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Alternative pathway regulation by factor H modulates *Streptococcus pneumoniae* induced proinflammatory cytokine responses by decreasing C5a receptor crosstalk

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ABSTRACT

Bacterial pathogens not only stimulate innate immune receptors, but also activate the complement system. Crosstalk between complement C5a receptor (C5aR) and other innate immune receptors is known to enhance the proinflammatory cytokine response. An important determinant of the magnitude of complement activation is the activity of the alternative pathway, which serves as an amplification mechanism for complement activation. Both alternative pathway activity as well as plasma levels of factor H, a key inhibitor of the alternative pathway, show large variation within the human population. Here, we studied the effect of factor H-mediated regulation of the alternative pathway on bacterial-induced proinflammatory cytokine responses. We used the human pathogen *Streptococcus pneumoniae* as a model stimulus to induce proinflammatory cytokine responses in human peripheral blood mononuclear cells. Serum containing active complement enhanced pneumococcal induced proinflammatory cytokine production through C5a release and C5aR crosstalk. We found that inhibition of the alternative pathway by factor H, with a concentration equivalent to a high physiological level, strongly reduced C5a levels and decreased proinflammatory cytokine production in human peripheral blood mononuclear cells. This suggests that variation in alternative pathway activity due to variation in factor H plasma levels affects individual cytokine responses during infection.

1. Introduction

The host innate immune system is activated immediately upon infection. Induction of inflammatory responses are essential for recruitment of immune cells and the control of adaptive immune responses. Pathogens, containing complex macromolecular surfaces, present multiple antigens to the host immune system that not only stimulate specific pattern-recognition receptors (PRRs), but also activate the complement system. Several studies indicate that there is crosstalk between the complement system, Toll-like receptors (TLRs) and Fcγ receptors, which modulates the proinflammatory cytokine responses [1–6]. It is known that complement activation product C5a is a potent inflammatory protein [7]. Addition of recombinant C5a to human peripheral blood mononuclear cells (PBMCs) stimulated with TLR ligands enhances cytokine production, whereas C5a alone has no effect [4]. In addition, it has been demonstrated that C5a binding to its receptor (C5aR) (also called CD88) modulates the inflammatory response induced by many bacterial pathogens, including *Escherichia coli*, *Staphylococcus aureus* and *Neisseria meningitides* [8–10]. Therefore it is important to study the mechanism by which complement activation contributes to the inflammatory response upon infection.

The complement cascade can be activated by three distinct pathways; (i) the classical pathway activated by C1q binding to antibody-antigen complexes; (ii) the lectin pathway activated by recognition of polysaccharide structures on pathogens; and (iii) the alternative pathway activated continuously at low levels by spontaneous hydrolysis of C3. In addition, the alternative pathway amplification loop plays a crucial role in the amplification of the initial activation of the classical and lectin pathway [11]. The alternative pathway may account for up to 80% of total complement activation, even if initially triggered by the classical pathway [12].

A key negative regulator of the alternative pathway is complement factor H (FH), which is essential for inhibiting alternative

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pathway activation in the fluid phase and on cellular surfaces [13]. FH acts as a co-factor for factor I mediated inactivation of C3b and also accelerates the decay of the alternative pathway C3 convertase [14]. Polymorphisms in the gene encoding complement FH (*CFH*) have been associated with human diseases such as hemolytic uremic syndrome, age related macular degeneration and dense deposit disease [13,15]. Polymorphisms may affect FH binding to host cells, regulation of alternative pathway activity, or FH expression levels [14–16]. FH plasma concentrations vary widely between individuals [17–21]. In the MRC Fenland population study of 1514 individuals, FH serum levels ranged from 63.5 to 847.6 μg/mL (median 226.6 μg/mL) [20]. The observed variation may be due to environmental factors (e.g. smoking) and genetic factors [13,19]. In addition, FH serum levels may vary depending on an individual's disease state. Reduced FH levels were observed during acute meningococcal disease compared to patients at convalescence. As noted above, the serum FH concentration in the healthy control group in this study varied widely, ranging from 31 to 953 μg/mL (median 395 μg/mL) [17]. We have also demonstrated the importance of FH levels and variation in alternative pathway activity on the host defense against *Streptococcus pneumoniae* [22].

