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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