scientific reports

Check for updates

OPEN

The specific contributions of factor H and factor I in controlling fluid phase activation of the alternative complement pathway

Nancy A. Turner✉ & Joel L. Moake

The immediate defense provided by the alternative complement pathway (AP) is under constant control by fluid phase regulators, factor H (FH) and factor I (FI), to prevent nonessential activation. Removal of either FI or FH from serum results in spontaneous AP activation, although the extent of activation caused by separate removal of each regulator has not been compared. In purified protein reactions with < 25% normal FH levels, additions of 6-100% normal FI levels, did not reduce C3a, Ba or FB cleavage. In reactions with 100% FH: C3a was not generated; Ba and FB cleavage was 3-fold lower; and C3 inactivation increased 2-fold as FI concentrations doubled. In reactions with 100% FI, FH levels ≥ 25% reduced C3a and Ba levels. AP activation levels were also compared in FI-depleted and FH-depleted serum. After magnesium addition to FI-depleted serum, C3a remained unchanged and Ba increased 3-fold, whereas in FH-depleted serum, C3a increased 13-fold and Ba increased 20-fold. Addition of 100% FI protein to FI-depleted serum did not change C3a and Ba, whereas, 100% FH added to FH-depleted serum prevented all activation. We conclude that normal levels of FH are sufficient to compensate for FI deficiencies and prevent unnecessary fluid phase AP activation.

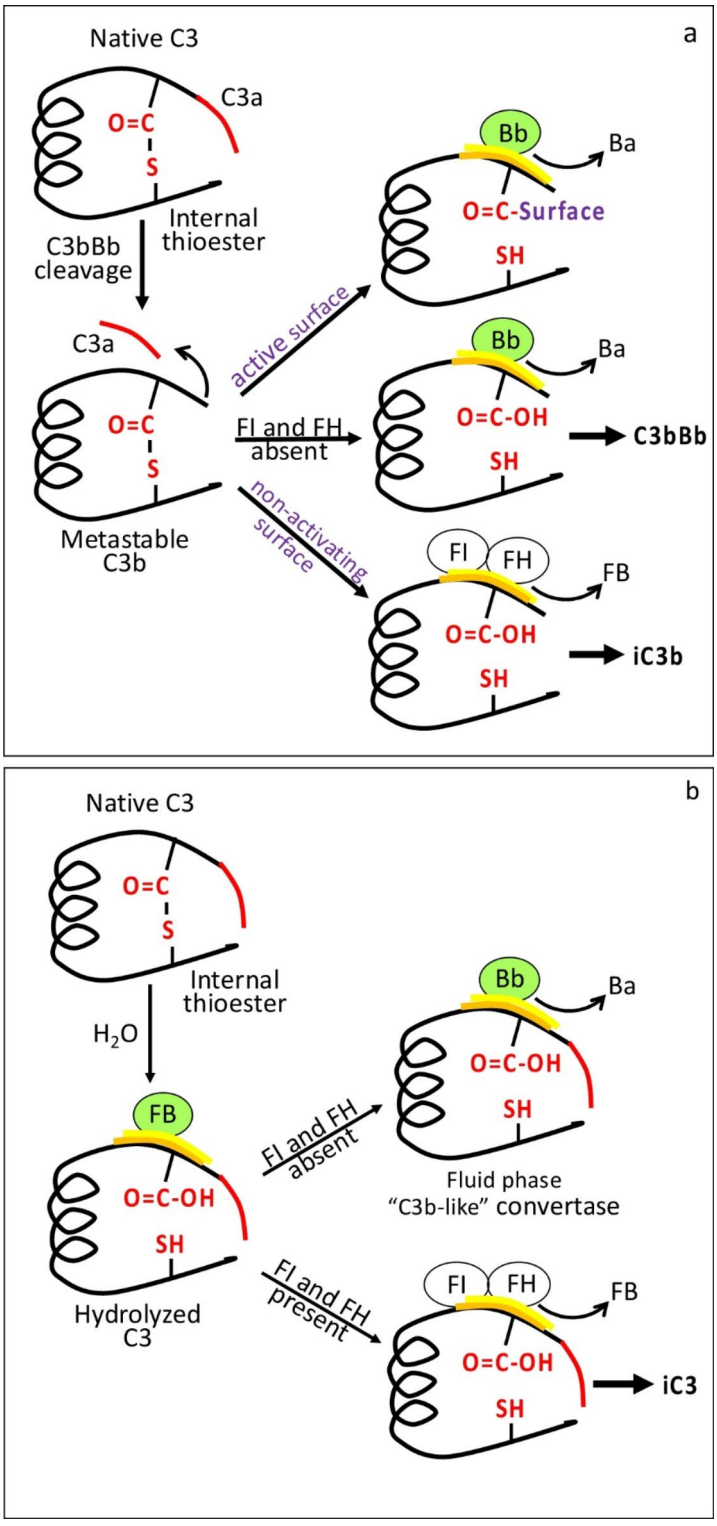
In plasma, the immediate defense against foreign proteins and microbes is provided by the alternate complement pathway (AP). The AP can also be activated inappropriately in response to harmless or host-produced proteins, under conditions of deficient or ineffective regulation by the AP fluid phase regulators, factor H (FH) and factor I (FI)[1,2]. FH is responsible for distinguishing between host and foreign proteins, whereas FI proteolytically inactivates C3 to prevent AP amplification.

After initiation of the AP, under conditions favorable for continued activation, amplification begins with factor B (FB) binding to cleave-activated C3b, generating C3bFB complexes. Factor D (FD) cleavage of FB releases Ba, and exposes the proteolytic domain of Bb to produce the fluid phase C3 convertase, C3bBb[3,4]. The Bb within C3 convertases proceeds to cleave additional C3 (releasing C3a) to generate C3b for further FB binding and escalate the formation of C3bBb complexes. C3b binds to surfaces through an internal thioester that is exposed in C3 after the release of C3a[5]. Surfaces with high densities of bound C3bBb complexes promote C5 binding and the formation of C5 convertases (C3bBbC3b). This induces the formation of terminal membrane attack complexes (C5b-9)[6].

The steady state of low AP activation depends on the regulatory functions of FI and FH. The internal thioester, within the a-chain of native C3, is exposed after the C3a domain is cleaved by C3bBb. This produces metastable C3b with surface-binding capabilities[3]. In the presence of an activator, Bb remains bound and C3b covalently binds to hydroxyl groups on the target surface. If FI and FH are absent, even without an activating protein, there is unrestricted formation of C3bBb complexes and uncontrolled AP activation. In the presence of FI and FH, along with the absence of a foreign target, FH preferentially displaces FB. Metastable C3b reacts with water and is quickly cleaved and inactivated by FI, producing iC3b (inactivated C3b) (Fig. 1a)[3].

An additional, distinct C3 inactivation fragment is also produced by the cleavage of C3 by FI, and cofactor FH, in the fluid phase. The hydrolysis of C3 occurs consistently, although at a slow rate[7]. Restricting hydrolyzed C3 [C3(H_2O)] from forming fluid phase C3(H_2O)-Bb convertases is a crucial regulatory function of FH and FI. The major distinction between C3bBb and C3(H_2O)-Bb complexes is that the later are functionally unable to bind to surfaces. The C3a domain remains intact in C3(H_2O) conformers concealing the internal thiol ester and, therefore, preventing any formation of ester bonds with surface proteins. In the absence of FI and FH, C3(H_2O) readily binds available FB, forming active fluid phase C3b-like convertases capable of cleaving C3 to

Department of Bioengineering, Rice University, Houston, TX, USA. ✉email: nturner@rice.edu



C3b. This leads to unrestricted AP activation. Under conditions of FH and FI regulation, FB is displaced by FH and C3(H_2O) is directly cleaved and inactivated by FI, resulting in iC3 (inactivated hydrolyzed C3) (Fig. 1b)[7,8].

In the fluid phase, FI is only functional as an AP regulator in the presence of FH[9]. Individuals with deficiencies in FI have low C3 levels and diminished FB levels. Recessive *CFI* mutations in individuals that result in complete FI deficiency have frequent infections; whereas patients with heterozygous *CFI* mutations have low levels of functional FI, and account for 4–8% of all atypical hemolytic uremic syndrome (aHUS) cases[10–12].

Individuals with moderate to severe reductions in functional FH have profound consumption of both C3 and FB, and regularly develop aHUS. *CFH* is the most frequently mutated gene in patients with aHUS, accounting for 20–30% of all aHUS cases. Patients with *CFH*-derived aHUS have the highest risk of developing kidney failure[12,13].

◀**Fig. 1**. Schematic diagram of fluid-phase activation and inactivation of C3. In the fluid phase, a conformer of C3 exists in a hydrolyzed configuration, structurally distinct from native C3, that is capable of participating in AP activation. (**a**) The internal thioester within the alpha-chain of native C3 is exposed after cleavage and release of C3a by the C3 convertase (C3bBb), producing metastable C3b. Three possibilities exist for the short-lived metastable C3b. In the presence of an activating surface, C3b covalently binds to hydroxyl groups on the target surface. If FI and FH are absent, there is formation of the fluid-phase C3 convertase (C3bBb) and the potential of uncontrolled AP activation. In the absence of a foreign target, C3b reacts with water and is quickly cleaved and inactivated by FI, with co-factor FH, producing iC3b. (**b**) Water hydrolysis of the internal thioester converts native C3 to hydrolyzed C3 [C3(H_2O)], a functionally C3b-like protein with FB binding capacity without previous cleavage of C3a. In the absence of FI and FH, C3(H_2O) binds FB, and after FB cleavage by FD, forms an active fluid phase C3 convertase capable of cleaving C3 to C3b, although unable to bind to surfaces because the thioester remains unexposed. In the presence of both FI and FH, FB is displaced by FH and C3(H_2O) is directly cleaved and inactivated by FI, resulting in iC3. Adapted from Pangburn and Muller-Eberhard[3].

Reaction proteins	Reaction concentrations, ng/µl				
	C3	FB	FD	FH	FI
C3, FB, FD, FH, FI	60	10	0.07	0, 1.5–25	0, 0.1–1.8

Table 1. Composition of purified protein reactions and range of protein concentrations in reactions. In additional control reactions, FD was omitted to assure specific AP cleavage of FB (Supplementary Table S1, Supplementary Fig. S2).

Reaction concentrations		% Normal serum levels
FH, ng/µl	FI, ng/µl	
25	1.8	100
12.5	0.9	50
6.25	0.4	25
3.1	0.2	12.5
1.5	0.1	6.25

Table 2. Protein concentrations of FI and FH in AP reactions using purified proteins and the corresponding normal serum level percentage.