In the current study, we aim to assess the role of FH levels and alternative pathway activity on bacterial-induced proinflammatory cytokine production. We used the human pathogen *S. pneumoniae* as a model stimulus to induce proinflammatory cytokine responses by PBMCs. Our results clearly show that complement activation enhances the inflammatory response through C5a release and C5aR-mediated crosstalk. Moreover, alternative pathway inhibition by exogenous soluble phase FH strongly reduces C5aR crosstalk and pathogen induced proinflammatory cytokine responses. Thus variation in alternative pathway activity due to variation in FH plasma levels may affect an individual's cytokine responses during infection.

2. Material and methods

2.1. Bacterial strains and growth conditions

Wild-type *S. pneumoniae* strain TIGR4 was used in all PBMC stimulation experiments [23]. In FH binding assays only, a TIGR4 Δ*pspC* deletion mutant was used as *S. pneumoniae* has been described to bind human FH by expressing pneumococcal surface protein C (PspC) [24]. The TIGR4 Δ*pspC* deletion mutant was constructed by allelic replacement of the target gene with an antibiotic resistance marker as described previously [25]. Briefly, overlap extension PCR was used to insert the spectinomycin resistance cassette of the pR412 plasmid between the two 500-bp flanking sequences adjacent of the target gene. The resulting PCR products were introduced by competence-stimulating peptide (CSP-2) induced transformation into TIGR4. Directed mutants were obtained by selective plating and were checked for correct integration of the antibiotic resistance cassette into the target gene by PCR using control primers located inside the gene. Subsequently, the TIGR4 wild-type strain was transformed with chromosomal DNA isolated from the mutants, to prevent the accumulation of inadvertent mutations elsewhere on the chromosome. The primer sequences are presented in Table 1 of the supplementary data.

Bacteria were grown on Columbia blood agar plates (Becton Dickinson) and in Todd-Hewitt broth supplemented with 5 g/L yeast extract at 37 °C and 5% CO_2 until an OD_{620} of 0.3 was reached. The number of colony forming units per milliliter was determined by plating serial 10-fold dilutions on blood agar plates. Subsequently, bacteria were heat killed at 65 °C for 30 min and stored at −80 °C. Heat killed pneumococci were used in order to avoid variation in bacterial numbers due to growth which could affect the host inflammatory response. Previous studies demonstrate that most TLR ligands remain functional after heat killing, although it has been shown that this can lower TLR9 dependent signaling [26].

2.2. Isolation of PBMCs and stimulation assays

After informed consent was obtained, a venous blood specimen was collected from the median cubital vein of healthy volunteers (age, 20–40 years; both males and females) into 10-mL EDTA tubes (BD). To isolate the PBMC fraction, blood was diluted in an equal volume of phosphate buffered saline (PBS), added onto 15 mL Lymphoprep (Axis Shield) and centrifuged at 800*g* for 20 min at room temperature. The PBMCs were harvested, washed three times in cold PBS and resuspended in culture medium (RPMI 1640 GlutaMAX-I medium, Invitrogen). Five hundred thousand cells in 100 μl were added to a round-bottom 96-well plate (Nunc) and incubated with 50 μl of stimuli and 50 μl of diluted serum resulting in a total volume of 200 μl/well. The stimuli were 10^5 heat-killed TIGR4 bacteria, or the TLR2 agonist Pam3Cys (Invivogen) (final concentration 1 μg/mL) or RPMI (negative control). The serum was diluted in RPMI to obtain a final concentration of 10% serum/well. Pooled normal human serum (NHS) (Sigma-Aldrich or GTI Diagnostics) or heat-inactivated serum (HI-NHS; 30 min at 56 °C) or RPMI (negative control) was used. Specific PBMC stimulations using 10% NHS were supplemented with 0.1 or 1 μM C5a receptor antagonist, PMX53 (R&D Systems) or with 5, 25 or 50 μg/mL purified human FH (Comp. Tech). The FH concentration in the pooled NHS was 460 μg/mL, which in diluted serum gave a final FH concentration of 46 μg/mL. Therefore adding 50 μg/mL of exogenous FH doubled the amount of FH already present in the serum. Each stimulation was prepared in duplicate. After 24 h at 37 °C and 5% CO2, the cells were pelleted by centrifugation at 650*g* at room temperature, after which the supernatants were pooled and stored at −20 °C for further analysis.

