C-Reactive Protein Increases Cytokine Responses to *Streptococcus pneumoniae* through Interactions with Fc γ Receptors

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C-Reactive Protein Increases Cytokine Responses to Streptococcus pneumoniae through Interactions with Fcγ Receptors

Carolyn Mold and Terry W. Du Clos

Streptococcus pneumoniae is the most common organism responsible for community acquired pneumonia and meningitis. In pneumococcal pneumonia, a strong local inflammatory cytokine response reduces the frequency of bacteremia and increases survival. The initiation of this cytokine response by innate recognition of bacterial cell wall components through TLR has been described, but the role of soluble innate mediators has received limited attention. C-reactive protein (CRP) is an acute phase protein that binds phosphocholine residues on S. pneumoniae cell walls. CRP interacts with phagocytic cells through FcγRI and FcγRII and activates the classical complement pathway. CRP is protective in mouse pneumococcal bacteremia by increasing complement-dependent clearance and killing of bacteria. We studied the cytokine response of PBMC stimulated with CRP-opsonized S. pneumoniae to determine the effect of CRP interaction with FcγRII. CRP dramatically increased the production of TNF-α and IL-1β in response to S. pneumoniae. These increases were blocked by phosphocholine, which inhibits CRP binding to S. pneumoniae, by inhibitors of FcγRII signaling, and by mAb to FcγRI and FcγRII. A mutated rCRP with decreased FcγR binding had a decreased ability to stimulate TNF-α release, compared with wild-type CRP. Individuals who were homozygous for the R-131 allele of FcγRII, which has a higher affinity for CRP, showed higher responses to CRP-opsonized bacteria than did individuals homozygous for the H-131 allele, further implicating this receptor. The results indicate that CRP recognition of S. pneumoniae and binding to FcγR may enhance the early protective cytokine response to infection. The Journal of Immunology, 2006, 176: 7598–7604.

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found that, although *S. pneumoniae* induce a strong IL-1β response, they are poor inducers of TNF-α in human PBMC (28). Thus, agents that would increase the TNF-α response to *S. pneumoniae* infection could potentially improve survival.

The importance of TLR2 in the cytokine response to *S. pneumoniae* cell wall components has been established (29). However, less is known about the role of soluble innate recognition molecules, which also mediate pathogen recognition. A recent report identified the acute phase protein, LPS binding protein (LBP) as a recognition molecule for pneumococcal cell walls and an important stimulus for the inflammatory response in pneumococcal meningitis (30). CRP binds to the teichoic acid and lipoteichoic acid components of the *S. pneumoniae* cell wall. CRP interacts with monocytes, macrophages, and neutrophils through FcγR (17, 18), protects mice from pneumococcal infection and facilitates *S. pneumoniae* clearance from the bloodstream (9, 12). Because cytokine responses, including TNF-α also may be triggered through FcγγR (31), we hypothesized that the binding of the innate opsonin, CRP, to pneumococci might alter the cytokine response as well as enhance clearance of the bacteria. CRP is likely to be available for early host defense in pneumococcal pneumonia, because it is not only rapidly synthesized in the liver (6, 7), but also is locally synthesized (8).

In the present study, we examined the effect of CRP on human mononuclear cell cytokine responses to *S. pneumoniae*. We found that CRP significantly enhances the TNF-α and IL-1β responses to these bacteria and that this effect is mediated by its binding to the bacteria and interaction with FcγRI and FcγRII. These results suggest another mechanism by which CRP could protect against invasive pneumococcal infection. In addition, this report is the first to directly demonstrate that FcγγR mediates the cytokine response elicited in human PBMC by CRP.

**Materials and Methods**

**Reagents**

Human CRP was purified from human pleural fluid as described previously (32). All preparations were examined on overloaded SDS-PAGE gels to ensure purity. No additional bands other than the major band at ~25 kDa were seen. The preparations were examined for endotoxin by a quantitative chromogenic Limulus amebocyte lysate assay (Cambrex). If needed, endotoxin was removed on an Etox Acticlean column (Sterogene) so that protein concentrations of six cytokines were determined by CBA (3, 28). Paired t tests were used to compare IL-1β, cytokine levels are presented as a percentage of the amount observed in cultures stimulated with R36a alone. This was done to allow multiple experiments to be combined. Paired t tests were used to compare effects of inhibitors on cytokine synthesis.

