by flow cytometry after staining with a goat anti-CFH antibody and a FITC-conjugated anti-human CD59 mAb to distinguish PNH RBCs.

For confocal microscopy analyses, RBCs from a PNH patient were first flow-sorted based on their CD59 expression, then the sorted $CD59^-$ and $CD59^+$ PNH RBCs were stained with the goat anti-CFH antibody following examinations by a Leica confocal microscope.

For ELISA, $CD59^-$ PNH RBCs were separated from CD59+ RBCs using streptavidin-coated magnetic beads, then cell lysates were prepared from the same numbers of the isolated CD59- and CD59+ RBCs. CFH levels in the lysates were measured by ELISA. For internal controls, CD235a levels in the same lysates were also measured by ELISA for normalization.

For western blot, CFH from the same cell lysates were first immunoprecipitated then loaded onto a SDS-PAGE and probed with the goat anti-CFH antibody.

Sialic acid and C3b/iC3b/C3d detection of PNH RBCs

For sialic acid detections on RBCs, PNH patient RBCs were incubated different lectins followed by flow cytometric analyses. For C3b/iC3b/C3d detection, RBCs of PNH patients with eculizumab treatment were stained with the goat anti-CFH antibody, an Alexa 674-labeled donkey anti-goat IgG, a FITC-conjugated goat anti-human complement C3 and a PE-conjugated anti-human CD59 mAb before being analyzed by a flow cytometer.

Neuraminidase treatment and sialic acid detection

Sialic acid was removed from RBCs by incubation with 250U α2–3,6,8 neuraminidase. Removal of sialic acids was confirmed by measuring levels of cell-bound lectin MAL-II before and after neuraminidase treatment, as described above.

CFH painting on RBCs and Hemolytic assays

RBCs from CD55/CD59 double KO mice, or $CD59^-$ RBCs purified from PNH patients were painted with purified human CFH, or heparin following an established protocol(18, 19).

Painted CD55/CD59 double KO mice RBCs were mixed with RBCs from wild type RBCs, then incubated with 10% NHS, 0.5 mM Mg-EGTA (or 10 mM EDTA) for 5 minutes at 37°C. The destruction of $CD59^-$ populations were analyzed by flow cytometry.

Painted $CD59^-$ human RBCs were mixed with RBCs from a healthy donor in a 7:3 ratio. The mixed RBCs were incubated with 20% acidified NHS (pH 6.4), 1.5 mM MgEGTA (or 10mM EDTA) for 20 minutes at 37°C. The destruction of $CD59^-$ populations were analyzed by flow cytometry.

Data Analysis

Data were analyzed via GraphPad Prism (GraphPad software Inc., La Jolla, CA). Student's unpaired t-tests, one-way ANOVA and Tukey post-hoc tests were used to determine the statistical difference among groups, $p<0.05$ was considered significant.

Results

PNH RBCs exhibit reduced levels of surface FH.

In accordance with common clinical practice, we used the expression of CD59 to distinguish the affected ($CD59^-$) and unaffected ($CD59^+$) populations of freshly collected RBCs from one PNH patient, and successfully detected the surface-bound FH on both GPI anchor-deficient and normal RBCs in a flow cytometric analysis. We also found significantly lower surface levels of FH on the $CD59^-$ RBCs than on the $CD59^+$ RBCs (Fig. 1A).

To confirm these intriguing data, after repeating the above-described flow cytometric analysis with the same patient sample, we separated $CD59^-$ RBCs from $CD59^+$ RBCs within by flow sorting, then analyzed the surface levels of FH on sorted cell populations by immunofluorescent staining. Consistent with the flow cytometry results, we observed detectable FH staining on the surfaces of RBCs under a fluorescence microscope, with stronger staining on the $CD59^+$ than that on the $CD59^-$ RBCs (Fig. 1B).

To further verify the results of the surface FH flow cytometric assay, we purified $CD59^-$ RBCs from another PNH patient by depleting $CD59^+$ RBCs via magnetic beads (>95% purity). The same flow cytometric analysis revealed lower surface FH levels on the purified $CD59^-$ RBCs than on normal $CD59^+$ RBCs from a healthy donor (Fig. 1C). We then prepared cell lysates from the same numbers of purified $CD59^-$ RBCs and normal $CD59^+$ RBCs, and measured the levels of FH in the lysates by ELISA. Again, FH was detectable in both RBC lysates using the combination of 2 different anti-FH antibodies, with lower levels of FH in lysates of $CD59^-$ RBCs relative to $CD59^+$ RBCs (Fig. 1D).

Finally, we evaluated the levels of FH in the above-prepared cell lysates by Western blotting. To avoid interference from the massive amounts of hemoglobin in the RBC lysates, we first pulled down FH using an excess of an anti-FH mAb, and subjected the pulled-down fraction

to a semi-quantitative Western blot analysis of FH. Again, we observed that FH was detectable in RBC lysates via Western blotting, and that a stronger FH band was obtained from the lysate of $CD59^+$ RBCs than from a lysate prepared using the same number of $CD59^-$ RBCs (Fig.1E).

Taken together, these data validated the flow cytometric measurement of surface-bound FH on the RBCs. We then examined freshly collected RBCs from 16 different PNH patients according to this protocol and determined that surface-bound FH could be detected on all examined RBCs. Importantly, we found that in these samples from PNH patients, the affected $CD59^-$ RBCs exhibited significantly lower levels of surface-bound FH than their $CD59^+$ counterparts (mean fluorescence intensity [MFI]: 236.0±34.9 vs. 860.8±170.3, $p<0.05$) (Fig. 1F).

PNH RBCs exhibit reduced surface levels of sialic acids.

FH binds to the cell surface through interactions with ligands such as sialic acids. Accordingly, we performed a flow cytometry analysis using different sialic acid-binding lectins, including MAL-I and MAL-II which favor α2,3-linked sialic acids, SNA which favors α2,6-linked sialic acids, and Jacalin which binds most O-GalNAc or mucin-type glycans, to measure the surface levels of sialic acids on RBCs from PNH patients. Notably, we observed significantly lower surface levels of sialic acids on the affected $CD59^-$ RBCs than on the unaffected $CD59^+$ RBCs (Fig.2A, B). These data suggest that mechanistically, the reduced levels of surface-bound FH on $CD59^-$ RBCs from PNH patients might be attributable to reduced surface levels of sialic acids. We further analyzed the correlations between the FH and sialic acid levels on $CD59^+$ and $CD59^-$ RBCs, and found the reduction in FH levels on $CD59^-$ RBCs was correlated to decreased surface sialic acid levels (Fig. 2C, D).