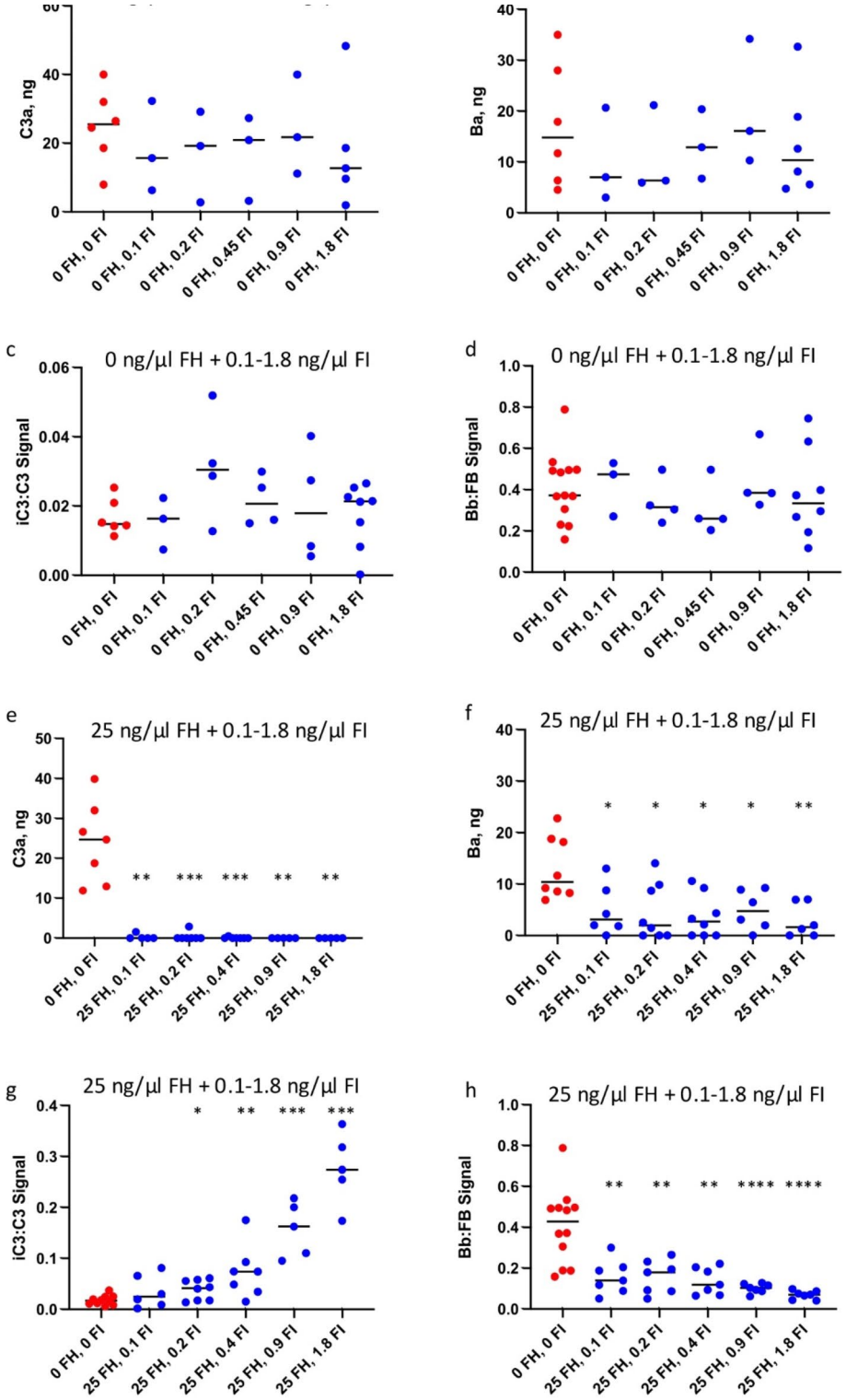
FI and FH function in tandem to inhibit unprovoked AP activation in the fluid phase, in order to prevent C3b deposition on host cells and avert terminal complex formation. Depletion of either one of these fluid phase regulators results in spontaneous AP activation[14–16]. In this study, AP activation was measured in fluid phase reactions over a range of FI and FH concentrations. These data enabled us to determine the combination of FI and FH levels necessary to prevent unprompted activation. The extent of spontaneous AP activation was also compared in serum that had been immunodepleted of either FI or FH.

Results

AP activation in fluid-phase reactions using purified proteins

AP reactions and activation determinants. AP activation products, C3a and Ba, and the extent of C3 inactivation and FB cleavage were measured in reactions composed of C3, FB, FD, FI, and FH proteins in Mg-EGTA buffer after 5 min at 22°C. Concentrations of C3 (60 ng/µl), FB (10 ng/µl), and FD (0.07 ng/µl) were constant at normal serum ratios, whereas concentrations of FI and FH were varied at 0, 6, 12, 25, 50, and 100% of normal serum levels (Tables 1 and 2). Determinants of activation were measured by Western blotting techniques. Levels of C3a and Ba were measured quantitatively by interpolation of standard curves, as previously described[17]. The extent of C3 inactivation was measured by the combined band intensities of C3 cleavage fragments (73 and 63 kD) divided by the combined band intensities of the alpha-chains from intact native C3 and C3(H_2O) (115 kD) (iC3:C3 signal ratio) (Supplementary Figs. S1, S2). FB cleavage was calculated by the band intensity of Bb (60 kD) divided by the band intensity of intact FB (93 kD) (Bb: FB signal ratio) (Supplementary Fig. S2).

Reactions using the range of FI concentrations without the addition of FH. In the absence of FH, similar amounts of C3a (18.9 ± 13.3 ng) were produced in AP reactions at each FI concentration (range 0.1–1.8 ng/µl), as were produced without either FI or FH (26.7 ± 14 ng) (Fig. 2a, Supplementary Table S2). Similarly, the Ba amounts generated without FI and FH (14 ± 8.8 ng) were within the range of Ba produced at each FI concentration (13.7 ± 9.3 ng) (Fig. 2b, Supplementary Table S2). In the absence of FH, the ratios of C3 inactivation (0 FH, 0 FI = 0.017 ± 0.007 vs. 0 FH, FI range = 0.021 ± 0.011) and FB cleavage (0 FH, 0 FI = 0.38 ± 0.18 vs. 0 FH, FI range = 0.37 ± 0.16) were also not affected by the FI concentration (Fig. 2c, d, Supplementary Table S2).



Reactions with 100% FH and the range of FI concentrations. In contrast to reactions without FH, only negligible amounts of C3a were generated in reactions containing 25 ng/µl FH (100%) at each FI concentration (25 FH, 0 FI = 0.66 ± 0.8 ng and 25 FH, FI range = 0.17 ± 0.60 ng) (Fig. 2e, Supplementary Table S2). In the presence of 100% FH and increasing concentrations of FI (6–100%), Ba levels were 3- to 5-fold lower (3.8 ± 4.3 ng) than in reactions without FH and FI (14 ± 8.8 ng) (Fig. 2f, Supplementary Tables S2, S3, Supplementary Fig. S2). Compared to reactions without FH and FI, C3 inactivation in reactions with 100% FH increased 2-fold with 12% FI, 4-fold with 25% FI addition, 9-fold with 50% FI addition, and 16-fold with 100% FI addition (Fig. 2g, Supplementary Table S3). Compared to the extent of FB cleavage in reactions in the absence of both FH and FI (0.38 ± 0.18), FB cleavage in reactions with 100% FH was 2-fold lower with FI additions of 6%, 12% and 25%, 3.5-fold lower with 50% FI, and 5-fold lower with 100% FI (Fig. 2h, Supplementary Tables S2, S3).

◀**Fig. 2**. AP activation in purified protein reactions with increasing FI concentrations in the absence of FH or presence of 100% FH. Activation in reactions using purified proteins C3, FB, FD in either the absence of FH or with 100% FH (25 ng/µl) and increasing FI concentrations (0.1–1.8 ng/µl) was measured by Western blotting. Measurements in (**a**-**d**) are from reactions in the absence of FH for: (**a**) C3a; (**b**) Ba; (**c**) C3 inactivation and (**d**) FB cleavage. Measurements in (**e**-**h**) are from reactions with 100% FH for: (**e**) C3a; (**f**) Ba; (**g**) C3 inactivation; and (**h**) FB cleavage. Graphs show individual values with the median indicated by the horizontal bar from the analysis of 3–12 reactions of each composition. Statistical significance is shown for values (blue circles) compared to measurements in reactions without either FH or FI (red circles). In (e) C3a: 0 FI vs. 0.1, 0.9, 1.8 ng/µl FI, $P = 0.0025$; 0 FI vs. 0.2 and 0.4 ng/µl FI, $P = 0.0006$; in (f) Ba: (0 FI vs. 0.1 ng/µl FI, $P = 0.0426$; 0 FI vs. 0.2 ng/µl FI, $P = 0.0373$; 0 FI vs. 0.4 ng/µl FI, $P = 0.0103$; 0 FI vs. 0.9 ng/µl FI, $P = 0.0293$; 0 FI vs. 1.8 ng/µl FI, $P = 0.0027$; in (g) C3 inactivation: 0 FI vs. 0.2 ng/µl FI, $P = 0.0343$, 0 FI vs. 0.4 ng/µl FI, $P = 0.0046$, 0 FI vs. 0.9, 1.8 ng/µl FI, $P = 0.0007$; and in (h) FB cleavage: 0 FI vs. 0.1 ng/µl FI, $P = 0.0026$, 0 FI vs. 0.2 ng/µl FI, $P = 0.0052$, 0 FI vs. 0.4 ng/µl FI, $P = 0.0018$, 0 FI vs. 0.9, 1.8 ng/µl FI, $P < 0.0001$.

Reactions with 6% or 12% FH and the range of FI concentrations. Additions of either 1.5 ng/µl FH (6%) or 3.1 ng/µl FH (12%) to reactions at each FI concentration tested did not reduce C3a, Ba or FB cleavage levels from the baseline levels measured in reactions without FH and FI (Supplementary Fig. S3, Supplementary Tables S2, S3). However, C3 inactivation was increased 2-fold with 0.9 ng/µl FI (50%) and 4-fold with 1.8 ng/µl FI (100%) in reactions with 6% FH (Supplementary Tables S2, S3, Supplementary Fig. S3).