2.3. Inflammatory response analysis

The concentrations of human interleukin-(IL)6, IL-1β, tumor necrosis factor (TNF-α) and IL-8 produced by the PBMCs were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Pelikine Compact, Sanquin) according to manufacturers' instructions. Levels of complement activation product C5a were measured using a commercial human C5a ELISA kit (HK349, Hycult).

2.4. Factor H binding assay

TIGR4 heat killed or alive bacteria (1×10^7) were pelleted in a 96-well plate and resuspended in 10% (vol/vol) pooled normal human serum (Sigma-Aldrich) in Hanks Buffered Salt Solution (HBSS) to a total volume of 100 μl. The bacterial suspension was incubated for 30 min at 37 °C in 5% CO_2. After incubation the bacteria were washed and labeled with polyclonal sheep anti-human factor H (Abcam). After a further 30 min incubation and washing, the bacteria were labeled with FITC-donkey anti-sheep IgG antibody (Jackson immunoresearch) followed by fixation in 2% paraformaldehyde. Factor H binding was measured using a FACScan flow cytometer (BD Biosciences). Data were analysed using FlowJo v10.1.

2.5. Statistics

Statistically significant differences were determined by the Wilcoxon or the Friedman test (nonparametric one-way ANOVA) followed by the Dunn's test to calculate multiplicity-adjusted P values. The data shown represents the mean ± standard error of

the mean (SEM) of two or three independent experiments using PBMCs isolated from 5 or 6 different donors.

3. Results

3.1. Complement activation enhances pneumococcal induced PBMC cytokine production

PBMCs were stimulated for 24 h with heat-killed *S. pneumoniae* or the TLR2 ligand Pam3Cys in the presence of normal human serum (NHS) or heat inactivated NHS (HI-NHS). HI-NHS is devoid of active complement since complement is a heat-labile component of human serum [27]. In the absence of active complement, using HI-NHS, PBMCs stimulated with *S. pneumoniae* produced proinflammatory cytokines IL-6, IL-1β, TNF-α and IL-8 (Fig. 1). The presence of active complement in NHS significantly increased the release of proinflammatory cytokines upon stimulation with *S. pneumoniae* thus indicating that complement activation augments cytokine release by PBMCs (Fig. 1). PBMCs stimulated with Pam3Cys induced IL-6, IL-1β, and IL-8 which was not affected by the absence or presence of active complement indicating that pneumococcal-induced complement activation was necessary for augmented cytokine release.

3.2. Pneumococcal induced PBMC cytokine production is enhanced through C5aR crosstalk

In order to study whether complement activation acts via the C5a-C5aR signaling pathway to enhance inflammatory cytokine release, PBMCs were stimulated with heat-killed *S. pneumoniae* in the presence of C5aR antagonist PMX53. Inhibition of C5aR in the presence of active complement led to decreased cytokine release in a dose-dependent manner to levels observed for HI-NHS. This demonstrates that the enhanced inflammatory response in the presence of active complement was largely C5aR mediated (Fig. 2).

3.3. Alternative pathway inhibition by exogenous factor H strongly reduces C5aR crosstalk

In order to study the role of alternative pathway activation on the pneumococcal induced inflammatory response, we used the natural alternative pathway inhibitor FH. PBMCs were stimulated with heat-killed *S. pneumoniae* in the presence of NHS with increasing concentrations of purified human FH. As observed previously, the presence of active complement in NHS significantly increased cytokine release by PBMCs compared to HI-NHS (Fig. 3). Adding exogenous FH decreased cytokine release in a dose-dependent manner, where addition of 50 μg/mL FH, which doubles the absolute FH serum concentration, reduced cytokine release to levels found with HI-NHS (Fig. 3). In order to demonstrate the effect of FH on complement activation, we measured C5a levels. Addition of exogenous FH inhibited complement activity in a dose-dependent manner, as demonstrated by decreased C5a levels (Fig. 4). Heat-killed *S. pneumoniae* TIGR4 used for the stimulations did not bind human FH when incubated in serum, as opposed to live *S. pneumoniae* which bound FH via the pneumococcal surface protein C (PspC) (supplementary data). Use of heat-killed *S. pneumoniae* allowed us to demonstrate that only soluble phase FH alternative pathway inhibition was sufficient to down-regulate pathogen induced proinflammatory cytokine release.