**Results**

**Bacteria**

*S. pneumoniae* R36a (a nonencapsulated variant of type 2 *S. pneumoniae*) was purchased from the ATCC. R36a were grown to mid-log phase in Todd-Hewitt broth containing 0.5% yeast extract. The concentration of R36a was estimated by absorbance at 600 nm and verified by plate counts. R36a were washed into PBS and heated at 60°C for 60 min. Heat treatment kills the bacteria and also destroys pneumolysin activity. For CRP opsonization, R36a were preincubated for 20 min at room temperature with 100 µg/ml CRP. The final concentration of CRP added to the wells was 25 µg/ml. In experiments using inhibitors of signaling, mAb to FcγγR and rCRP, the bacteria were washed after treatment with CRP. Washing did not affect the cytokine responses observed.

**Peripheral blood cells**

Blood from normal volunteers was drawn into heparinized tubes. Genomic DNA extracted from whole blood was used to determine the presence or absence of the H-131/R-131 alleles of FcγRIIA as described previously (17). Donors heterozygous for FcγRIIA alleles were used in all experiments, except where otherwise indicated. PBMC were obtained by gradient separation using Mono-Poly Resolving Medium (ICN). PBMC were washed three times in RPMI 1640 medium and cultured in 96-well plates at a concentration of 0.5 × 10⁷ cells per well in 200 µl of complete RPMI 1640 medium (containing 10% heat-inactivated FBS and 10–20 µg/ml polymyxin B). PBMC were incubated for 2 h with or without inhibitors before the addition of stimuli. Unless otherwise indicated, PBMC were cultured an additional 24 h after the addition of stimuli. After 24 h, supernatants were collected, centrifuged to remove any cells, and stored at −80°C for cytokine determinations.

**Cytokine determinations**

Cell culture supernatants were assayed for cytokines by flow cytometry using the human inflammation Cytometric Bead Array (CBA) kit from BD Biosciences. TNF-α, IL-1β, IL-10, and IL-8 proteins were also determined individually by ELISA using BD BioSciences reagents.

Levels of cytokine mRNA were determined by RT-PCR using cells collected after 4 h incubation with stimuli. Samples were prepared and analyzed using the hCK-2 or hCK-3 MultiProbe template sets and developing reagents from BD BioSciences.

**Data analysis**

Graphical and statistical analyses were performed using GraphPad Prism software (GraphPad). For experiments testing the inhibition of TNF-α and IL-1β, cytokine levels are presented as a percentage of the amount observed in cultures stimulated with R36a alone. This was done to allow multiple experiments to be combined. Paired t tests were used to compare effects of inhibitors on cytokine synthesis.

**Results**

CRP increases the PBMC cytokine response to *S. pneumoniae* R36a

Protein concentrations of six cytokines were determined by CBA in 24-h culture supernatants from PBMC stimulated with different concentrations of R36a or CRP and R36a. The results are shown in Fig. 1. The addition of CRP greatly enhanced the production of the early proinflammatory cytokines, TNF-α and IL-1β, cytokine levels are presented as a percentage of the amount observed in cultures stimulated with R36a alone. This was done to allow multiple experiments to be combined. Paired t tests were used to compare effects of inhibitors on cytokine synthesis.
alone (10–100 μg/ml) (Table I). PBMC incubated with CRP alone produced IL-6 and IL-8, but at levels much lower than those seen in the presence of R36a. In additional experiments using PBMC incubated with CRP in the absence of bacteria, we have found significant induction of IL-1β, IL-10, and IL-1RA by CRP concentrations ≥100 μg/ml, but low responses to the 25 μg/ml CRP used in the experiments with R36a. These results are consistent with previous reports of CRP-induced cytokine responses in PBMC but do not represent a significant contribution to the levels of cytokines produced in the presence of R36a (34).

The effect of CRP on mRNA levels of these cytokines was examined by RPA (Fig. 2). The combined results of four experiments are shown for a single concentration of R36a (4 × 10^7 CFU) in the presence or absence of CRP and for cells treated with the equivalent concentration of CRP alone (25 μg/ml). The results are consistent with the protein analysis with significant increases in IL-1β and IL-6 in the cells treated with CRP and R36a, compared with R36a alone. Treatment with CRP alone increased mRNA for IL-1, IL-6, IL-1RA, and IFN-γ. The probe for TNF-α was not included in the data shown in Fig. 2 but was analyzed in three additional experiments using a different probe set. CRP did not significantly affect the level of TNF-α mRNA, compared with R36a alone at any concentration of bacteria at the 4-h time point (not shown). This may indicate rapid and transient TNF-α transcription, as seen in mast cells stimulated through FceRI (35), or may indicate that some or all of the increased TNF-α in the 24-h supernatants is released from preformed stores.