Enzymatic desialylation does not affect the surface FH levels on PNH and normal RBCs.

To clarify the role of sialic acids in the binding of FH to the surfaces of RBCs from PNH patients, we treated these cells with neuraminidase to remove the surface-bound sialic acids, then performed flow cytometry to measure the levels of surface FH. A flow cytometry analysis of lectin-stained cells revealed that neuraminidase treatment almost completely removed the surface-bound sialic acids from the RBCs (Fig. 3A). Surprisingly, however, the surface levels of FH remained lower on the $CD59^-$ PNH RBCs relative to the unaffected $CD59^+$ RBCs, even after neuraminidase treatment (Fig. 3B,C). These results suggest that sialic acids might not be critical for the surface binding of FH to the surfaces of RBCs, or, the sialic acids bound with FH are resistant to the neuraminidase treatment.

Correlation between the surface levels of FH and C3b/iC3b/C3d on PNH RBCs.

Deposits of C3b/iC3b/C3d on cell surfaces represent another major type of ligand for FH. We analyzed blood samples from PNH patients who were responsive to eculizumab therapy and observed significantly higher levels of deposited C3b/iC3b/C3d on some $CD59^-$ RBCs from these patients (Fig. 4A), consistent with previous reports. Moreover, although we generally observed significantly lower FH levels on the $CD59^-$ RBCs relative to $CD59^+$ RBCs in these patients, the surface FH levels were correlated positively with the deposited

C3b/iC3b/C3d levels on the $CD59^-$ RBCs (Fig.4A,B,C). These results suggest that deposited C3b/iC3b/C3d on the surfaces of RBCs contributes significantly to the recruitment and maintenance of FH on these cells in PNH patients.

Human complement selectively lyses RBCs from CD55/CD59 double KO mice in a mixture with wild type (WT) RBCs.

In PNH patients, affected RBCs lack surface CD55 and CD59 and are selectively lysed by activated complement. To develop an animal model to mimic this well-observed clinical phenomenon, we mixed RBCs from CD55/CD59 double KO mice and WT mice and incubated the cell mixture with NHS to induce complement-mediated hemolysis. We then assessed the survival of the CD55/CD59 double KO RBCs by flow cytometry. Similar to the clinical observations, the CD55/CD59 double KO mouse RBCs were selectively lysed by activated human complement, as indicated by the significant reduction (~60%) of CD55/CD59-deficient RBCs in the cell mixtures containing RBCs from both WT and CD55/CD59 double KO mice (Fig. 5A).

Augmentation of surface FH levels on RBCs from CD55/CD59 double KO mice protects against complement-mediated hemolysis.

The above-described pilot experiments confirmed previous reports by others in which mouse CD55 and CD59 were shown to regulate human complement activity without species restrictions(20, 21). Moreover, our results also demonstrated that this mixed mouse RBC assay could be used to model the intravascular hemolysis that occurs in PNH patients. To determine the significance of lower levels of surface-bound FH on affected RBCs from PNH patients, we augmented the surface levels of FH on CD55/CD59 KO mouse RBCs either by painting FH directly onto the cells or by a two-step indirect process in which the cells were painted with heparin and subsequently incubated with purified FH, according to our previously published protocols(18). After confirming the increased surface levels of FH on the painted RBCs via flow cytometry (Fig.5B), we then mixed the painted or control CD55/CD59 KO RBCs with WT RBCs and incubated the cell mixtures with NHS to enable complement-mediated hemolysis. Finally, we assessed the survival of the CD55/CD59 double KO RBCs using flow cytometry. These experiments showed that the direct or indirect augmentation of surface FH levels significantly protected CD55/CD59 double KO mouse RBCs from complement-mediated hemolysis in the mixtures (Fig. 5C,D). Our results demonstrate a critical protective role for surface-bound FH on CD55/CD59-deficient RBCs in this *in vitro* mouse model of PNH.

Augmentation of surface FH levels on human PNH RBCs protects against complement-mediated hemolysis.

To determine whether augmenting surface FH levels on PNH RBCs protects the cells from complement attack, we again purified $CD59^-$ RBCs from a PNH patient by depleting $CD59^+$ RBCs using magnetic beads. Next, we painted the $CD59^-$ PNH RBCs with heparin and incubated these painted RBCs in the presence or absence of purified FH, according to the same protocol described above in the mouse PNH intravascular hemolysis model. Subsequent flow cytometric analyses confirmed that the surface levels of FH on the painted PNH RBCs increased by 13-fold after incubation with purified FH (Fig. 6A). We then mixed

the $CD59^-$ PNH RBCs containing different surface levels of FH with $CD59^+$ RBCs from a normal donor and incubated the cells with acidified human sera to induce complement-mediated hemolysis. Finally, we assessed the total hemolysis by measuring the concentrations of released free hemoglobin in the supernatants (OD_{414}) and quantified the survival of $CD59^-$ PNH RBCs using flow cytometry. Similar to our observations in the mixed CD55/CD59 double KO and WT mouse RBCs, we found that augmenting the surface levels of FH on the $CD59^-$ PNH RBCs generally reduced the complement-mediated hemolysis of these cells in the free hemoglobin measurement assays (Fig. 6B). Moreover, surface augmentation of FH significantly improved the survival of $CD59^-$ PNH RBCs in the cell mixtures, as demonstrated by flow cytometry (Fig. 6C,D). These results demonstrated that surface FH critically protects affected PNH RBCs from complement-mediated intravascular hemolysis.