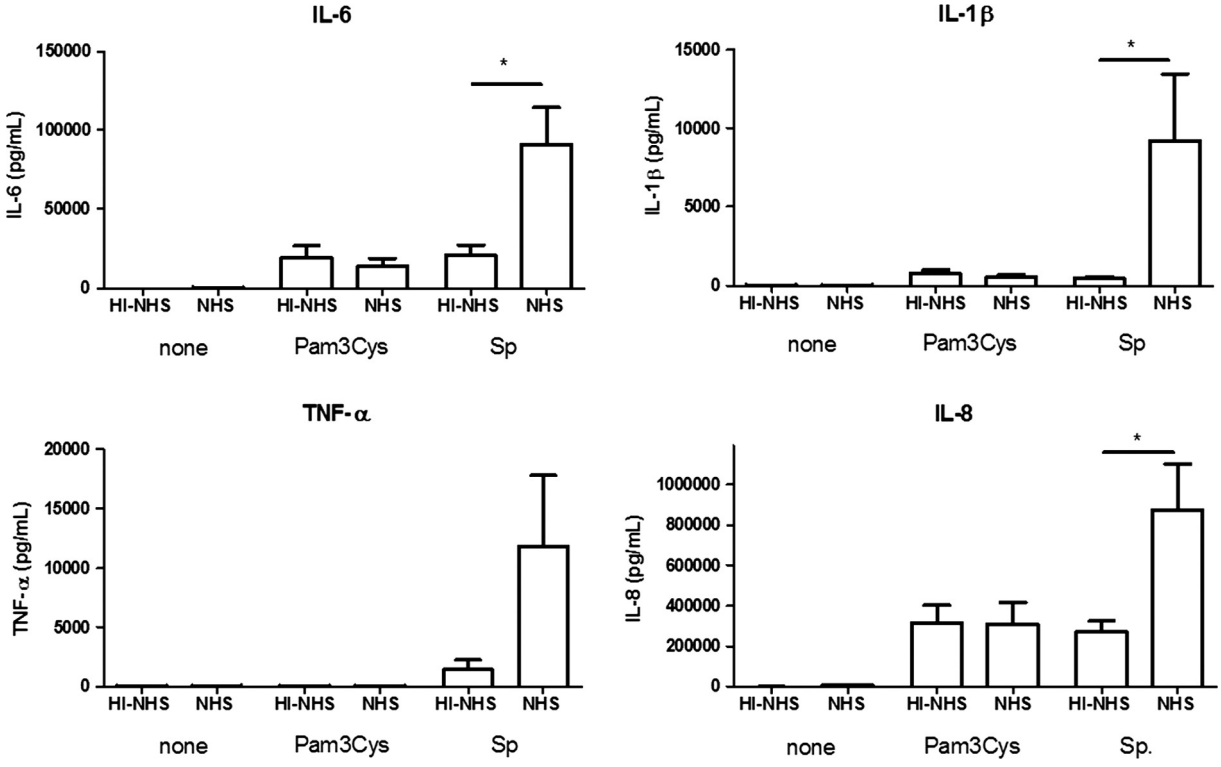

Fig. 1. Complement enhances pneumococcal induced cytokine responses. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp), Pam3cys or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 h. Supernatants were collected and IL-6, IL-1β, TNF-α and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 5 donors. Statistically significant differences were determined by the paired Wilcoxon test. $^{*}P < 0.05$.