CRP binding to ligand is required for the enhanced PBMC TNF-α response to R36a

Additional experiments focused on the ability of CRP to enhance the TNF-α and IL-1β responses to R36a, because these cytokines were most affected by CRP opsonization and both contribute to resistance to pneumococcal infection in vivo (19–22, 24). To determine whether CRP binding to R36a was required for increased TNF-α secretion, CRP was incubated with R36a in the presence of 0.1 mM PC, which blocks CRP binding to S. pneumoniae (Fig. 2).

### Table I. Cytokine responses of PBMC to CRP

<table>
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<th>CRP (μg/ml)</th>
<th>IL-12p70 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
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<td>23.2</td>
<td>183.0</td>
<td>16.7</td>
<td>14.8</td>
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</table>

*PBMC were cultured with CRP for 24 h and cytokine concentrations in the supernatants determined by CBA.

IL-8 concentrations were determined by ELISA.
In the presence of PC, CRP had no effect on the TNF-α response to R36a. In addition, when soluble CRP was added in different concentrations to PBMC with 10^6 CFU/ml R36a, the increased TNF-α response saturated at ~25 μg/ml CRP (Fig. 3B). The binding of CRP to R36a at saturation is 20 μg of CRP/10^6 CFU R36a (36). These results indicate that only CRP bound to R36a was effective in stimulating TNF-α release.

To determine whether CRP bound to a nonbacterial ligand would also induce TNF-α release, complexes of CRP and snRNP particles were tested for stimulation of PBMC. CRP has been shown to precipitate snRNP particles from nuclear extracts, to bind to purified snRNP through the 70 K and D proteins and to react with snRNP particles in cell nuclei (32, 37). The binding of CRP to snRNP particles is similar to binding to S. pneumoniae in that it is calcium-dependent and can be inhibited by PC. PBMC incubated with CRP-snRNP complexes released TNF-α in a dose-dependent manner, although the response was lower than that observed with R36a (Fig. 3C). The release of TNF-α in response to aggIgG is shown in comparison. These results suggest that aggregation of CRP by ligand is sufficient for the induction of TNF-α release.

**Inhibitors of FcγR signaling block the enhanced TNF-α and IL-1β responses to CRP-opsonized R36a**

To determine the role of FcγR in CRP enhancement of the TNF-α and IL-1β responses to R36a, two inhibitors of FcγR signaling pathways were used. Pretreatment of PBMC with 10 ng/ml wortmannin, a PI3K inhibitor, or 10 μg/ml piceatannol, a Syk inhibitor, substantially blocked the effect of CRP on TNF-α and IL-1β release in the presence of 4 × 10^7 CFU R36a (Fig. 4). We determined previously that these inhibitor concentrations block CRP-mediated phagocytosis (38). The IL-8 response to R36a or CRP-treated R36a in the same supernatants was not affected by wortmannin or piceatannol (not shown).

Monoclonal Ab to FcγRI and FcγRIIa block the enhanced TNF-α and IL-1β responses to CRP-opsonized R36a

CRP binds to FcγRI and FcγRIIa on human monocytes (17). To determine the roles of these two receptors in enhanced cytokine release by CRP-R36a, we tested the ability of mAb to each receptor to inhibit the response. We found that the anti-FcγRI (CD64) mAb 10.1, but not an IgG1 isotype control, when added to the cultures at 5 μg/ml inhibited the CRP-dependent increase in TNF-α and IL-1β without affecting the baseline response to R36a (Fig. 5, A and B). However, an F(ab′)_2 of this mAb was not inhibitory at either 5 μg/ml (Fig. 5A) or 10 μg/ml (data not shown). This suggested that an additional interaction with FcγRIIa might be involved, and that the whole anti-CD64 might be inhibiting both receptors by binding to FcγRIIa through its Fc region.