Discussion

In this report, we demonstrate that surface-bound FH can be detected on human RBCs by different methods using different anti-FH antibodies. In addition, we observed that the surface levels of this conventionally fluid-phase complement inhibitor were significantly lower on affected $CD59^-$ RBCs than on unaffected $CD59^+$ RBCs from PNH patients. Moreover, this reduced surface level of FH was associated with reduced surface levels of sialic acids, although enzymatic desialylation did not significantly alter the levels of surface-bound FH. The surface levels of FH were also positively correlated with the levels of deposited C3b/iC3b/C3d on $CD59^-$ RBCs from PNH patients with anti-C5 treatments. We further developed an *in vitro* mouse PNH model by mixing RBCs from CD55/CD59 double KO and WT mice, and found that augmenting the surface FH levels on the CD55/CD59 double KO RBCs protected these cells from complement-mediated hemolysis. Finally, we demonstrated that augmentation of the surface FH levels on $CD59^-$ RBCs from PNH patients also protected these cells from hemolysis. Taken together, our results demonstrate that affected RBCs from PNH patients exhibit significantly reduced surface levels of FH, and provide strong evidence suggesting that, in addition to the lack of CD55 and CD59, reduction in surface FH is another major mechanism underlying the increased sensitivity of the affected RBCs to complement-mediated intravascular hemolysis in PNH. Conversely, cell-bound FH may explain the low-level persistence of residual GPI-deficient RBCs in patients with PNH. In the past, this targeted inhibitory activity of FH has been exploited to design recombinant CR2-FH fusion protein (TT30) as a potential therapeutic for PNH, which showed efficacy in the initial studies.(22)

As noted previously, CD35, CD55 and CD59 are three conventional cell surface complement inhibitors expressed on human RBCs to protect against complement attacks. CD35, a receptor of C3b/C4b, not only facilitates the clearance of complement-opsonized cells and immune-complexes, but also inhibits complement by accelerating the decay of the C3 and C5 convertases and by serving as a co-factor to factor I in the inactivation of C3b to iC3b(23). CD55 inhibits complement activation by accelerating the decay of the C3 and C5 convertases(24), while CD59 directly inhibits MAC formation by preventing the insertion of C9 into the C5b-8 complex during MAC assembly(24). Apparently, the lack of any one of these cell surface complement inhibitors on RBCs will render the cells more susceptible to

complement-mediated damage. As noted earlier, affected RBCs from PNH patients lack both CD55 and CD59 due to a genetic mutation-based GPI anchor pathway deficiency, which is thought to be the mechanism underlying the pathogenesis of PNH(14).

In addition to the established significance of CD55 and CD59 deficiency in the pathogenesis of PNH, researchers have proposed a role for the soluble complement inhibitor FH as a critical protector of RBCs in PNH (15, 16). FH is composed of 20 CCPs, of which CCP19–20 is primarily responsible for the binding of this factor to C3b and polyanions (e.g., sialic acids) on the cell surface(25–27). The FH in the fluid phase can be converted to a cell surface complement inhibitor after attachment, and studies have shown that treatment with recombinant FH19–20 inhibits the binding of FH in the plasma to RBCs during complement activation and increases the susceptibility of these cells to complement-mediated hemolysis, particularly when PNH patient RBCs were used(15). Although these earlier studies concluded that the recruitment of FH to RBCs during complement activation represented a critical step in hemolysis prevention, the surface levels of FH on RBCs, especially on RBC freshly collected from PNH patients before these in vitro assays have never been examined. Besides, it is also possible that the recombinant FH19–20 might have removed already-bound FH from the RBCs, thus rendering them more sensitive to complement attack in these previously reported in vitro assays. In our study, we used various assays, including flow cytometric analyses, immunofluorescent staining, western blot, and ELISA with different anti-FH antibodies, to demonstrate that FH remained detectable on the RBC surface even after extensive washing, and that surface FH levels are lower on affected $CD59^-$ RBCs than on unaffected $CD59^+$ RBCs from PNH patients, thus providing direct evidence to support the potentially integral involvement of FH in protecting affected RBCs in PNH in vivo. One could stipulate that variability of FH surface binding may be responsible for a great deal of heterogeneity of clinical courses observed in PNH patients. To that end, hemolytic crises may, apart from the acute increase in complement activation, be triggered/augmented by a drop in surface FH levels. In addition, lack (or variability) of the FH (either directly or via low levels of C3b/iC3b/C3d and sialic acids) on the surface of GPI-deficient RBCs may be responsible to extravascular RBC destruction in patients treated eculizumab in whom various degrees of anemia persists despite the presence of anti-C5 antibody excess.

We further observed lower surface sialic acid levels on $CD59^-$ RBCs than on $CD59^+$ RBCs from PNH patients. As sialic acids are present on most (if not all) glycosylated proteins, and on certain GPI anchors such as those attached to CD59(28) and to cellular prion protein(29), the absence of any GPI anchors or GPI-anchored proteins on PNH RBCs could explain the overall reduced surface levels of sialic acids on these cells. Interestingly, although sialic acids are established ligands of FH(26), our treatment of RBCs from PNH patients with neuraminidase removed most of the surface-bound sialic acids but paradoxically did not significantly reduce the level of surface-bound FH on these cells. These results, together with previous studies showing that affinity of sialic acids to FH is quite weak and that there is no direct evidence showing FH binds to sialic acids in physiological buffers(26, 30), suggest that although sialic acid may recruit FH in concert with C3b during complement activation to protect susceptible RBCs, other FH ligands, including C3b/iC3b/C3d, might be sufficient to bind FH to the RBC surface in the absence of sialic acids at the basal level. In support of this hypothesis, we indeed observed that in PNH patients treated with

eculizumab, the surface levels of FH were positively correlated with the levels of deposited C3b/iC3b/C3d on the affected $CD59^-$ RBCs that have low levels of sialic acids on their surface. Another possible explanation for this apparent discrepancy is that the sialic acids that FH binds were resistant to the neuraminidase-mediated removal because the bound FH served as a physical barrier denying the access of the neuraminidase while the other accessible sialic acids are removed, leading to overall decreased levels of sialic acids on the cell surface as we observed without affecting the surface levels of FH. The detailed underlying mechanism certainly warrants further studies.

To demonstrate that cell-surface bound FH on RBCs is a critical protector against complement-mediated hemolysis, we first established a model using RBCs from WT and CD55/CD59 double KO mice, and augmented the levels of FH on the double KO RBCs directly by painting the cells with purified human FH or indirectly by incubating heparin-painted RBCs with purified FH. Both approaches successfully increased the surface levels of FH on the CD55/CD59-deficient RBCs, and these modified RBCs exhibited increased resistance to complement-mediated hemolysis. Our experiments thus directly demonstrate an important role for surface FH as a protective agent against the hemolysis of CD55/CD59-deficient RBCs in this *in vitro* mouse model of PNH, and suggest that augmenting surface levels of FH on the RBCs might be another approach for treating PNH. In addition, given the difficulty in obtaining PNH patient samples, this convenient mouse model has value in evaluating novel therapeutics for PNH.