Reactions with 25% or 50% FH and the range of FI concentrations. C3a levels in reactions with 6.25 ng/µl FH (25%) at FI concentrations 0.4, 0.9, and 1.8 ng/µl were 6.6 ± 2.5, 11.1 ± 7.0, 7.8 ± 7.8 ng, respectively. These significantly lower levels were 2.4- to 4-fold lower than C3a levels in reactions without FH and FI (26.7 ± 14 ng) (Fig. 3a, Supplementary Tables S2, S3, S4, Supplementary Fig. S4). In reactions with 50% FH, C3a levels were reduced 4.9-fold with 0.1 ng/µl FI (5.5 ± 6.2 ng) and reduced 7.3- to 11.4-fold with 0.2–1.8 ng/µl FI) (Fig. 3b, Supplementary Tables S2, S3, S4, Supplementary Fig. S4). Compared to baseline Ba levels (14 ± 8.8 ng), additions of 50% FH reduced Ba levels 2.7-fold in reactions containing 50% FI and 3.3-fold in reactions with 100% FI (Fig. 3c, d, Supplementary Tables S2, S3, Supplementary Fig. S4).

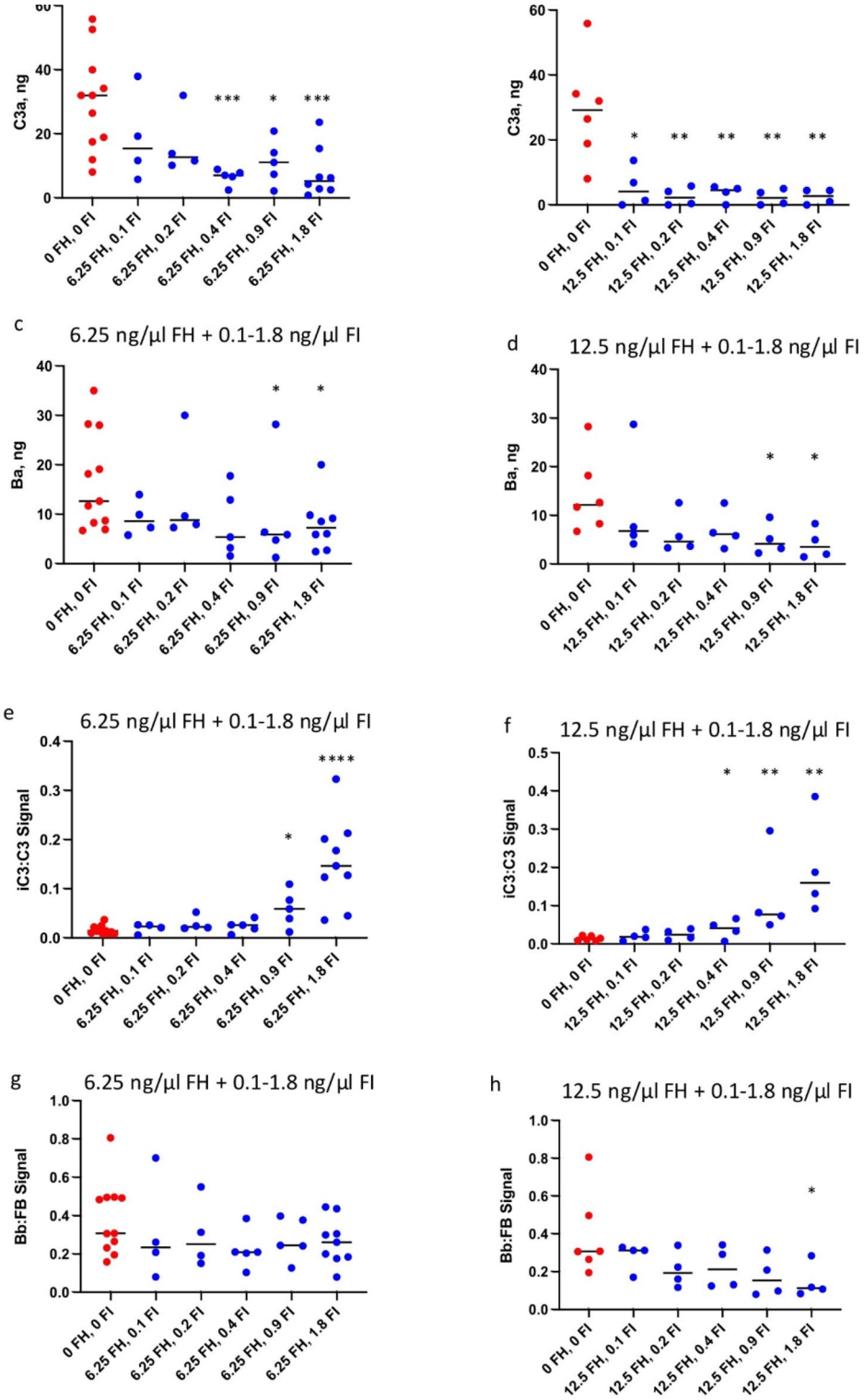
The extent of C3 inactivation with the addition of 6.25 ng/µl FH (25%) was increased 3.5-fold in reactions with 50% FI, and 9.1-fold in reactions with 100% FI, over baseline levels of C3 inactivation (Fig. 3e, Supplementary Table S3). In reactions with the addition of 50% FH, the C3 inactivation ratios were 2.3-fold higher with 25% FI, 7.4-fold higher with 50% FI and 11.7-fold higher with 100% FI (Fig. 3f, Supplementary Table S3, Supplementary Fig. S4). The extent of FB cleavage in reactions containing 25% FH was similar to baseline levels at each FI concentration (0 FH, 0 FI = 0.38 ± 0.18 vs. 6.25 FH, FI range = 0.27 ± 0.14) (Fig. 3g, Supplementary Table S2). In reactions with 50% FH added to the range of FI concentrations, FB cleavage was only significantly reduced at the highest level of FI (2.6-fold lower with 100% FI) (Fig. 3h, Supplementary Tables S3, S4, Supplementary Fig. S4).

Reactions with 100% FI and the range of FH concentrations. The C3a generated in the absence of both FI and FH (26.7 ± 14 ng) was substantially reduced in reactions with 100% FI (1.8 ng/µl) with FH additions above 25%. The addition of 25% FH reduced C3a amounts 3.4-fold (7.8 ± 7.8 ng), the addition of 50% FH reduced C3a amounts 10.7-fold (2.5 ± 2.3 ng), and with the addition of 100% FH, there was no detection of C3a generation (Fig. 4a, Supplementary Tables S2, S3, S4). In the presence of 100% FI, addition of 50% FH reduced Ba amounts 3.3-fold (4.2 ± 3.1 ng) and addition of 100% FH reduced Ba amounts 4.9-fold (2.9 ± 3.3 ng) from amounts produced in reactions without FH and FI (14 ± 8.8 ng) (Fig. 4b, Supplementary Tables S2, S3, S4). There was increased C3 inactivation with 100% FI at each FH concentration. C3 inactivation increased 4-fold with 6–12% FH, 9.1-fold with 25% FH, 11.7-fold with 50% FH, and 16.3-fold with 100% FH (Fig. 4c, Supplementary Table S3). In reactions with 100% FI, the extent of FB cleavage was reduced 2.6-fold with addition of 50% FH and 5.5-fold with addition of 100% FH, compared to the FB cleavage in reactions in the absence of both FH and FI (Fig. 4d, Supplementary Table S3).

The correlation of hydrolyzed C3 and AP activation measurements. The extent of C3 inactivation was determined by band intensity ratios of iC3 fragments and intact C3 (Supplementary Figs. S1, S2). The value for iC3 in the iC3:C3 ratio is predominantly the 73-kD band intensity derived from the FI-mediated cleavage of $C3(H_2O)$, whereas the denominator is the combined band intensities from the 115-kD alpha-chains of native C3 and $C3(H_2O)$. (Activation product, C3a, results exclusively from the convertase cleavage of native C3.) In order to determine if the amount of C3 inactivation, derived primarily from $C3(H_2O)$, was directly related to the C3a, Ba and FB cleavage produced by the native C3 convertase, the data were analyzed statistically using Pearson's correlation coefficient. The analysis showed that the increased amounts of C3a, Ba, and FB cleavage were highly correlated with the decreased C3 inactivation resulting from reactions with 100% FI and decreasing FH concentrations (Fig. 4e, f, g). There was also a strong linear association of C3 inactivation and FB cleavage in reactions with 100% FH and increasing reaction concentrations of FI (Fig. 4h).

AP activation in fluid-phase reactions using human serum depleted of FI or FH

Characterization of FI-depleted (FI-D) serum and FH-depleted (FH-D) serum. The absence of FI protein in FI-D serum was verified by Western blotting. The blots were analyzed for FI, and also for the abundant serum protein, vitronectin[18]. Simultaneous detection of vitronectin allowed the serum to be visualized in the lanes that appeared blank for FI detection (Supplementary Fig. S5). The absence of FH protein in FH-D serum was shown previously using the same techniques[17]. Blot analysis was also used to determined levels of FH in FI-D serum, FI in FH-D serum and FB levels in both depleted serum types. FH-D serum contained 30.5 ng/µl FI and 252 ng/µl FB; and



FI-D serum had 262 ng/µl FH and 242 ng/µl FB (Supplementary Fig. S6). Normal ranges from recent reports are: 338–462 ng/µl FH, 19–100 ng/µl FI, and 70–317 ng/µl FB[11,19,20].

C3a and Ba produced in FI-D serum and FH-D serum over 30 min. Levels of activation products C3a and Ba were measured in FI-D or FH-D serum at baseline and at 5 min intervals for 30 min after the addition of Mg-EGTA buffer. Initial C3a amounts were the same in both types of depleted serum (FI-D = 0.3 ± 0.44 ng, FH-D = 0.27 ± 0.41 ng) (Supplementary Table S5). The addition of magnesium ions to FI-D serum did not substantially increase C3a levels at any time point. In contrast, C3a levels in FH-D serum after activation reached levels (5.7 ± 3.1 ng) that were 20-fold higher than initial values. For comparison, C3a levels measured 5 min after activation, were 8-fold higher in FH-D serum (4.6 ± 2.3 ng) than in FI-D serum (0.58 ± 0.61 ng) (Fig. 5a, Supplementary Table S5).