We tested several different mAb specific for FcγRIIa (CD32) and the corresponding isotype controls. As shown in Fig. 6, mAb...
IV.3 (an IgG2b that binds to FcγRIIa), inhibited TNF-α and IL-1β responses to CRP-R36a, but not the responses to R36a alone. mAb AT10 (an IgG1) did not inhibit, and mAb FLI8.26 (an IgG2b which binds to both FcγRIIa and FcγRIIb) inhibited the cytokine responses to both R36a and CRP-R36a (data not shown). Isotype control Abs of the IgG1 (Fig. 5A), IgG2b, and IgG2a subclasses were tested at 5 μg/ml and did not inhibit the TNF-α response to R36a or CRP-R36a. These results suggest that CRP binding to both FcγRI and FcγRIIa contributes to the enhanced cytokine response. The addition of mAb 10.1 and IV.3 together had the same inhibitory effect as either added separately (data not shown).

**Figure 6.** Treatment of PBMC with mAb to FcγRII (CD32) blocks the enhanced TNF-α and IL-1β responses to CRP-treated R36a. PBMC were incubated for 24 h with 4 × 10⁸ CFU R36a or CRP-treated R36a. The anti-CD32 mAb IV.3 or an IgG2 isotype control mAb was added to cultures at 5 μg/ml. TNF-α concentrations (A) and IL-1β concentrations (B) in culture supernatants were measured by ELISA. Values are normalized to R36a alone (n = 3). Significant differences are indicated by *, p < 0.05, or **, p < 0.01, compared with the R36a + CRP control.

**Figure 7.** FcγRIIa allelic polymorphisms affect the enhanced TNF-α response to CRP-treated R36a. PBMC from different individual donors who were homozygous for either the H-131 allele (HH, n = 3) or the R-131 (RR, n = 6) allele of FcγRIIa were incubated for 24 h with 4 × 10⁸ CFU R36a or CRP-treated R36a. TNF-α concentrations in culture supernatants were measured by ELISA and responses of individual donors to R36a and CRP-treated R36a are shown.

**Figure 8.** Mutagenesis of CRP to decrease FcγR binding decreases its ability to enhance the TNF-α response to R36a. PBMC were incubated for 24 h with 4 × 10⁸ CFU R36a or R36a treated with 100 μg/ml CRP and washed. Recombinant wild-type (wt) and T173 mutant CRP purified from T. ni larvae were used. Values from two experiments are combined. *, p < 0.05 for mutant vs wt CRP.

**Discussion**

The results presented here characterize the cytokine response of PBMC to CRP and demonstrate the dependence of this response on FcγR. The ability of CRP to induce proinflammatory cytokines like TNF-α and IL-1β has been previously established by several investigators (34, 41–43). Similar to results presented here, CRP in the absence of ligand produces relatively low levels of TNF-α, IL-1β, and IL-6 (34). In some studies, CRP also induced the anti-inflammatory cytokines IL-1RA and IL-10 (41, 44, 45). CRP also has been shown to markedly up-regulate the IL-1β and IL-1RA
response to LPS (41, 46). Unlike previous studies, we have determined that CRP binding to a natural ligand, S. pneumoniae, can substantially up-regulate the production of proinflammatory cytokines. The levels of cytokines produced are much higher than those found with CRP alone. Because CRP also increases LPS-induced responses, we carefully removed LPS from CRP and added poly-L-lysine to cultures. CRP also was shown to induce a proinflammatory cytokine response to an unrelated multivalent ligand, the snRNP particle, to which CRP binds with high affinity. Similar results were obtained with CRP bound to a polyvalent cation, poly-L-lysine (data not shown). Thus, it seems likely that optimal interaction of CRP with FcγR to induce cytokine responses requires interactions with multiple receptors and therefore requires binding to a multivalent ligand. The ability of soluble PC to inhibit the response to CRP-treated R36a supports this model, which also is consistent with the requirement for cross-linking in FcγR signaling (47).

This study demonstrates that the induction of proinflammatory cytokine synthesis (TNF-α, IL-1β) in PBMC cultures by CRP is dependent on FcγR activation. CRP binds to both FcγRI and FcγRIIa on human monocytes (17), and these receptors signal pathways that include Syk and PI3K (47). Treatment of cultures with inhibitors of these kinases decreased the cytokine response to CRP-opsonized R36a back to the level of bacteria alone. We confirmed the requirement for FcγR binding by showing that a CRP mutant that lacks receptor binding also decreased TNF-α stimulating ability (33). The wild-type CRP was fully active in inducing TNF-α further demonstrating that other human proteins that could potentially contaminate the purified CRP were not required.