We also tested these FH painting procedures using affected $CD59^-$ RBCs purified from PNH patients. However, the direct painting of purified FH onto the enriched $CD59^-$ PNH RBCs exerted minimal protection on the PNH RBCs (data not shown), in contrast to our observations in mouse RBCs. Only the indirect two-step approach to FH augmentation protected the human RBCs from PNH patients against complement attacks. The different results observed between mouse and human cells might be attributed to the random pattern of FH binding to the RBC surface as a result of the direct painting approach; consequently, some (most) of the FH molecules are detectable by flow cytometry but are no longer functional. In contrast, the indirect approach enables the binding of all FH molecules to heparin on the RBC surface in a physiological manner, and the molecules retain their full complement-inhibiting function. Our results also clearly demonstrate the significant differences between mouse and human RBCs in human complement-mediated hemolysis assays, which may be related to differences in the characteristics of the RBC membrane between these species.

In conclusion, we discovered that FH can be detected on the surfaces of RBCs from PNH patients, with significantly lower levels of this factor present on the surfaces of the affected $CD59^-$ RBCs relative to the unaffected $CD59^+$ RBCs in these patients. Although the surface levels of sialic acids were also lower on $CD59^-$ RBCs than on $CD59^+$ RBCs, these sialic acids did not appear to contribute significantly to FH binding to the surfaces of these RBCs. Potentially, other FH ligands on RBCs, such as deposited C3b/iC3b/C3d, are also important for FH binding. We further demonstrated that augmenting the FH levels on the surfaces of CD55/CD59-deficient mouse or human RBCs provided significant protection against complement-mediated destruction. These new experimental data derived from both mouse

and PNH patient samples provide solid evidence demonstrating a critical role for surface-bound FH on RBCs in the pathogenesis of PNH, suggesting that augmenting FH levels on the RBCs would be another therapeutic approach for the management of PNH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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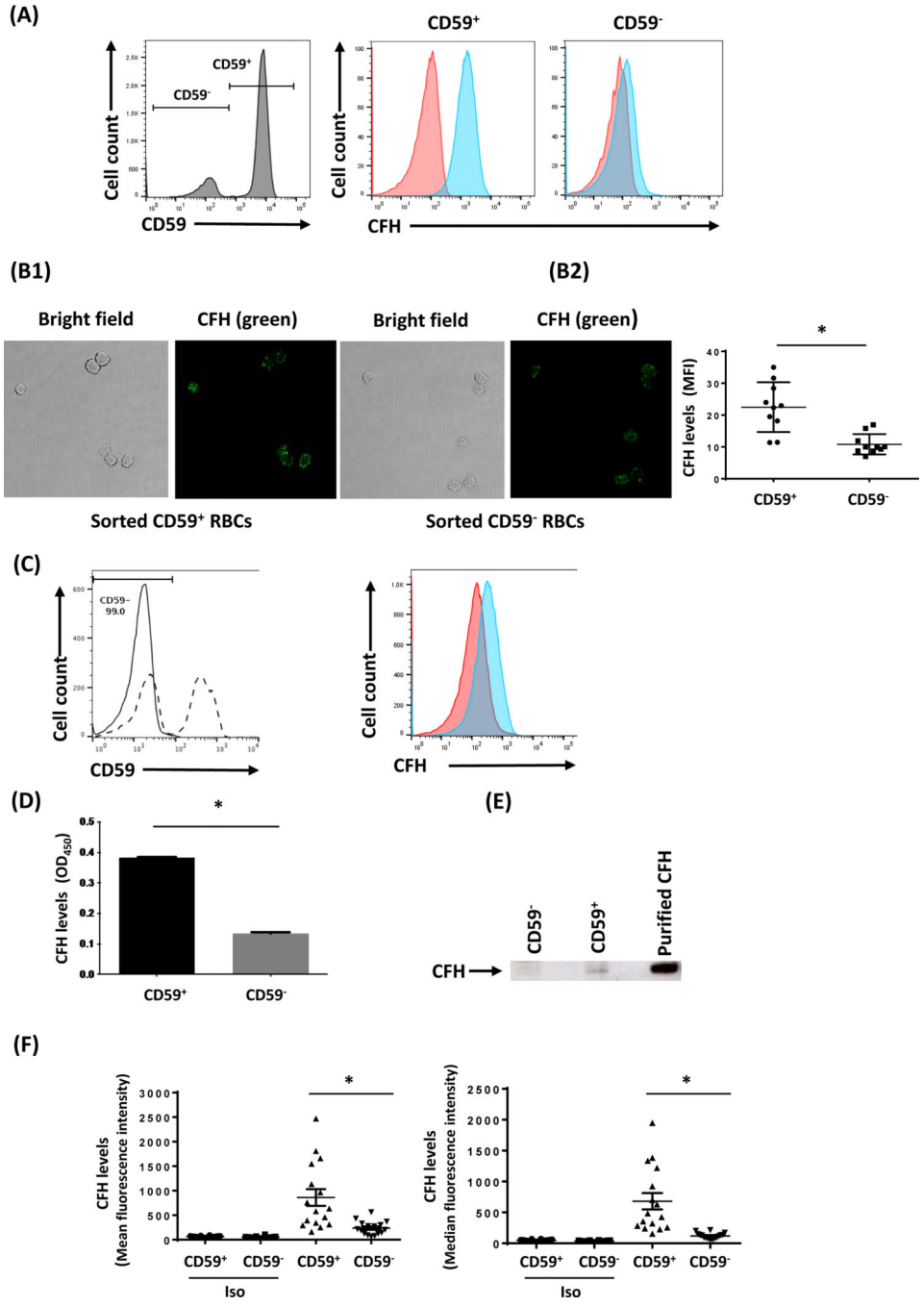

Figure 1. PNH RBCs exhibit reduced levels of surface FH.

The presence of FH on PNH patient RBCs and its levels were evaluated by different assays. **(A)** Surface FH on PNH patient RBCs were detected by a goat anti-human FH antibody and analyzed by a flow cytometer. red: isotype controls, blue: FH staining. **(B)** RBCs from the same patient in panel A were flow sorted according to their CD59 expression. Surface FH on the sorted $CD59^+$ or $CD59^-$ RBCs were detected by immunofluorescent staining followed by confocal microscopy analysis. FH mean fluorescence intensities (MFI) of 10 randomly selected $CD59^+$ and $CD59^-$ RBCs were analyzed. $p^*<0.05$, unpaired t-test**. (C)** $CD59^-$ RBCs

were first negatively purified using magnetic beads and the $CD59^-$ RBC purity was 99% (solid line). Dash line: original CD59 profile of PNH RBCs. FH levels on the purified $CD59^-$ RBCs (red) and normal RBCs (blue) from a healthy donor were evaluated by flow cytometry. FH levels in the cell lysates that were prepared from the same number of the purified $CD59^-$ RBCs and the normal RBCs are detected both by ELISA **(D)** and western blotting **(E)**. Iso: isotype controls. $p^*<0.05$, unpaired t-test. **(F)** Summary of surface levels of FH on affected ($CD59^-$) and unaffected ($CD59^+$) RBCs from PNH patients, presented by mean fluorescence intensities (left) and median fluorescence intensities (right) as quantitated by the flow cytometric analyses. Iso: isotype controls. N=16. $p^*<0.05$, one-way ANOVA.