◀**Fig. 3**. AP activation in purified protein reactions with either 25% or 50% FH and increasing FI concentrations. Activation in reactions using purified proteins C3, FB, FD with either 25% FH (6.25 ng/µl) or 50% FH (12.5 ng/µl) and increasing FI concentrations (0.1–1.8 ng/µl) was measured by Western blotting. Graphs show measurements for: (**a**) C3a with 25% FH (**b**) C3a with 50% FH; (**c**) Ba with 25% FH; (**d**) Ba with 50% FH; (**e**) C3 inactivation with 25% FH; (**f**) C3 inactivation with 50% FH; (**g**) FB cleavage with 25% FH; and (**h**) FB cleavage with 50% FH. Graphs show individual values with the median indicated by the horizontal bar from 4–11 reactions of each composition. Statistical significance is shown for values (blue circles) compared to measurements in reactions without either FH or FI (red circles). In (a) 25% FH: 0 FI vs. 0.4 ng/µl FI, $P = 0.0009$; 0 FI vs. 0.9 ng/µl FI, $P = 0.0181$, 0 FI vs. 1.8 ng/µl FI, $P = 0.0007$; in (b) 50% FH: 0 FI vs. 0.1 ng/µl FI, 0 FI vs. 0.2, 0.4, 0.9 and 1.8 ng/µl FI, $P = 0.0095$; in (c) 25% FH: 0 FI vs. 0.9 ng/µl FI, $P = 0.0381$, 0 FI vs.1.8 ng/µl FI, $P = 0.0409$; in d) 50% FH: 0 FI vs. 0.9, 1.8 ng/µl FI, $P = 0.0381$; in (e) 25% FH: 0 FI vs. 0.9 ng/µl FI, $P = 0.0133$, 0 FI vs. 1.8 ng/µl FI, $P < 0.0001$; in (f) 50% FH: 0 FI vs. 0.4 ng/µl FI, $P = 0.0286$, 0 FI vs. 0.9, 1.8 ng/µl FI, $P = 0.0095$; and in (h) 50% FH: 0 FI vs. 1.8 ng/µl FI, $P = 0.0381$.

Unlike initial C3a levels, baseline Ba amounts in FI-D serum (1.8 ± 1 ng) were 5-fold higher than initial Ba amounts in FH-D serum (0.37 ± 0.49 ng) (Supplementary Table S5). Following magnesium addition, Ba levels in FI-D serum increased 2.7-fold after 5 min and were 3.5-fold higher after 30 min. After activation in FH-D serum, Ba levels had increased 16-fold by 5 min and were 20-fold higher than initial levels at later time points. Overall, the levels of Ba in activated FH-D serum were 30% higher than the Ba levels in FI-D serum after activation (Fig. 5b, Supplementary Table S5).

C3a and Ba produced in FI-D and FH-D serum in the presence of heat-inactivated FB. FB purified from human plasma was inactivated by controlled heating to produce heat-inactivated FB (HFB)[17]. The heating process impaired the enzymatic function of HFB, although the binding capacity of HFB to C3b and C3(H_2O) was preserved. HFB inhibits AP activation by competing with FB for binding to C3b and C3(H_2O) and forming complexes incapable of supporting further activation. HFB was added to the depleted serum prior to the addition of Mg-EGTA buffer. The final concentration of HFB was 2.4-fold higher than FB levels present in each serum type (FI-D serum: 252 ng/µl FB; and FH-D serum: 242 ng/µl FB; Supplementary Fig. S6).

The sub-nanogram C3a levels in FI-D serum increased slightly with HFB addition, whereas the Ba amounts generated with HFB addition were similar to the amounts generated in FI-D serum alone (Fig. 5c, d, Supplementary Table S5). In contrast, the addition of HFB to FH-D serum suppressed generation of both C3a and Ba. Five min after activation, C3a levels in FH-D serum alone had increased 17-fold, whereas in the FH-D serum containing HFB, C3a levels were only 5.6-fold higher than initial values (Fig. 5e, Supplementary Table S5). Baseline Ba levels were increased 12-fold in FH-D serum with HFB (compared to a 16.2-fold increase in FH-D serum alone) after 5 min of activation. Generally, Ba levels in FH-D serum in the presence of HFB were 2-fold lower (although not statistically significant) than in FH-D serum alone at each time point (Fig. 5f, Supplementary Table S5).

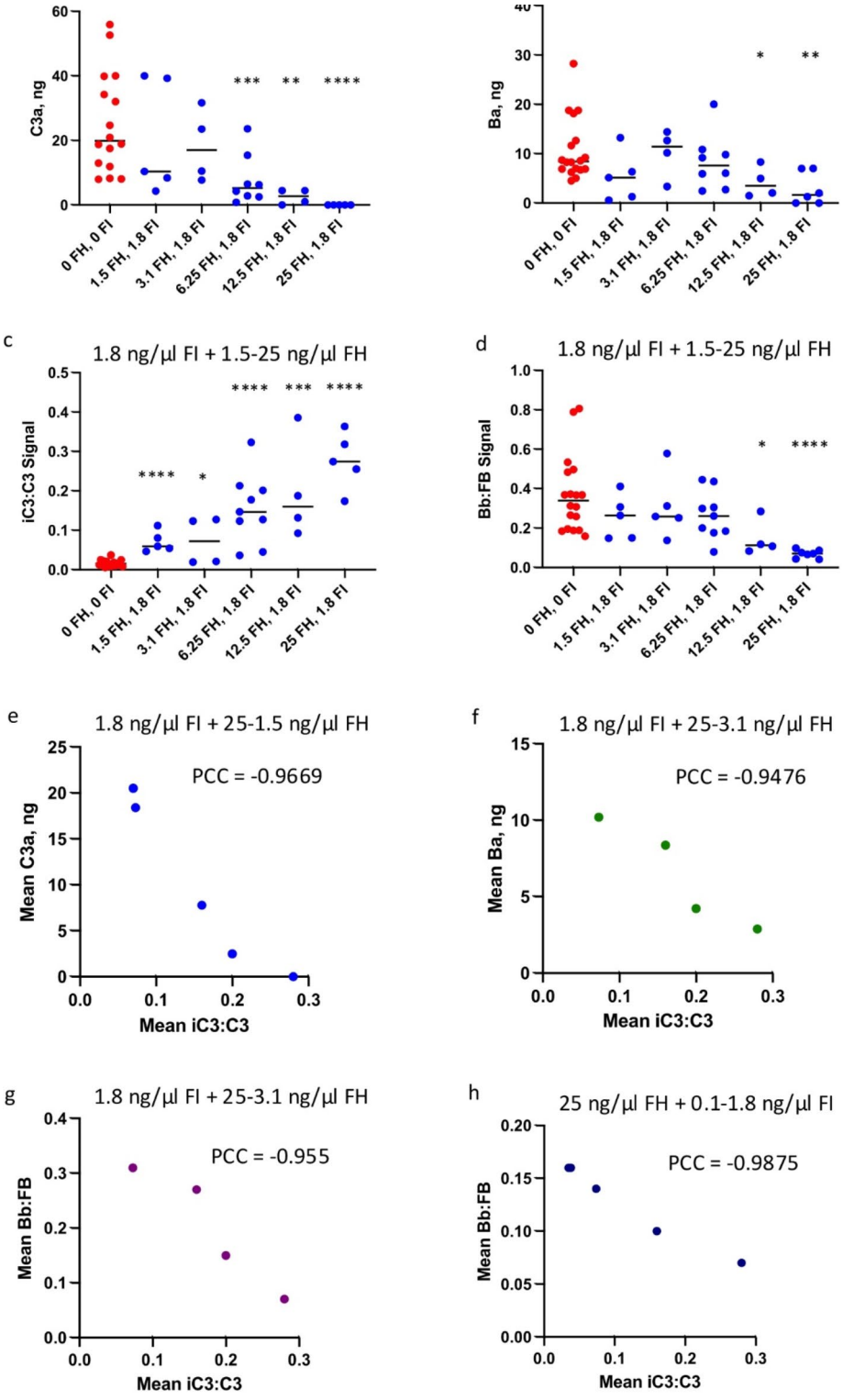
Additions of 100% FI to FI-D serum and 100% FH to FH-D serum. AP activation was measured at 5 min intervals over 15 min in each depleted serum type with and without the addition of 100% of the appropriate depleted protein. The addition of 100% FI (1.8 ng/µl) to the FI-D serum did not significantly lower production of C3a or Ba (Fig. 6a, c, Supplementary Table S6). In FI-D serum alone, activation increased FB cleavage 20.4-fold at 5 min, 25.5-fold at 10 min, and 35.7-fold at 15 min. The addition of 100% FI protein to the FI-D serum reduced FB cleavage by 2-fold at each point (Fig. 6e, Supplementary Table S6).

Unlike FI-D serum, the addition of 100% FH (25 ng/µl) protein to the FH-D serum clearly reduced each parameter of AP activation. Levels of C3a, Ba and FB cleavage in FH-D serum with FH addition were unchanged from initial values at each time point after activation (Fig. 6b, d, f, Supplementary Table S6). C3a levels were 4-fold lower in FH-D serum with the added FH, than in FH-D serum alone, after 5 and 10 min of activation (Fig. 6b, Supplementary Table S6). Ba amounts were 13-fold lower at 5 min, and 4.8-fold lower at later time points with FH addition (Fig. 6d, Supplementary Table S6). The extent of FB cleavage initially measured in FH-D serum increased substantially (> 400-fold) after activation at each time point. The FB cleavage was completely prevented with the addition of 100% FH protein to FH-D serum. In the FH-D serum with FH, the FB cleavage was 52-fold lower at 5 min, 72-fold lower at 10 min, and 69-fold lower at 15 min than in FH-D serum alone at these time points (Fig. 6f, Supplementary Table S6).

Discussion

Depletion of either FH or FI from magnesium-containing serum has been known to result in spontaneous AP activation. However, the extent of activation caused by the separate removal of each regulator has not previously been compared[14–16].

The discovery of FI and its role in controlling AP activation was a paramount advance and prompted the "tick-over" mechanism of continuous C3b production and its regulated breakdown[15]. FI deficiency was first recognized in a patient with frequent infections, low C3 levels and sustained C3b production[21]. The absent protein was identified as FI (known at the time as KAF) by an antibody that had been recently developed by Lachmann, et al.[22] Subsequently, the investigators immunodepleted normal serum of FI using F(ab')2 fragments of the FI antibody, and found that after the serum was rewarmed and replenished with Ca^{+2}/Mg^{+2}ions, spontaneous complement activation occurred. Using the available techniques, AP activation was confirmed by full conversion of FB to Bb and conversion of C3 to an inactive lower molecular weight form. These experimental results were interpreted as a demonstration that the instigation of the AP was continuous, did not require a specific activator, and would be fully activated if an inhibitor (FI) was removed[15]. This suggested mechanism for



continuous, controlled AP activation was further refined by the convincing experimental evidence of Pangburn, et al., who proposed that the hydrolysis of the presumed thioester within C3 could produce a conformer of C3 capable of binding FB and initiating the AP[3,7].

After its initial discovery, FI deficiency was also identified in additional patients with recurring severe infections[23–25]. Radial immunodiffusion assays revealed the absence of FI, severely reduced C3 and FB levels and circulating C3b. In 1994, Vyse et al. reported four new individuals with FI deficiency, bringing the worldwide total to 23 documented cases[26]. This account included a tabulated summary of previously reported FI deficient patients. In the cases where FH levels were also evaluated, 9 out of 9 individuals with FI deficiency had FH values of ~ 50% of normal levels.