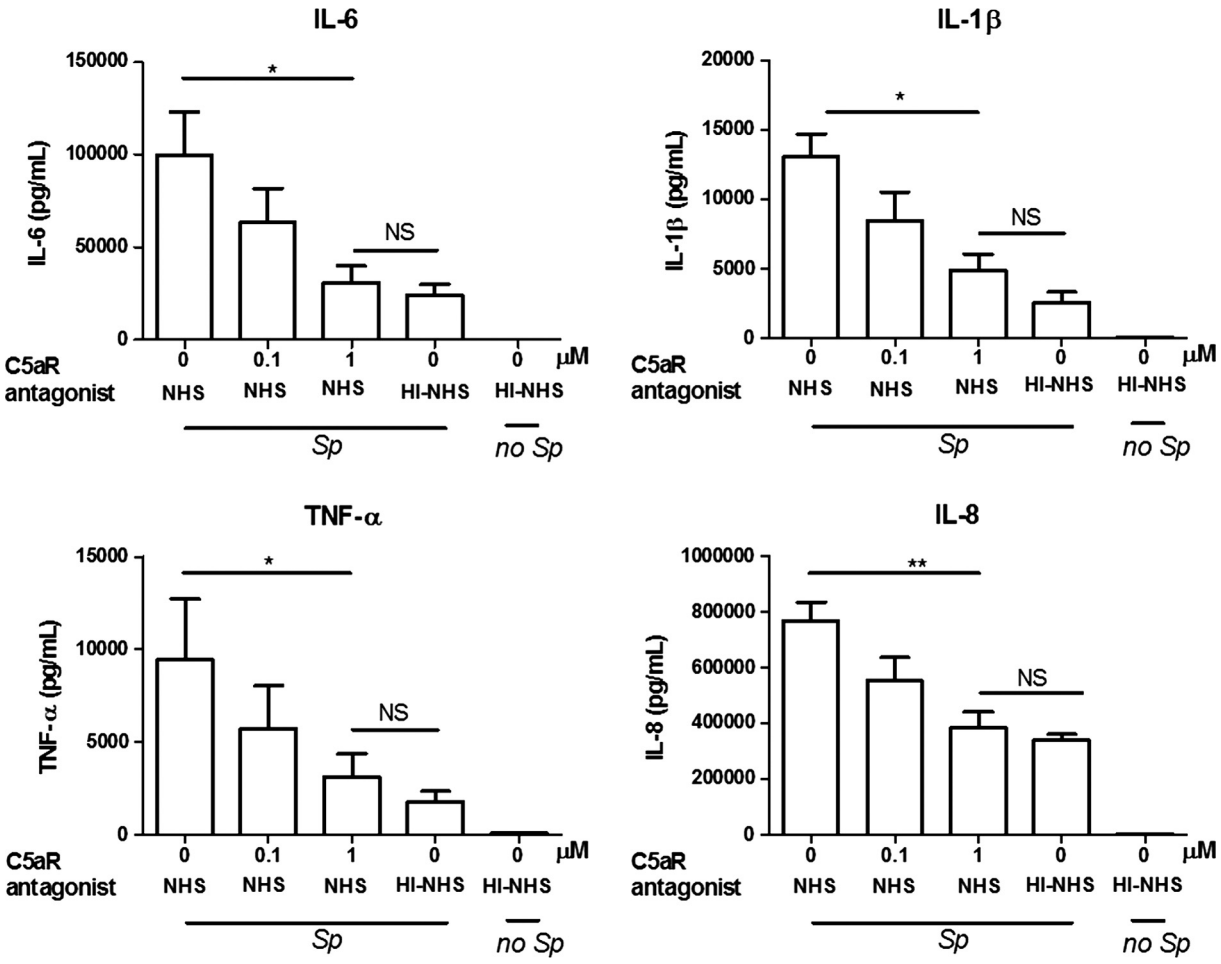

Fig. 2. Complement mediated enhancement of cytokine induction is mediated by C5aR crosstalk. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp) or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 h. 0.1 or 1 μM of C5aR inhibitor (PMX53) was added to stimulations with 10% NHS. Supernatants were collected and IL-6, IL-1β, TNF-α and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 5 different donors. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. $^{*}P < 0.05$, $^{**}P < 0.01$. NS = not significant.

4. Discussion

This study demonstrates a critical role of fluid phase FH on pneumococcal induced proinflammatory cytokine responses. Induction of proinflammatory cytokines IL-6, IL-1β, TNF-α and IL-8 were measured upon stimulation of human PBMCs with *S. pneumoniae* in the absence of active complement. This pathogen contains multiple antigens that are recognized by multiple innate immune receptors such as TLR2, TLR4, TLR9, NOD-like receptors (NLRs) and DNA sensors [26,28]. Several reports demonstrate that signaling by these innate immune receptors induces cytokine secretion [26,28,29]. We showed in our study that PBMC stimulation with the TLR2 ligand Pam3Cys induced IL-6, IL-1 β and IL-8 release to the same extent as *S. pneumoniae* in the presence or absence of complement active serum.