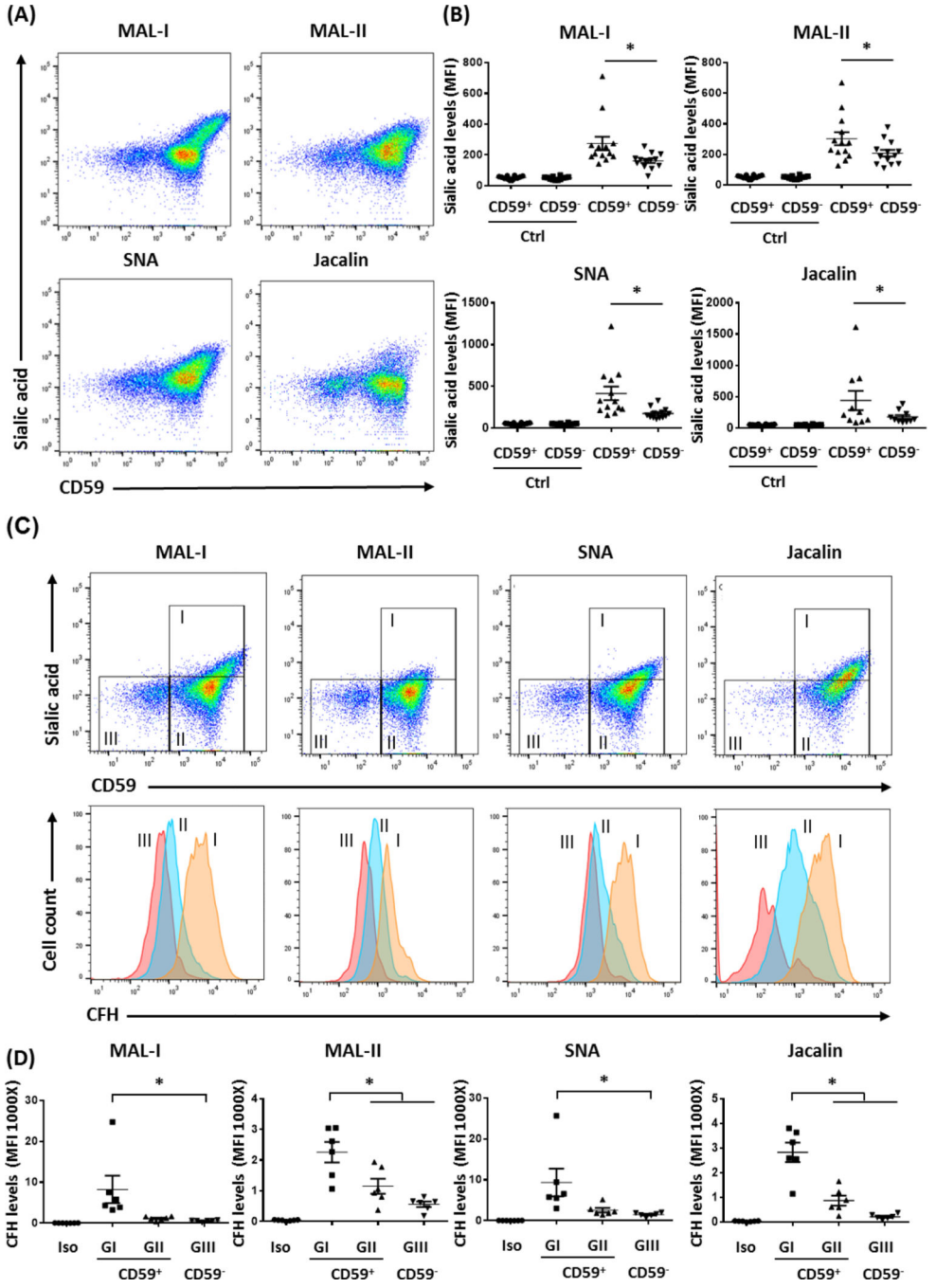

Figure 2. PNH RBCs exhibit reduced surface levels of sialic acids.

Levels of sialic acids on $CD59^+$ and $CD59^-$ RBCs from different PNH patients were examined by staining the cells with different lectins MAL-I, MAL-II, SNA or Jacalin followed flow cytometric analyses. **(A)** Representative results of lectin staining of RBCs from one PNH patient. **(B)** Surface sialic acid levels (median fluorescence intensities, MFI) from different patient samples as indicated by lectin staining. MAL-I, N=13. MAL-II, N=13. SNA, N=13. Jacalin, N=10. $p^*<0.05$, one-way ANOVA. **(C)** Representative results of lectin staining and their correlations with surface levels of FH on RBCs from one PNH patient.

The orange peak: $CD59^{+}$ Sialic acid ^{high} RBCs, Gate I. The blue peak: $CD59^{+}$, Sialic acid ^{low} RBCs, Gate II. The red peak: $CD59^{-}$ RBCs, Gate III. **(D)** Summary of surface FH levels (median fluorescence intensities, MFI) on different RBC populations of multiple PNH patients based on lectin staining. GI: Gate I. GII: Gate II. GIII: Gate III. Iso: isotype controls. N=6. $p^{*}<0.05$, one-way ANOVA.