In more recent reports of FI deficiency, the combined analysis of 13 patients, evaluated for complement disorders after multiple severe infections, also revealed a high frequency of reduced FH levels. In addition to

◀**Fig. 4**. AP activation measured in purified protein reactions with 100% FI and increasing concentrations of FH and correlated with C3 inactivation. Levels of activation in reactions of purified proteins C3, FB, FD containing 100% FI (1.8 ng/µl) and increasing concentrations of FH (1.5–25 ng/µl) were measured by blot analysis. Graphs in (**a**-**d**) show individual values of (**a**) C3a; (**b**) Ba; (**c**) C3 inactivation; and (**d**) FB cleavage from the analysis of 4–16 reactions of each composition. The horizontal bars indicate the median. Statistical significance in (a-d) is shown for values (blue circles) compared to measurements in reactions without either FH or FI (red circles). In (a) C3a: 0 FH vs. 6.25 ng/µl FH, $P = 0.0013$, 0 FH vs. 12.5 FH, $P = 0.0004$, 0 FH vs. 25 ng/µl FH, $P < 0.0001$; in (b) Ba: 0 FH vs. 12.5 ng/µl FH, $P = 0.0238$, 0 FH vs. 25 ng/µl FH, $P = 0.0031$; in (c) C3 inactivation: 0 FH vs. 1.5 ng/µl FH, $P < 0.0001$; 0 FH vs. 3.1 ng/µl FH, $P = 0.0257$, 0 FH vs. 6.25 ng/µl FH, $P < 0.0001$, 0 FH vs. 12.5 ng/µl FH, $P = 0.0003$, 0 FH vs. 25 ng/µl FH, $P < 0.0001$; and in (d) FB cleavage: 0 FH vs. 12.5 ng/µl FH, $P = 0.0104$, 0 FH vs. 25 ng/µl FH, $P < 0.0001$. Correlation plots in (**e**-**g**) compare C3 inactivation with (**e**) C3a (blue circles); (**f**) Ba (green circles); and (**g**) FB cleavage (purple circles) measured in reactions of 1.8 ng/µl FI and decreasing concentrations of FH. In (**h**), C3 inactivation was compared to FB cleavage (dark blue circles) in reactions of 25 ng/µl FH and increasing concentrations of FI. Data in (e-h) (mean values measured in 4–8 reactions under each condition) was analyzed for interdependence using Pearson's correlation coefficient (PCC). In (e) PCC = −0.9669, $r^2 = 0.9348$, $P = 0.0072$, in (f) PCC = −0.9476, $r^2 = 0.8979$, $P = 0.0524$; in (g) PCC = −0.955, $r^2 = 0.9120$, $P = 0.045$; and in (h) PCC = −0.9875, $r^2 = 0.9751$, $P = 0.0017$.

the features of over-activation of the AP, low C3 and (often undetectable) FB levels, plasma analysis of the FI deficient patients detected FH levels ranging from 14 to 57% of normal FH (Table 3)[27–31]. Our experimental results are consistent with these clinical reports (Table 3) of reduced FH levels often being associated with elevated activation in patients with FI deficiencies. Our data (Fig. 3), measured in purified protein reactions with low FI levels (< 25% of normal or < 0.4 ng/µl), also showed that FH levels above 50% (> 12.5 ng/µl) of normal were required to suppress fluid phase activation. Our results from reactions with 100% FH were also compatible with the conclusion from the study by Alba-Dominguez, et al., who additionally evaluated 9 relatives of the reported FI deficient patients. None of these individuals with normal FH levels and partial FI deficiencies (20–50%) had experienced symptoms or had reduced levels of C3 or FB[27]. In our protein reactions containing subnormal FI levels (6–50%) and normal FH levels (100%), most AP activation was suppressed. In the presence of 100% FH, only the reduced C3 inactivation in reactions with the lowest FI levels (6–12%), indicated a decrease in AP regulation (Fig. 2g). These reduced FI levels were 3- to 4-fold lower than the FI levels measured in the relatives with partial FI deficiencies[27].

The recurrent bacterial infections with complete FI deficiency partially result from the absence of iC3b formation. Only iC3b fragments, produced by FI cleavage, bind to CR3 (CD11b/CD18) on neutrophiles and enable immune cell recognition and phagocyte removal[32,33]. C3b primarily binds to CR1 (for immune complex clearance), and has low binding affinity for CR3[32,34]. In partial FI deficiency, the limited generation of iC3b allows some immune cell recruitment through CR3, and infections are reduced[33].

FH is responsible for distinguishing between host and foreign proteins to prevent unnecessary AP activation in the fluid phase[16,35]. FH is composed of 20 flexible domains divided functionally to adjust either the intensity of activation or priority of target recognition. The N-terminal domains 1–4 modulate the AP amplification response, whereas the remaining 16 domains contain diverse binding sites with varying affinities to host-associated patterns of polyanionic molecules[36,37]. Despite the multiple binding sites, FH may have reduced affinity for binding to host proteins altered by mutations or presented in an unrecognizable conformer as, for example, improperly cleaved ultra large VWF[17,38].

Supplementary to distinguishing between foreign and host-specific proteins, FH also functions as the cofactor for FI-mediated cleavage/inactivation of C3 convertases. FI can only proteolytically inactivate soluble forms of C3b or C3(H_2O) after the binding of FH induces conformational changes to expose the FI binding site[39]. In the absence of FH, FI can also inactivate C3b within C3 and C5 convertases that are bound to cell surface CR1 and CD46 receptors[1,40].

In this study, Western blot analysis was used to quantify the activation products, C3a and Ba. For Ba measurements, this technique enabled us to quantify Ba in the presence of high levels of HFB that interfere with Ba measurement in immunoassays. We have also found that C3a levels are more accurately measured in reaction samples prepared for Western blot analysis. In these samples, AP activation was instantly stopped, and further activation was prevented, by the addition of protein-denaturing buffer (containing EDTA) and rapid heating. In typical immunoassays, measurement samples are exposed for extended periods to mild conditions (in order to promote antibody binding and substrate detection). These latter conditions also allow further activation. Although excessive EDTA added to samples prevents further convertase formation; existing convertases continue to cleave C3 and generate C3a.

The variability in the levels of activation measured in the reactions without FI and FH is expected because the large number of regulatory proteins and receptors, normally present to control AP activation, are absent. How rapidly the initial convertases are assembled, formed by random collisions of C3(H_2O) and FB, likely determines extent of amplification and the levels of activation products. In the purified protein reactions, as the levels of FH and FI approached normal serum levels, the increased AP regulation resulted in more reproducible data (Figs. 2e-h and 4a-d). Similarly, there was less variability in the activation levels measured in the FH-D serum replenished with normal levels of FH protein (Fig. 6b, d, f).

We have previously published experimental data showing the effectiveness of HFB in inhibiting AP activation in FH-D serum supplemented with 3.1 ng/µl FH (12.5%).[17] Based on these results, the current experiments using both types of depleted serum ± HFB were used to separately compare its inhibitory effectiveness in the absence