CRP-dependent cytokine responses were inhibited by mAb to both FcγRI and FcγRIIa. Inhibition was equally effective using mAb to either one or both receptors, suggesting a cooperative response. Others have demonstrated cooperation between FcγRI and FcγRIIa in the uptake of CRP-opsonized particles (39). It is likely that multiple factors determine which of these receptors plays a dominant role in CRP-mediated responses. FcγRI expression is increased by cytokines such as IFN-γ, and this treatment dramatically increases CRP binding to monocytes (17). Other factors such as protease activation of the receptors increase the ability of CRP to bind to FcγRII (18). CRP also shows higher affinity binding to the R-131, compared with the H-131 allelic form of FcγRIIa (39) and neutrophil responses to CRP as measured by calcium influx studies required the R-131 allele (17, 39). Other investigators did not find a difference in responses to CRP between donors homozygous for the R-131 and H-131 allele (48). That study examined neutrophil IL-8 release, phagocytosis, and respiratory burst to CRP-opsonized S. pneumoniae, which may account for the different results. The preferential binding of CRP to the R-131 allele is a difference in affinity and not an absolute difference between the two forms of the receptor. The higher response of donors homozygous for the R-131 allele to CRP-opsonized R36a is consistent with a role for FcγRIIa in the TNF-α response of monocytes to S. pneumoniae.

This study also demonstrates an important role for soluble pattern recognition molecules such as CRP, LBP, and mannose binding protein in the innate response of monocytes and macrophages to bacteria. S. pneumoniae infection induces a strong cytokine response by the host. This response is inflammatory and is both required for host defense and responsible for many of the clinical characteristics of pneumococcal infection. Peptidoglycan and lipoteichoic acid are the primary inflammatory S. pneumoniae cell wall components that interact with TLR2 to induce cytokine release (29). The intracellular protein peumolysin, which is released following bacterial lysis, also is inflammatory and has been reported to stimulate responses through TLR4 (49). Recently, the acute phase protein, LBP, was shown to bind to the peptidoglycan component and enhance the response to S. pneumoniae cell walls through TLR2 (30). Human monocytes respond to S. pneumoniae by producing TNF-α, IL-1β, IL-8, and NO, but the amount of TNF-α produced in response to pneumococcal cell wall components is low, compared with the response to LPS (28). This cytokine response is crucial for mobilization of the innate immune response to the infection. TNF-α increases cellular infiltration into tissue sites of infection, increases bactericidal activity of phagocytic cells, and may protect against systemic damage in pneumococcal infection (50). Agents that enhance the early production of these danger signals are expected to increase the ability to fight the infection. CRP is a part of the innate immune response and is secreted rapidly in response to IL-6 and IL-1. The findings presented here indicate that CRP is capable of ramping up this early innate response. A protective role for CRP in S. pneumoniae bacteremia has been described previously by our laboratory and others. In these infection models, CRP protection is, for the most part, complement dependent and FcγR independent. Based on the current study and studies of the role of TNF in S. pneumoniae infection, we propose that CRP interaction with FcγR may be beneficial in a pulmonary infection model. However, this type of response also would be expected to have deleterious effects in pneumococcal meningitis where the inflammatory response is a major cause of morbidity and mortality.

CRP binds to PC moieties of lipoteichoic acid and teichoic acid of the pneumococcal cell wall. These PC residues are important binding sites for several choline-binding proteins that are virulence factors for the organism and also may interact with platelet-activating factor receptors on cells to facilitate transport of bacterial across cell barriers (51). Lipoteichoic acid lacking PC is less stimulatory for monocytes (28). However, the effect of LBP on cytokine responses to pneumococcal cell walls was independent of PC (30). Thus, the effects of the two acute phase reactants, CRP and LBP, are likely to be additive because they act through different binding sites on the bacterial cell wall and different receptors on the responding cells.

S. pneumoniae produces an acute, fulminant systemic disease in man. The induction of a rapid response by the innate immune system is crucial for the protective, lifesaving response by the host. Recognition of S. pneumoniae by CRP in a pattern recognition manner before the adaptive immune response may provide for a more rapid and intense response by the host, laying the foundation for the full response by the immune system. CRP binding to the organism, at concentrations below those found during the full acute phase response can trigger FcγR-bearing cells to produce protective, inflammatory cytokines. Studies from our laboratory and others indicate that, once acute phase levels of CRP are achieved, it plays a regulatory role in the inflammatory response (45, 52, 53).

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Disclosures

The authors have no financial conflict of interest.

References