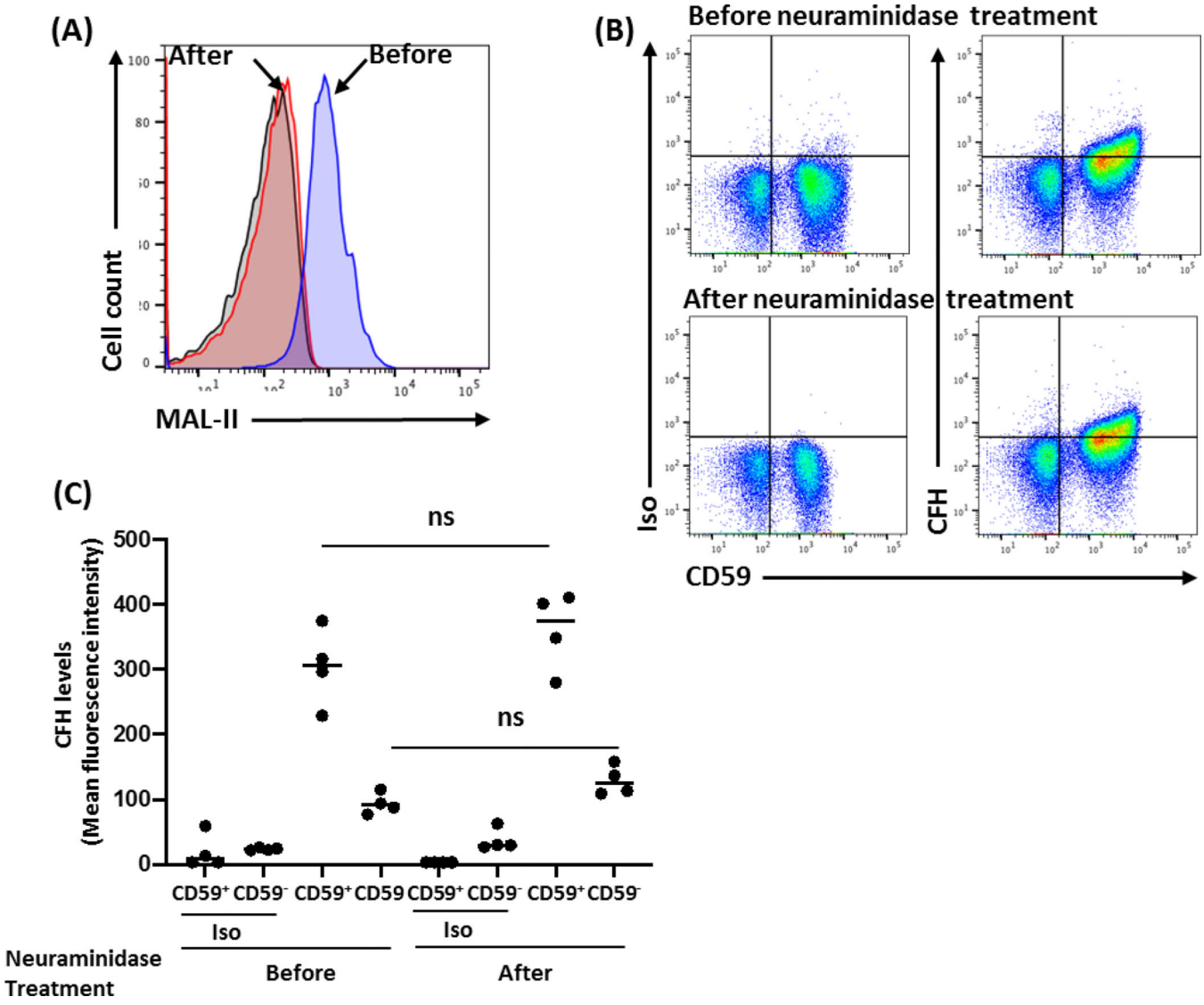

Figure 3. Enzymatic desialylation does not affect surface FH levels on PNH and normal RBCs.

(A) Surface sialic acid levels before (blue) and after (red) neuraminidase treatment were measured by staining with a biotinylated MAL-II followed by flow cytometry analyses. Black line: no staining control. **(B)** RBCs from PNH patients were treated with neuraminidase, and FH levels were measured by a goat anti-FH antibody and analyzed by flow cytometry. **(C)** Summary of surface levels of FH on neuraminidase-treated $CD59^-$ and $CD59^+$ RBCs. Iso, isotype controls. N=4. ns, no significance, one-way ANOVA.