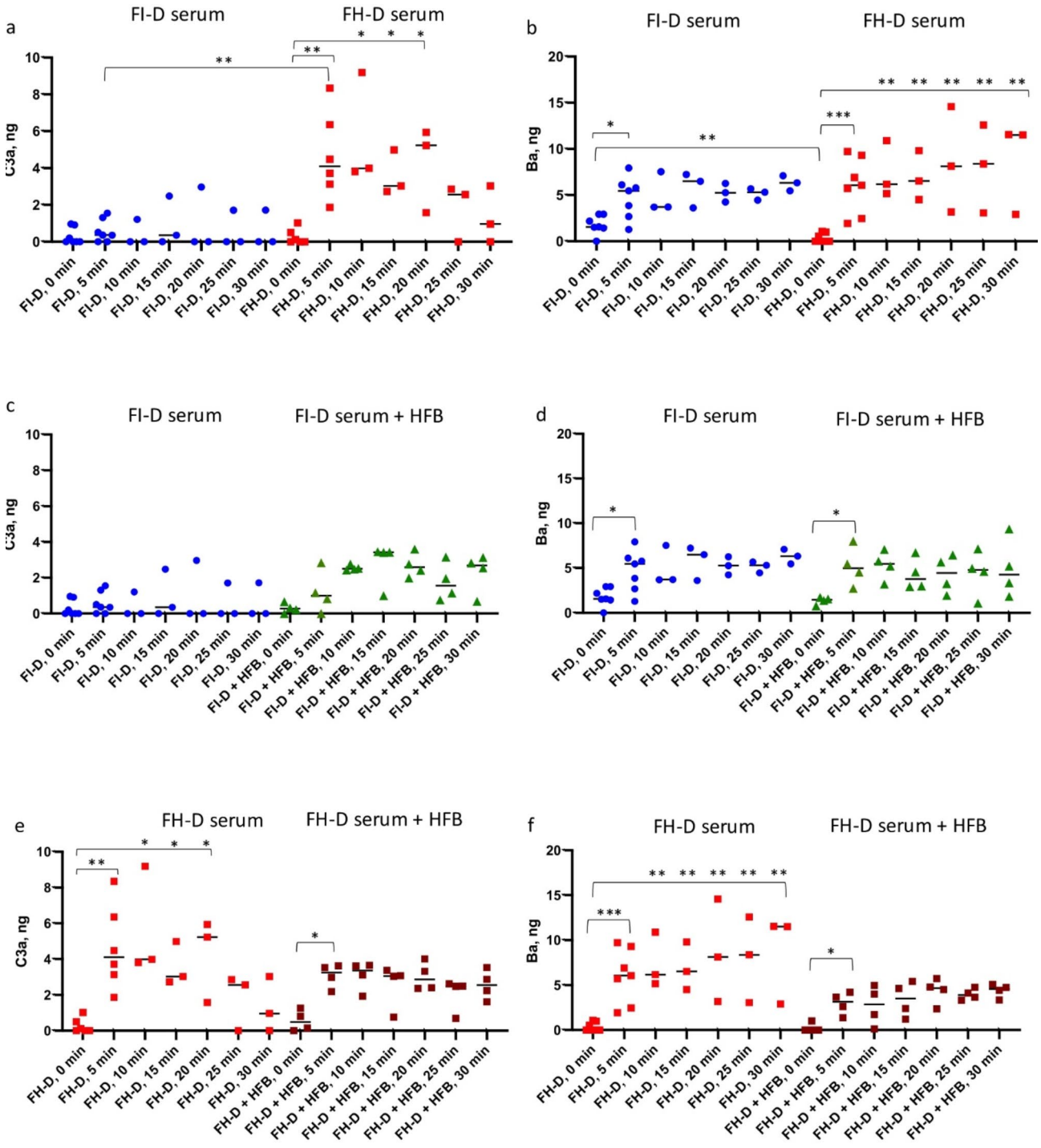


Fig. 5. AP activation in either FI-D or FH-D serum with/without HFB addition. Activation products C3a and Ba were measured (by Western blotting) at 5 min intervals over 30 min in both depleted serum types in the presence and absence of HFB. Reactions were initiated at time = 0 by the addition of 5 mM Mg^{+2} ions. The HFB concentration (30 ng/µl) was 2.4-fold higher than the FB levels in both depleted serum types. Graphs show comparison levels of: (**a**) C3a in FI-D and FH-D serum alone, (**b**) Ba in FI-D and FH-D serum alone, (**c**) C3a in FI-D serum ± HFB, (**d**) Ba in FI-D serum ± HFB, (**e**) C3a in FH-D serum ± HFB; and (**f**) Ba in FH-D serum ± HFB. Each graph shows individual values measured in: FI-D serum (blue circles); FI-D serum + HFB (green triangles); FH-D serum (red squares); and FH-D serum + HFB (brown squares). Horizontal bars indicate the median value from 3–7 measurements for each experimental condition. Statistical significance in (a) 5 min FI-D serum vs. 5 min FH-D serum, $P = 0.0012$; FH-D serum, 0 min vs. 5 min, $P = 0.0022$, 0 min vs. 10, 15, 20 min, $P = 0.0238$; (b) FI-D serum, 0 min vs. 5 min, $P = 0.0379$, FI-D vs. FH-D at 0 min, $P = 0.0099$, FH-D serum, 0 min vs. 5 min, $P = 0.0006$, 0 min vs. 10–30 min, $P = 0.0083$; (d) FI-D serum, 0 min vs. 5 min, $P = 0.0379$ and FI-D + HFB, 0 min vs. 5 min, $P = 0.0286$; (e) FH-D serum, 0 min vs. 5 min, $P = 0.0022$, 0 min vs. 10, 15, 20 min, $P = 0.0238$ and FH-D + HFB, 0 min vs. 5 min, $P = 0.0286$; and (f) FH-D serum, 0 min vs. 5 min, $P = 0.0006$, 0 min vs. 10–30 min, $P = 0.0083$; and FH-D + HFB, 0 min vs. 5 min, $P = 0.0286$.

of FH and in the absence of FI. HFB was prepared by controlled heating of plasma-purified FB. This heating process impairs the enzymatic function of HFB, although the binding capacity of HFB to C3b is preserved. On our Western blots, the migration pattern of HFB indicates that it is composed of dimers (186 kD) and tetramers (372 kD) of FB monomers (93 kD). HFB inhibits AP activation by competing with FB for binding to C3b and forming complexes (C3b-HFB) incapable of supporting further activation. HFB was determined to be inactivated if activation products, C3a and Ba, were not generated in reactions consisting of C3, HFB and FD, without the addition of FB[17].

In the FI-D serum experiments, the low levels of C3a generation and the moderate (3-fold) increase in Ba resulting from the addition of magnesium ions, showed that the FH levels present in FI-D serum were sufficient to suppress most of the AP activation. Consequently, the addition of 100% FI protein to the FI-D serum did not substantially reduce C3a or Ba levels, although the FI protein addition decreased the extent of FB cleavage by half (Fig. 6a, c, e). Conversely, the addition of 100% FH completed prevented the increases in C3a (4-fold), Ba (15-fold), and FB cleavage (>400-fold) measured in FH-D serum after activation (Fig. 6b, d, f and Supplementary Table S6). As a further comparison, the addition of HFB to activated FH-D serum reduced Ba generation in half (Fig. 5f), and the resulting levels were similar to the Ba levels in FI-D serum with or without HFB addition after activation (Fig. 5d). Together, these results (Fig. 5a, b) indicate that only minimal AP activation may occur in FI-D serum containing FH levels within the normal range, and suggest that HFB would be an effective inhibitor of AP activation under the combined conditions of partial FI deficiency and subnormal FH levels (Figs. 5a, b and 6d, f).

In a previous report by Ekdahl, et al., the authors proposed that other mechanisms for initiating the AP were more likely than the hydrolysis of C3 forming the first convertase[41]. The report questioned whether hydrolyzed C3 as the "C3b-like" fluid phase convertase was functional under physiological conditions. The authors challenged the necessity of adding Ni^{+2} instead of Mg^{+2}, the use of C3 convertase autoantibodies to amplify the AP response, and the addition of preparations of properdin enriched for aggregates to induce complement activation in serum. In our experiments, only the addition of 5 mM $MgCl_2$ (required for FB binding to C3b) was used to initiate the AP response in both the purified protein and depleted serum reactions. In the reactions composed of purified proteins, exogenous properdin was not included and AP activation did not occur without FD addition (Supplementary Table S1). The reactions contained EGTA, in order to chelate the calcium and prevent any possible contributions of the classical and lectin pathways. This was done because our aim was to study only AP activation. Our report supports the conclusion by Ekdahl, et al., that in the presence of (sufficient) FH and FI, there is no detectable C3 convertase activity. Our interpretation, instead of reasoning that the "C3b-like" convertase is a poor version of C3b, is that FH is very efficient at recognizing and restricting unprovoked AP fluid phase activation that is continuously initiated by the hydrolysis of C3.

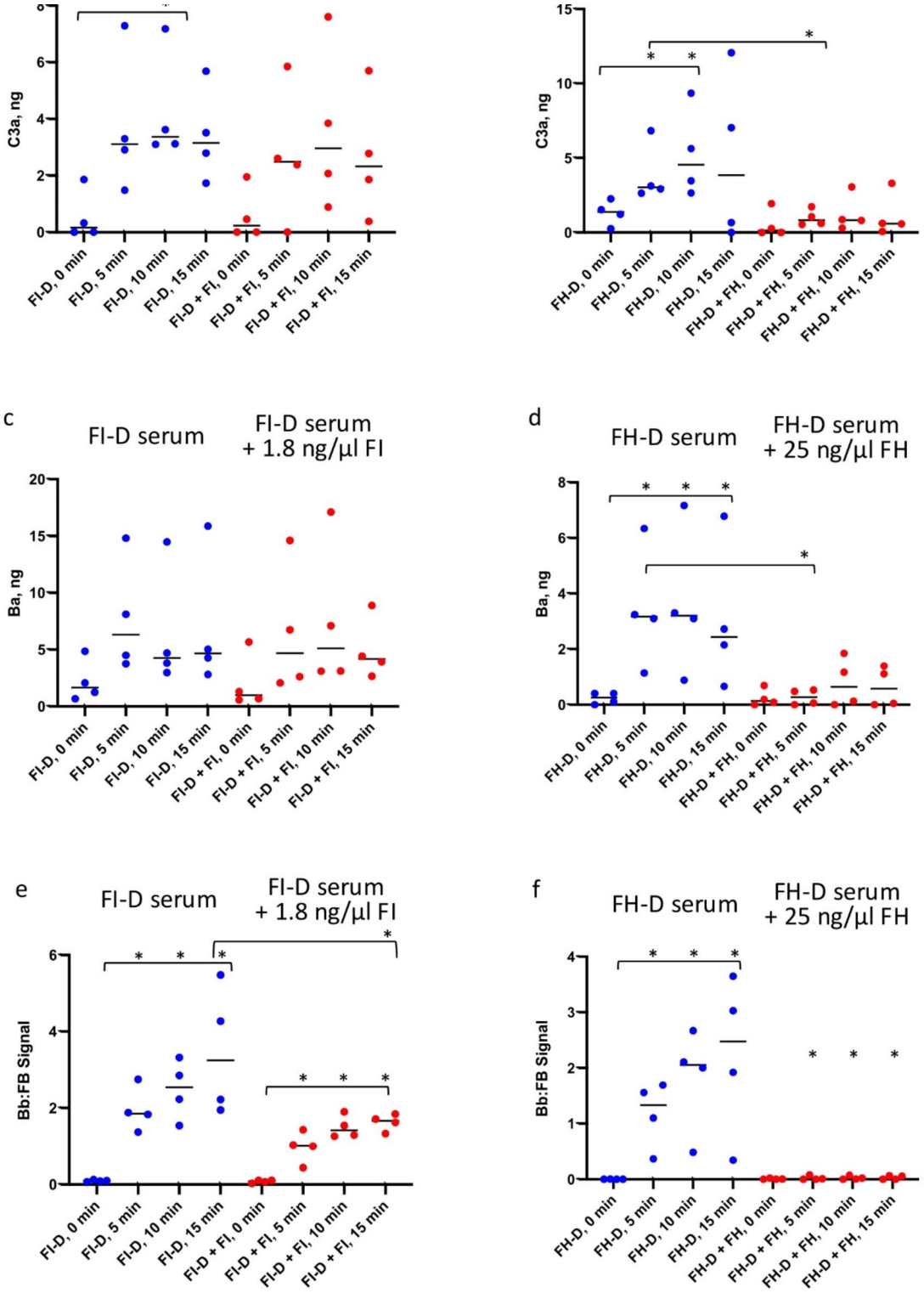
In the reactions composed of purified proteins, the extent of C3 inactivation was measured by ratios of C3 inactivation fragments to intact C3. These inactivated forms of C3 are unable to support further complement activation, although iC3b fragments remain important for cell signaling through receptor binding. The C3 inactivation fragments quantified by Western blot analysis were primarily produced by FI cleavage of $C3(H_2O)$ because the detection antibody predominantly recognizes forms of C3 with intact C3a domains (Supplemental Fig. S1). The correlation analysis showed that the amounts of $C3(H_2O)$ inactivation were directly related to the measured amounts of C3a, Ba and FB cleavage resulting from the activation of native C3 and C3 convertase formation. These data measured in reactions showed the significance of suppressing hydrolyzed C3 from instigating AP activation. In the fluid phase, in the absence of foreign particles, the combined functions of FH and FI prevent $C3(H_2O)$ from forming possible "C3b-like" convertases and the cascading AP activation that follows. However, in purified protein reactions containing 100% FH, there was no detectable production of C3a, and levels of Ba and FB cleavage were substantially reduced, regardless of the FI concentration (Fig. 2e-h). These results suggest that if levels of FH are sufficient to prevent formation of initial fluid phase convertases, then C3 inactivation by FI is less consequential for reducing inadvertent AP activation, although iC3b formation is essential for preventing infections.

Materials and methods

AP activation measured in reactions using purified complement proteins

Complement reagents. Complement proteins and antibodies were purchased from Complement Technology. C3 (A113); C3a-desArg (A119); Ba, (A154); FB (A135); FD (A136); FH (A137); FI (A138); rabbit anti-human C3a (A218); goat anti-human FB (A235); goat anti-human FH (A237); goat anti-human FI (A238); rabbit anti-human vitronectin (A260); FH-depleted serum (A337); and FI-depleted serum (A338). Complement protein aliquots were stored at −80°C until use.