Interaction of *S. pneumoniae* with human serum leads to complement activation by the classical, lectin and alternative pathway resulting in a cascade of reactions and the release of the complement activation product C3a and C5a [28,30]. Our results show the importance of complement activation and C5aR crosstalk on *S. pneumoniae* induced inflammatory cytokine responses in PBMCs. These results are in line with previous studies that demonstrated the effect of recombinant C5a on TLR ligand induced cytokine responses [4,5]. Other studies, where the inflammatory response was induced by *Neisseria meningitidis, Candida albicans* or *Staphylococcus aureus*, also demonstrate an important role for C5aR crosstalk [4,9,10].

We are the first to show that the presence of relative high FH levels strongly reduce whole pathogen induced inflammatory responses from human PBMCs, as measured by proinflammatory cytokine production. As the heat-killed *S. pneumoniae* strain TIGR4 failed to bind human FH to its surface (supplemental figure), our assessment focused solely on soluble phase FH alternative pathway regulation. Previous studies demonstrated that exogenous purified FH acted on fluid phase complement activity as determined by haemolytic activity of serum [31].

In line with our findings, other studies have demonstrated that alternative pathway inhibition by anti-factor D reduced the oxidative burst of monocytes and granulocytes in a human whole blood model of meningococcal sepsis [10]. Furthermore, inhibition of the alternative pathway by anti-factor D in PBMCs stimulated with *C. albicans* reduced proinflammatory cytokine production [4]. Our study shows that FH, in a dose-dependent manner, reduced C5a release and subsequent proinflammatory cytokine production, which indicated that alternative pathway-mediated C5aR crosstalk is essential for the release of cytokines by PBMCs.

Our results may help to understand inter-individual differences in inflammatory responses. Human FH concentrations vary greatly between individuals (range, 63.5–847.6 μg/mL) and may affect an individual's alternative pathway activity [17–21]. Furthermore,