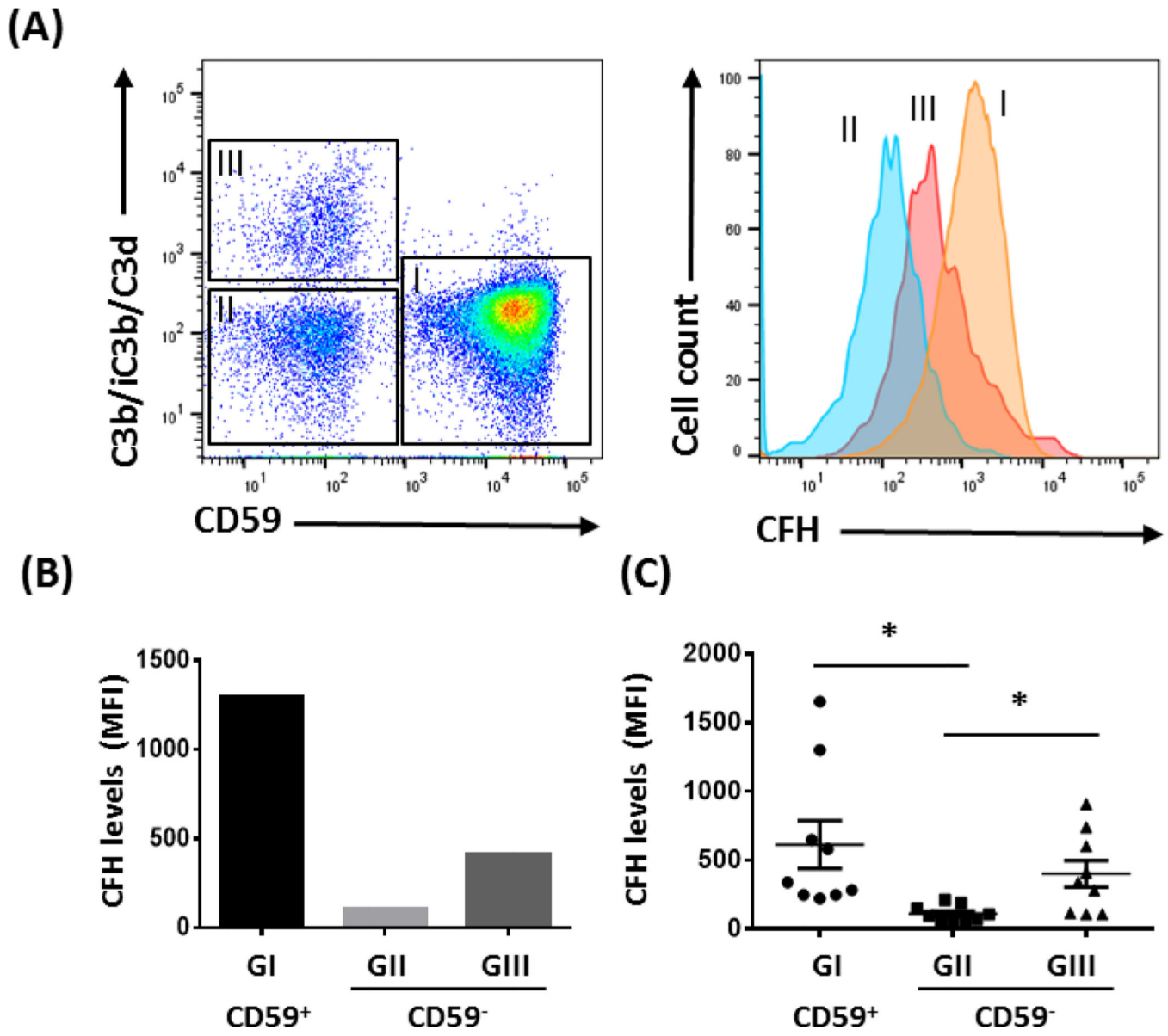

Figure 4. Correlation between the surface levels of FH and C3b/iC3b/C3d on RBCs of PNH patients under anti-C5 treatments.

Levels of FH and C3b/iC3b/C3d on RBCs of PNH patients with anti-C5 mAb treatments were detected by flow cytometry. **(A)** Representative figures of C3b/iC3b/C3d levels of one PNH patient. FH levels of different RBC populations were analyzed. The orange peak: $CD59^{+}$ RBCs, Gate I. The blue peak: $CD59^{-}$, $C3b/iC3b/C3d^{low}$ RBCs, Gate II. The red peak: $CD59^{-}$, $C3b/iC3b/C3d^{high}$ RBCs, Gate III. **(B, C)** FH median fluorescence intensities (MFI) of different RBC populations. GI: Gate I. GII: Gate II. GIII: Gate III. **(B)** N=1. **(C)** N=9. $p^{*}<0.05$, one-way ANOVA.

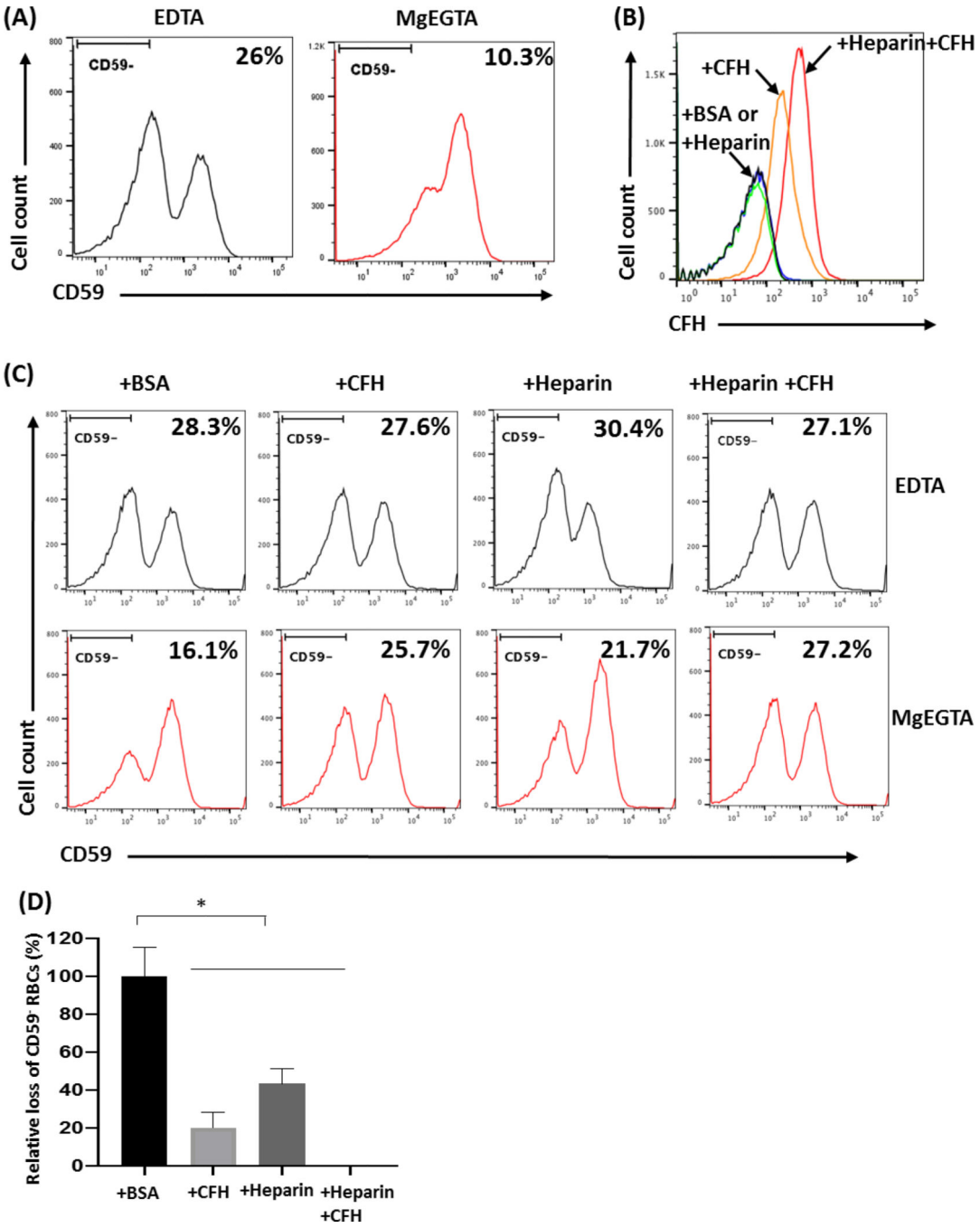

Figure 5. Augmentation of surface FH levels on RBCs from CD55/CD59 double KO mice protects against complement-mediated hemolysis.

(A) CD55/CD59 double KO mouse RBCs were mixed with wild type RBCs in a 7:3 ratio and were selectively lysed by NHS. The unlysed cells were evaluated by an anti-CD59 antibody and flow cytometry. The $CD59^-$ population% was reflected on the right corner. Black line, EDTA group that inhibits complement; red line, MgEGTA group with complement activation. **(B)** FH was painted to CD55/CD59 double KO murine RBCs directly through EDC or indirectly through heparin. FH levels were measured by a goat anti-

human FH antibody and flow cytometry. **(C)** The painted RBCs were mixed with RBCs from wild type mice in a 1:1 ratio. The mixed RBCs were incubated with NHS and MgEGTA (or EDTA) and the evaluation was performed as in **(A)**. **(D)** Calculation of loss of $CD59^-$ RBCs in each MgEGTA group in **(C)** relative to the BSA group.