Reaction composition and experimental conditions. AP activation experiments were conducted on ice using pre-cooled reagents with 25 µl reaction volumes. Proteins were diluted in 20 mM Tris, pH 7.4, 5 mM EGTA, and 5 mM $MgCl_2$ (Mg-EGTA). Reaction concentrations of C3 (60 ng/µl), FB (10 ng/µl) and FD (0.07 ng/µl) remained constant (at normal serum ratios), whereas reactions included a range of FH and FI concentrations. FH concentrations were 25, 12.5, 6.25, 3.1 and 1.5 ng/µl (representing 100, 50, 25, 12.5 and 6.25% of normal serum levels). FI concentrations were 1.8, 0.9, 0.4, 0.2, and 0.1 ng/µl (representing 100, 50, 25, 12.5, and 6.25% of normal serum levels) (Tables 1 and 2). After C3 addition (the last protein added), the tubes were mixed and incubated at 22°C for 5 min. Reactions were stopped by the addition of 25 µl of Laemmli sample buffer (BioRad,161–0737), containing 10 mM EDTA and 0.7 M 2-mercaptoethanol, followed by heating at 95°C for 5 min. AP reaction proteins were analyzed using fluorescent Western blotting techniques. C3 aliquots were only used once. Room temperature incubation (22°C) was chosen to allow quick access to the samples in order to precisely stop the reactions. Each of the 33 different purified protein reactions was repeated 3 to 8 times. The



positive control reaction, composed of C3, FB, FD in the absence of FI and FH, was included on each of the 22 Western blots used for the analysis.

Fluorescent Western blot analysis. Proteins were separated by 4–15% Tris-Glycine SDS-PAGE (BioRad, 456–1086) under reducing conditions for purified protein experiments and under non-reducing conditions for experiments using depleted serum. Gels included standard lanes of C3a-desArg (32–2.5 ng protein/lane) and Ba (20–2.5 ng protein/lane) for quantification and a pre-stained protein ladder (LI-COR, 928–6000) for approximating size. Gels were transferred using a semi-dry method (BioRad Trans-Blot,170–3940) onto low-fluorescence PVDF membranes (IPFL 10100). Remaining steps followed protocols recommended by LI-COR using TBS-based Intercept Blocking Buffer (LI-COR, 927-00001) for membrane blocking and antibody dilution. Primary antibodies were diluted in Intercept buffer containing 0.2% Tween-20 and diluent for fluorescent secondary antibodies contained 0.2% Tween-20 plus 0.01% SDS. Ba protein was detected using goat anti-

◀**Fig. 6**. AP activation in FI-D serum and FH-D serum with addition of each depleted protein. The extent of FB cleavage and production of C3a and Ba were measured (by Western blot analysis) in both types of depleted serum after addition of 100% of the relevant depleted protein. Graphs of C3a levels in: (**a**) FI-D serum ± 1.8 ng/µl FI (100%) and (**b**) FH-D serum ± 25 ng/µl FH (100%). Graphs of Ba levels in: (**c**) FI-D serum ± 1.8 ng/µl FI (100%) and (**d**) FH-D serum ± 25 ng/µl FH (100%). Graphs showing the extent of FB cleavage in: (**e**) FI-D serum ± 1.8 ng/µl FI (100%) and (**f**) FH-D serum ± 25 ng/µl FH (100%). Individual values are shown for depleted serum values alone (red circles) and FI-D serum plus 100% FI or FH-D serum plus 100% FH (blue circles). Horizontal bars indicate the median value and statistical significance is shown for values compared to measured values in reactions as noted. $N = 4$ for each experimental condition. In (a) FI-D serum, 0 min vs. 10 min, $P = 0.0286$; (b) FH-D serum, 0 min vs. 5, 10 min, $P = 0.0286$ and FH-D at 5 min vs. FH-D + FH at 5 min, $P = 0.0286$; (d) FH-D serum, 0 min vs. each time point, $P = 0.0286$ and FH-D at 5 min vs. FH-D + FH at 5 min, $P = 0.0286$; (e) FI-D serum, 0 min vs. each time point, $P = 0.0286$; FI-D + FI, 0 min vs. each time point, $P = 0.0286$ and FI-D at 15 min vs. FI-D + FI at 15 min, $P = 0.0286$; and (f) FH-D serum, 0 min vs. each time point, $P = 0.0286$, there were no significant differences in FH-D + FH at 0 min vs. each time point, and FH-D vs. FH-D + FH, at each time point except time = 0, $P = 0.0286$.

Reference	Patient	FI, µg/ml	C3, µg/ml	FB, µg/ml	FH, µg/ml
Amadei, et al., 2001[28]	Pt 1	<<	127	<<	93
2 siblings	Pt 2	<<	259	<<	105
	Normal	51–77	1300–1500	NS	330–578
Grumach, et al., 2006[29]	Pt 1	0	270	NS	245
3 related patients	Pt 2	0.31	280	NS	240
	Pt 3	0.23	280	NS	212
	Normal	25–44	550–1100	NS	360–680
Naked, et al., 2000[30]	Pt 1	<<	290	19	151
	Normal	38–100	1300–1500	206–588	228–827
Alba-Dominguez, et al., 2012[27]	Pt 1	0.7	228	<<	82
	Pt 2	0	226	<<	84
5 unrelated patients	Pt 3	0	344	<<	194
	Pt 4	1.4	315	<<	48
	Pt 5	0	384	<<	94
	Normal	25–40	750–1350	75–280	120–560
Ugrinovic, et al., 2020[31]	Pt 1	< 2.4	320	< 38	256
2 siblings	Pt 2	< 2.4	180	< 38	178
	Normal	38–58	750–1650	295–400	345–590

Table 3. Plasma levels of FI, C3, FB and FH reported in select studies of 13 patients with complete deficiency of FI. Patients were evaluated for complement disorders after multiple severe infections, except for Patient 2 in the article by Ugrinovic, et al., who was asymptomatic[31]. These studies of FI deficiency were chosen because the reports included FH levels. Mean patient FH levels were 34% of normal plasma levels, (ranging 14–57%), using the average value in the normal FH range reported. Normal = normal range in study; << = below limits of detection; NS = not stated in study.

human FB (4.3 µg/ml) plus donkey anti-goat IRDye-680RD (1:20,000, LI-COR, 926–68074). C3a protein was detected using rabbit anti-human C3a (6.9 µg/ml) plus donkey anti-rabbit IRDye-800CW (1:20,000, LI-COR, 926–32213). Dried membranes were scanned on a LI-COR Odyssey DLx Imager (9142-01P), intensities were measured at 700 and 800 nm, and data were analyzed using Empiria Studio software (LI-COR, 2000-000).

*AP activation determinants.*AP activation in reactions was determined by amounts of generated C3a and Ba, FB cleavage to Bb, and C3 inactivation. C3a and Ba levels were measured by interpolation of standard curves incorporated into the Western blots[17], whereas levels of FB cleavage and C3 inactivation were calculated from band intensity ratios. The extent of C3 inactivation (iC3:C3 signal ratio) was measured (in reduced samples only) by the combined band intensities of C3 fragments, iC3 alpha$_1$-chain (73 kD) and iC3b alpha$_1$-chain (63 kD), divided by band intensities of the intact C3 alpha-chain (115 kD) (Supplementary Fig. S1). The antibody against C3a, used in the Western blot analysis, primarily detects C3a and forms of C3 with intact C3a, while not detecting C3b, and only faintly detects the 63 kD fragment of iC3b (Supplementary Figs. S1, S2). C3 inactivation was not analyzed in the depleted serum experiments. The extent of FB cleavage (Bb: FB signal ratio) was measured on Western blots by dividing band intensities of Bb (60 kD) by band intensities of intact FB (93 kD). Higher Bb: FB values are indicative of increased FB cleavage (Supplementary Figs. S2, S4).

AP activation measured in serum depleted of either Factor H or Factor I

Human factor H-depleted and factor I-depleted serum. Factor H-depleted serum (FH-D serum) and factor I-depleted serum (FI-D serum) were prepared from human serum by immuno-depletion of either FH or FI. Both depleted serum types were supplied in 0.1 mM EDTA and contained normal levels of each complement protein, except FH or FI. C3 levels in both depleted serum types were estimated at 1200 µg/ml, based on supplier AP functional measurements. The depleted serum was further diluted (20 µl serum + 4 µl 0.1 mM EDTA) to obtain 1 µg/µl C3. Reaction volumes of 25 µl with 1.5 µl of diluted serum produced 60 ng/µl C3. AP activation was initiated with the addition of Mg-EGTA to a final concentration of 5 mM $MgCl_2$. FH-D and FI-D serum aliquots were used only once. Each experimental condition using the FI-D and FH-D serum was repeated 3–5 times and required 27 Western blots for the analysis.

FI levels in FI-D serum. FI levels in FI-D serum were measured by fluorescent Western blot analysis. FI-D serum was diluted 20-, 30-, 40-, 60-, 80-, and 120-fold directly into Laemmli (non-reducing) sample buffer containing 5 mM EDTA. FI protein standards in gel lanes ranged from 15, 30, 45, 60, 75, 90 and 120 ng. Blots were detected for FI using goat anti-FI plus donkey anti-goat IRDye-680RD, and for vitronectin using rabbit anti-vitronectin plus donkey anti-rabbit IRDye-800CW.

Heat-inactivated factor B. Heat-inactivated factor B (HFB) was prepared from human plasma-purified FB protein and was inactivated by controlled heating at 56°C for 30 min using a thermocycler. HFB was stored at 4°C until use[17]. HFB was determined to be inactivated if activation products, C3a and Ba, were not generated in reactions consisting of C3, HFB and FD, without the addition of FB[17].

AP activation measured in FI-D or FH-D serum with/without HFB. Single-use aliquots of both depleted serum types were diluted 1:1.2 in 0.1 mM EDTA to produce 1 µg/µl C3 (final reaction concentrations of 60 ng/µl C3). In reactions with HFB addition, 30 ng/µl HFB (2.4-fold higher than serum FB levels) was added to the depleted serum types prior to initiation of activation. Activation was initiated with 5 mM $MgCl_2$. Samples were collected at 5 min intervals over 30 min into tubes of Laemmli sample buffer containing 10 mM EDTA, immediately heated at 95°C for 5 min, and analyzed by Western blotting for C3a, Ba and FB cleavage.