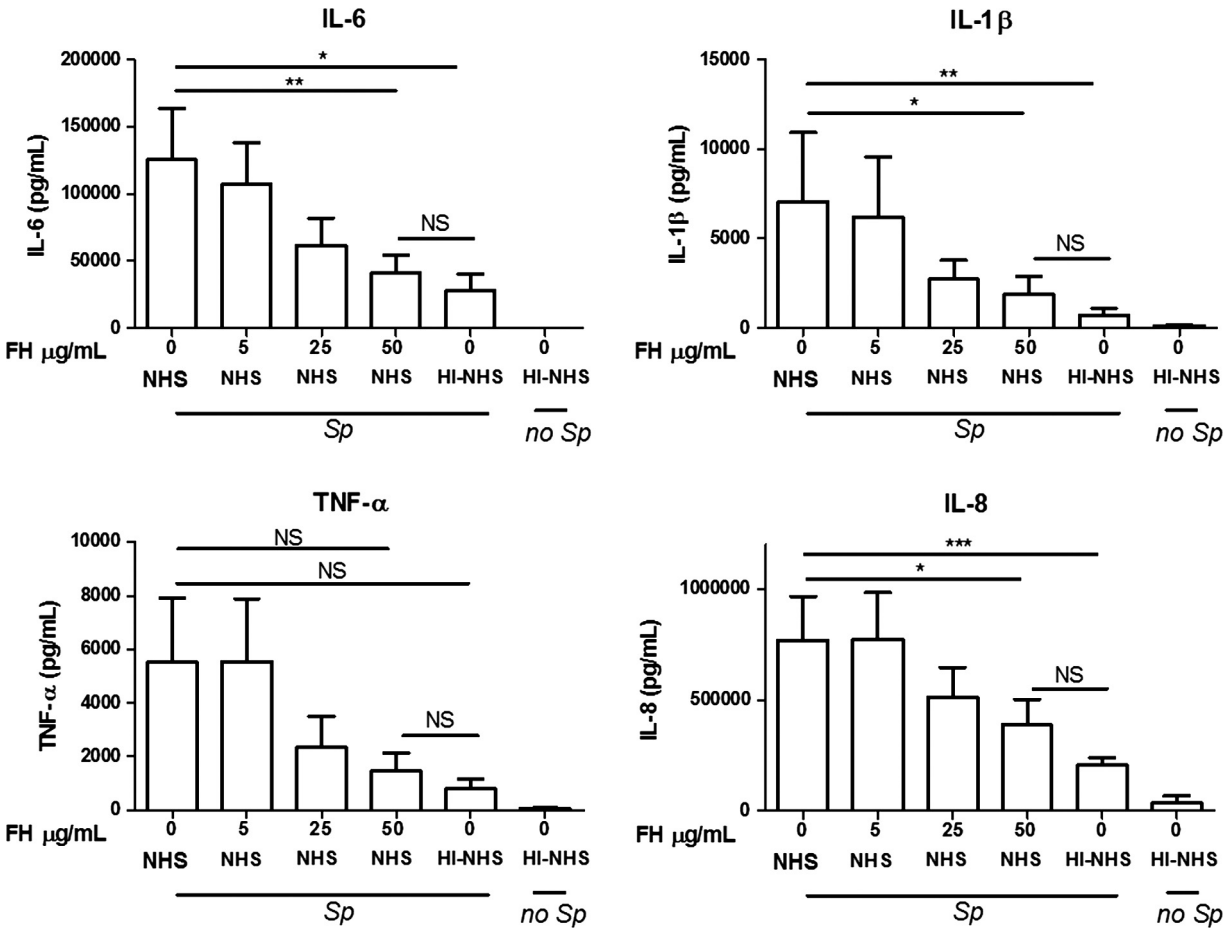

Fig. 3. Alternative pathway inhibition by exogenous FH strongly reduces pneumococcal cytokine responses. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4 (Sp) or culture media alone, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 h. Increasing concentrations of purified human FH were added to the PBMC stimulation in 10% NHS. Supernatants were collected and TNF-α, IL-1β, IL-6 and IL-8 measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 6 different donors. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = not significant.

combinations of polymorphisms in alternative pathway proteins have been described to influence alternative pathway activity [15]. Particular combinations of polymorphisms may result in a hyper-inflammatory state and predispose for chronic inflammatory diseases, such as haemolytic uremic syndrome, age-related macular degeneration and dense deposit disease [15]. In contrast, other combinations may result in a hypo-inflammatory state and may predispose to infections by ineffective immune activation [15]. An association between FH plasma levels, plasma C5a levels and inflammatory activity has been observed for clinical diseases such as anti-neutrophil cytoplasmic antibody associated vasculitis and age-related macular degeneration [16,32,33].

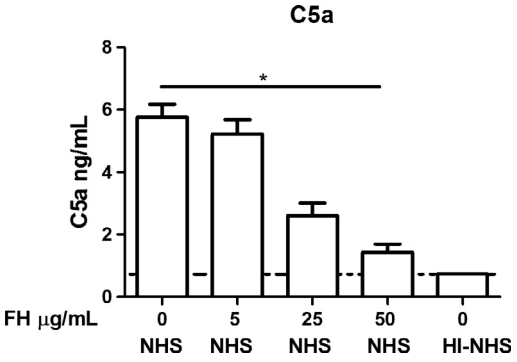

Fig. 4. Exogenous FH reduces C5a generation. Human PBMCs were stimulated with heat-killed *S. pneumoniae* TIGR4, in the presence of 10% pooled normal human serum (NHS) or heat-inactivated pooled normal human serum (HI-NHS) for 24 h. Increasing concentration of purified human FH were added to the PBMC stimulation in 10% NHS. Supernatants were collected and C5a measured by ELISA. The data shown represent the mean and SEM of three independent experiments using PBMCs isolated from 6 different donors. Dash line indicates lower limit of detection. Statistically significant differences were determined with the Friedman test (non-parametric one-way ANOVA) followed by Dunn's test to calculate multiplicity-adjusted P values. $^{*}P < 0.05$.

In conclusion, we have found that FH, the alternative pathway inhibitor, modulates pneumococcal induced proinflammatory cytokine responses by inhibiting C5aR crosstalk. Variation in FH levels within the physiological range of serum affected C5aR cross-talk in PBMCs stimulated with *S. pneumoniae*. This may explain the mechanism by which an individual's alternative pathway activity not only affects the susceptibility to chronic inflammatory diseases, but also the extent of the inflammatory response during infectious diseases.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.09.025.

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