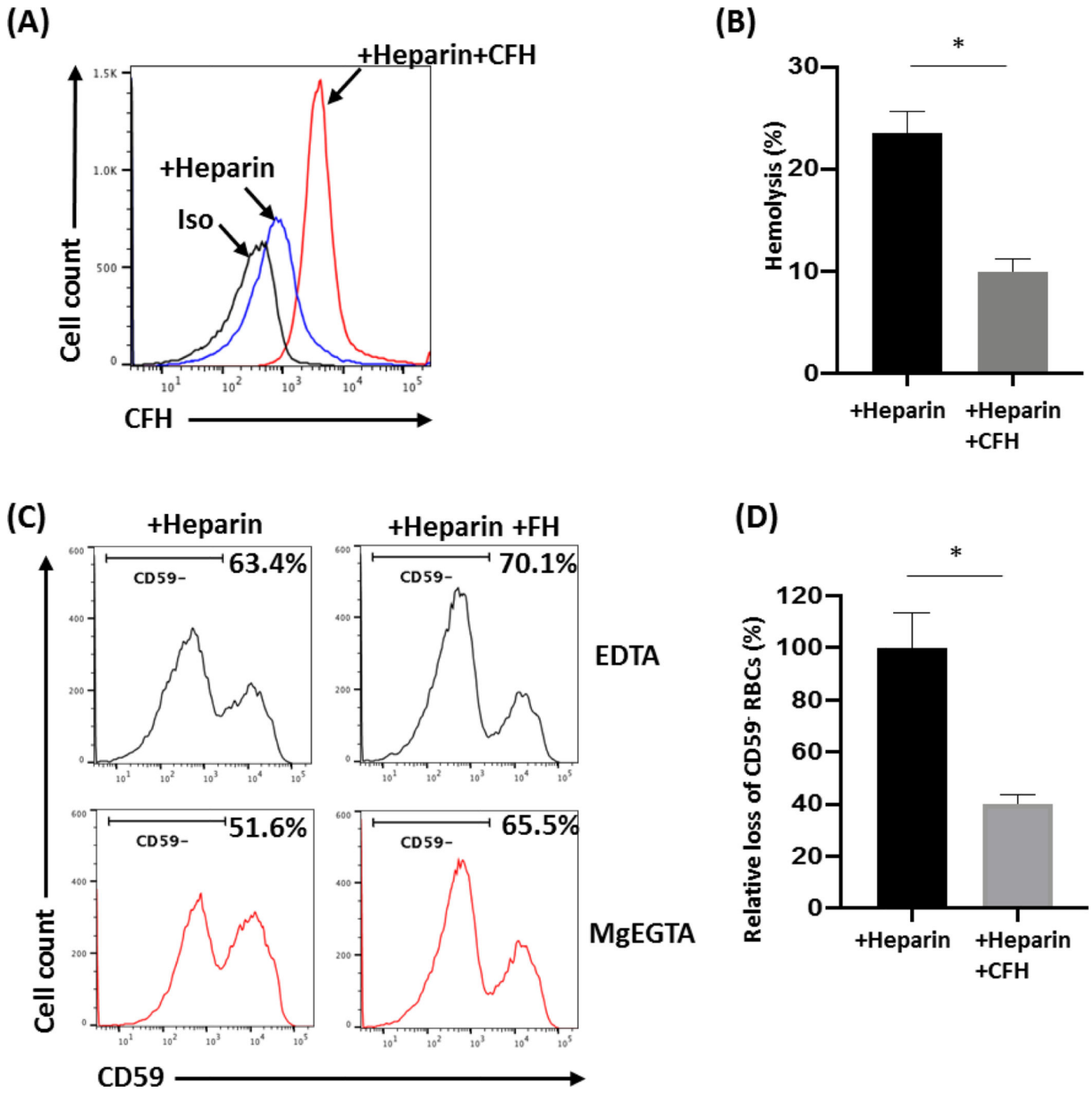

Figure 6. Augmentation of surface FH levels on human PNH RBCs protects against complement-mediated hemolysis.

(A) FH was painted to CD59$^-$ RBCs that were purified from one PNH patient indirectly through heparin. FH levels were measured by a goat anti-human FH antibody and flow cytometry. **(B)** The painted RBCs were mixed with RBCs from a healthy donor in a 7:3 ratio. The mixed RBCs were incubated with NHS and MgEGTA (or EDTA), and hemolysis was evaluated by measuring released hemoglobin in supernatant (OD_{414})**. (C)** The survived CD59$^-$ RBC were evaluated by an anti-CD59 antibody and flow cytometry. The remaining CD59$^-$ population% in each group was reflected on the right corner. Black line, EDTA group

that inhibits complement; red line, MgEGTA group with complement activation. **(D)** Calculation of loss of $CD59^-$ RBCs in +Heparin+FH (MgEGTA) group relative to +Heparin group. $p^*<0.05$, unpaired t-test.

Table 1

Information of patients studied in this project

ID	DX	Age	Gender	PNH WBC Clone (%)	PNH Type III Clone (%)	PNH Type II Clone (%)	Treated with Eculizumab
Patient2	AA/PNH	45	M	59.87	12.91	1.06	X
Patient8	AA/PNH	39	M	37.86	16.12	1.38	
Patient10	PNH	33	M	70.48	21.7	0.38	
Patient12	AA/PNH	33	M	82.87	5.91	42.43	
Patient18	PNH	19	M	NA	NA	NA	X
Patient19	AA/PNH	77	M	1.71	0.73	NA	
Patient20	PNH	25	M	91.19	13.12	40.75	
Patient21	PNH	34	M	81.54	14.87	3.5	X
Patient22	AA/PNH	38	F	98.05	9.08	3.21	X
Patient23	AA/PNH	32	M	66.07	17.13	0.97	X
Patient24	AA/PNH	71	M	58.86	10.41	0.82	
Patient25	AA/PNH	66	F	75.35	15.6	1.48	X
Patient27	AA/PNH	51	F	65.75	7.79	2.6	X
Patient36	PNH	56	F	74.41	23.33	0.94	X
Patient37	AA/PNH	21	F	98.77	15.19	5.39	
Patient38	PNH	31	F	97.15	21.56	9.3	X