AP activation in FI-D serum and FH-D serum with addition of each depleted protein. Single-use aliquots of both depleted serum types were diluted 1:1.2 with 0.1 mM EDTA to produce 1 µg/µl C3 (final reaction concentrations of 60 ng/µl C3). Activation was initiated with the addition of Mg-EGTA to FI-D serum ± 1.8 ng/µl FI protein (100%) and to FH-D serum ± 25 ng/µl FH protein (100%). Samples were collected into tubes of Laemmli sample buffer containing 10 mM EDTA at 5 min intervals over 30 min, immediately heated at 95°C for 5 min, and analyzed by Western blotting for C3a, Ba and FB cleavage.

Levels of FH protein in FI-D serum and levels of FI protein in FH-D serum. Western blots with lanes containing non-reduced samples of FI-D serum diluted 20-, 30-, 40-, 60-, 80-, and 120-fold, and lanes with 125, 62.5, 31.3, 15.7 and 7.8 ng of FH protein, were analyzed for FH detection using goat anti-FH plus donkey anti-goat IRDye-680. FH levels in the FI-D serum were measured by interpolation of the standard curve generated from the FH protein band intensities. For measurements of FI levels in FH-D serum, blots with non-reduced samples of FH-D serum diluted 10-, 20-, 30-, 40-, and 60-fold and lanes containing 17.5, 8.8, 5.8, 4.4, and 2.9 ng of FI protein were analyzed for FI detection using goat anti-FI plus donkey anti-goat IRDye-680. FI levels in the FH-D serum were measured by interpolation of the standard curve generated from the FI protein band intensities.

Levels of FB protein in FI-D serum and FH-D serum. Western blots with lanes containing 40, 20, 10, 5, and 2.5 ng of FB protein and diluted samples of FI-D serum and FH-D serum were detected for FB using goat anti-FB plus donkey anti-goat IRDye-680. The depleted serum samples were treated as in reactions except the $MgCl_2$ was replaced with 10 mM EDTA: (a) initial dilution 20 µl serum + 4 µl 0.1 mM EDTA; (b) 1.5 µl of diluted serum + 23.5 µl Tris/10 mM EDTA; or (c) addition of 25 µl of Laemmli sample buffer and heating at 95°C. Duplicate samples of each depleted serum type with lanes containing 2.5 µl and 5 µl of sample, were analyzed for levels of FB protein by interpolation of the FB standard curve.

Statistical Analysis. GraphPad Prism v 10 software (GraphPad.com) was used for standard curve interpolation, Pearson's correlation coefficient measurements, and for determining significance of differences using Mann Whitney 2-tail t-test comparison with an alpha value of 0.05.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 24 January 2025; Accepted: 21 March 2025
Published online: 29 March 2025

References

1. Pangburn, M. K. & Müller-Eberhard, H. J. Kinetic and thermodynamic analysis of the control of C3b by the complement regulatory proteins factors H and I. *Biochemistry* **22**, 178–185 (1983).
2. Pangburn, M. K., Pangburn, K. L., Koistinen, V., Meri, S. & Sharma, A. K. Molecular mechanisms of target recognition in an innate immune system: interactions among factor H, C3b, and target in the alternative pathway of human complement. *J. Immunol.* **164**, 4742–4751 (2000).
3. Pangburn, M. K. & Muller-Eberhard, H. J. Relation of putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. *J. Exp. Med.* **152**, 1102–1114 (1980).
4. Schreiber, R. D., Pangburn, M. K., Lesavre, P. H. & Müller-Eberhard, H. J. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *PNAS* **75**, 3948–3952 (1978).
5. Law, S. K. & Dodds, A. W. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Sci.* **6**, 263–274 (1997).

6. Rawal, N. & Pangburn, M. Formation of high-affinity C5 convertases of the alternative pathway of complement. *J. Immunol.* **166**, 2635–2642 (2001).
7. Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J. Exp. Med.* **154**, 856–867 (1981).
8. Pangburn, M. K. Initiation of the alternative pathway of complement and the history of tickover. *Immunol. Rev.* **313**, 64–70 (2023).
9. Pangburn, M. K., Schreiber, R. D. & Müller-Eberhard, H. J. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *J. Exp. Med.* **146**, 257–270 (1977).
10. Bienaime, F. et al. Mutations in components of complement influence the outcome of factor I-associated atypical hemolytic uremic syndrome. *Kidney Int.* **77**, 339–349 (2010).
11. Hallam, T. M., Sharp, S. J., Andreadi, A. & Kavanagh, D. Complement factor I: regulatory nexus, driver of immunopathology, and therapeutic. *Immunobiology* **228**, 152410 (2023).
12. Lemaire, M., Noone, D., Lapeyraque, A. L. & Licht, C. Frémeaux-Bacchi, V. Inherited kidney complement diseases. *Clin. J. Am. Soc. Nephrol.* **16**, 942–956 (2021).
13. Kavanagh, D., Goodship, T. H. & Richards, A. Atypical hemolytic uremic syndrome. *Semin Nephrol.* **33**, 508–530 (2013).
14. Lachmann, P. J. The story of complement factor I. *Immunobiology* **224**, 511–517 (2019).
15. Nicol, P. A. & Lachmann, P. J. The alternate pathway of complement activation. The role of C3 and its inactivator (KAF). *Immunology* **24**, 259–275 (1973).
16. Pangburn, M. K. Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J. Immunol.* **169**, 4702–4706 (2002).
17. Turner, N. A. & Moake, J. L. Heat-inactivated factor B inhibits alternative pathway fluid-phase activation and convertase formation on endothelial cell-secreted ultra-large von Willebrand factor strings. *Sci. Rep.* **13**, 5764 (2023).
18. Høgåsen, K., Mollnes, T. E., Tschopp, J. & Harboe, M. Quantitation of vitronectin and clusterin. Pitfalls and solutions in enzyme immunoassays for adhesive proteins. *J. Immunol. Methods*. **160**, 107–115 (1993).
19. Ansari, M. et al. Genetic influences on plasma CFH and CFHR1 concentrations and their role in susceptibility to age-related macular degeneration. *Hum. Mol. Genet.* **22**, 4857–4869 (2013).
20. Keith, R., Gilliam, B., Pepin, D. & Xiao, Q. Complement factor serum and plasma levels measured by a MILLIPLEX® magnetic bead panel: A reply. *Scand. J. Immunol.* **93**, e13036 (2021).
21. Alper, C. A., Abramson, N., Johnston, R. B., Jandl, J. H. & Rosen, F. S. Studies in vivo and in vitro on an abnormality in the metabolism of C3 in a patient with increased susceptibility to infection. *J. Clin. Invest.* **49**, 1975–1985 (1970).
22. Lachmann, P. J. The purification of specific antibody as F(ab')2 by the Pepsin digestion of antigen-antibody precipitates, and its application to Immunoglobulin and complement antigens. *Immunochemistry* **8**, 81–88 (1971).
23. Barrett, D. J. & Boyle, M. D. Restoration of complement function in vivo by plasma infusion in factor I (C3b inactivator) deficiency. *J. Pediatr.* **104**, 76–81 (1984).
24. Møller Rasmussen, J. et al. Three cases of factor I deficiency: the effect of treatment with plasma. *Clin. Exp. Immunol.* **74**, 131–136 (1988).
25. Wahn, V., Rother, U., Rautherberg, E. W., Day, N. K. & Laurell, A. B. C3b inactivator deficiency: association with an Alpha-migrating factor H. *J. Clin. Immunol.* **1**, 228–233 (1981).
26. Vyse, T. J. et al. Hereditary complement factor I deficiency. *QJM* **87**, 385–401 (1994).
27. Alba-Domínguez, M. et al. Complement factor I deficiency: a not so rare immune defect: characterization of new mutations and the first large gene deletion. *Orphanet J. Rare Dis.* **7**, 42 (2012).
28. Amadei, N. et al. Inherited complete factor I deficiency associated with systemic lupus erythematosus, higher susceptibility to infection and low levels of factor H. *Scand. J. Immunol.* **53**, 615–621 (2001).
29. Grumach, A. S., Leitão, M. F., Arruk, V. G., Kirschfink, M. & Condino-Neto, A. Recurrent infections in partial complement factor I deficiency: evaluation of three generations of a Brazilian family. *Clin. Exp. Immunol.* **143**, 297–304 (2006).
30. Naked, G. M. et al. Deficiency of human complement factor I associated with Lowered factor H. *Clin. Immunol.* **96**, 162–167 (2000).
31. Ugrinovic, S. et al. Primary Pneumococcal peritonitis can be the first presentation of a Familial complement factor I deficiency. *Clin. Exp. Immunol.* **202**, 379–383 (2020).
32. Vandendriessche, S., Cambier, S., Proost, P. & Marques, P. E. Complement receptors and their role in leukocyte recruitment and phagocytosis. *Front. Cell. Dev. Biol.* **9**, 624025 (2021).
33. Xu, S., Wang, J., Wang, J. H. & Springer, T. A. Distinct recognition of complement iC3b by integrins $\alpha_X\beta_2$ and $\alpha_M\beta_2$. *Proc. Natl. Acad. Sci. U S A*. **114**, 3403–3408 (2017).
34. Boero, E. et al. Purified complement C3b triggers phagocytosis and activation of human neutrophils via complement receptor 1. *Sci. Rep.* **13**, 274 (2023).
35. Perkins, S. J., Nan, R., Li, K., Khan, S. & Miller, A. Complement factor H-ligand interactions: self-association, multivalency and dissociation constants. *Immunobiology* **217**, 281–297 (2012).
36. Ferreira, V. P., Herbert, A. P., Hocking, H. G., Barlow, P. N. & Pangburn, M. K. Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. *J. Immunol.* **177**, 6308–6316 (2006).
37. Pangburn, M. K., Ferreira, V. P. & Cortes, C. Discrimination between host and pathogens by the complement system. *Vaccine* **26** (Suppl 8), I15–21 (2008).
38. Turner, N. A. & Moake, J. Assembly and activation of alternative complement components on endothelial cell-anchored ultra-large von Willebrand factor links complement and hemostasis-thrombosis. *PLoS One*. **8**, e59372 (2013).
39. Soames, C. J. & Sim, R. B. Interactions between human complement components factor H, factor I and C3b. *Biochem. J.* **326**, 553–561 (1997).
40. Merle, N. S., Church, S. E., Fremeaux-Bacchi, V. & Roumenina, L. T. Complement system part I - Molecular mechanisms of activation and regulation. *Front. Immunol.* **6**, 262 (2015).
41. Ekdahl, K. N. et al. Is generation of C3(H_2O) necessary for activation of the alternative pathway in real life. *Mol. Immunol.* **114**, 353–361 (2019).

Acknowledgements

The Mary R. Gibson Foundation and the Mabel and Everett Hinkson Memorial Fund at Rice University support JLM and NAT.

Author contributions

The study design, experiments, original manuscript draft, figures and diagrams were done by NAT. NAT and JLM reviewed and edited the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-95533-6.

Correspondence and requests for materials should be addressed to N.A.T.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.