# C-Reactive Protein Enhances Immunity to *Streptococcus pneumoniae* by Targeting Uptake to FcγR on Dendritic Cells[1]

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**C-reactive protein (CRP) is an acute phase reactant with roles in innate host defense, clearance of damaged cells, and regulation of the inflammatory response. These activities of CRP depend on ligand recognition, complement activation, and binding to FcγR. CRP binds to phosphocholine in the *Streptococcus pneumoniae* cell wall and provides innate defense against pneumococcal infection. These studies examine the effect of this early innate defense molecule on the development of Abs and protective immunity to *S. pneumoniae*. Dendritic cells (DC) initiate and direct the adaptive immune response by integrating innate stimuli with cytokine synthesis and Ag presentation. We hypothesized that CRP would direct uptake of *S. pneumoniae* to FcγR on DC and enhance Ag presentation. CRP opsonization of the R36a strain of *S. pneumoniae* increased the uptake of bacteria by DC. DC pulsed with untreated or CRP-opsonized R36a were transferred into recipient mice, and Ab responses were measured. In mice challenged with free R36a, CRP opsonization resulted in higher secondary and memory IgG responses to both phosphocholine and pneumococcal surface protein A. Furthermore, mice immunized with DC that had been pulsed with CRP-opsonized R36a showed increased resistance to intranasal infection with virulent *S. pneumoniae.* The effects of CRP on Ag uptake, Ab responses, and protection from infection all required FcR γ-chain expression on DC. The results indicate that innate recognition by CRP enhances effective uptake and presentation of bacterial Ags through FcγR on DC and stimulates protective adaptive immunity. *The Journal of Immunology,* 2007, 178: 7283–7291.**

*Streptococcus pneumoniae* is the most common cause of community-acquired pneumonia in the United States and an important cause of otitis, meningitis, and bacteremia (1, 2). Colonization with *S. pneumoniae* is found in 40% of individuals in the general population, and invasive infection is initiated when colonizing organisms spread to the lungs, paranasal sinuses, or middle ear. If the infection is not effectively cleared, bacteremia or meningitis may follow. The innate immune system provides the stimulus for an early inflammatory response, which is essential in preventing invasive infection, but also contributes to morbidity and mortality in pneumococcal meningitis and sepsis (3). The risk of invasive pneumococcal disease is greatest among the very young, the elderly, and immunocompromised individuals. Protective adaptive immunity to *S. pneumoniae* is conferred by Abs specific for bacterial polysaccharide and protein Ags. Current vaccines induce Abs to capsular polysaccharides and are serotype specific. A recent large retrospective study of older adults found that the pneumococcal polysaccharide vaccine did not reduce the risk of community-acquired pneumonia (4). Thus, there is a need for new approaches to immunization against pneumococcal infection. We used intranasal infection of mice under anesthesia to simulate human pneumococcal pneumonia, which is usually initiated by aspiration of organisms from the nasopharynx.

C-reactive protein (CRP)[3] was discovered >75 years ago in the serum of patients acutely ill with pneumococcal pneumonia as a precipitin for the C-polysaccharide of the *S. pneumoniae* cell wall (5). The reactivity of CRP for C-polysaccharide is calcium dependent and specific for phosphocholine (PC) groups on the teichoic and lipoteichoic acids of the cell wall (6). CRP is not specifically induced by *S. pneumoniae* infection, but is a part of the acute phase response. CRP is normally present in the blood at <5 μg/ml, but is rapidly synthesized in the liver following injury, infection, or trauma to reach acute phase levels as high as 100–500 μg/ml (7, 8). CRP interacts with the immune system by activating the classical complement pathway leading to opsonization (9, 10) and by direct binding to receptors on leukocytes leading to phagocytosis and cytokine synthesis.

We established the identity of the leukocyte receptors for CRP as FcγRI and II for both human and mouse cells (11–13). FcγRI is an activating receptor that signals through the associated FcR γ-chain (14). FcγRII is a single-chain receptor. Mice have a single form of this receptor, FcγRIIb, which contains an inhibitory motif in its cytoplasmic tail. In humans, there are three FcγRII forms with highly homologous extracellular regions (14). Human FcγRIIa and c are activating receptors and FcγRIIb is an inhibitory receptor.

CRP is expressed only at low levels in the mouse (<2 μg/ml). However, human and rabbit CRP activate mouse complement and human CRP binds to mouse FcγRI and FcγRIIb (15, 16). For these reasons, passive administration or transgenic expression of human or rabbit CRP in mice has been used extensively for in vivo studies of CRP effects on infection, inflammation, and autoimmunity (17–24). CRP, either injected or produced from a transgene, is protective

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[3] Abbreviations used in this paper: CRP, C-reactive protein; DC, dendritic cell; BMDC, bone marrow-derived DC; PC, phosphocholine; Pn3, *S. pneumoniae* serotype 3; PspA, pneumococcal surface protein A; CM-DiI, chloromethylbenzamido derivative of 3,3′-dioctadecylindocarbocyanine; PC-BSA, PC conjugated BSA.

in mouse models of pneumococcal bacteremia (23, 24). The mechanism of protection supported by most studies is CRP binding to *S. pneumoniae* resulting in increased complement-dependent opsonization and clearance (25, 26). However, other activities of CRP, such as regulation of the cytokine response, may also play a role (27–29). A polymorphism in the promoter of the *CRP* gene is associated with a nearly 10-fold increase in mortality in patients with pneumococcal bacteremia, suggesting that CRP influences the course of invasive *S. pneumoniae* infection in humans (30). We recently reported that CRP increases human PBMC secretion of TNF-$\alpha$ and IL-1$\beta$ in response to *S. pneumoniae* (28).

Although both innate and adaptive immunity play roles in protection against pneumococcal pneumonia and bacteremia, little is known about the contribution of the innate response to the development of adaptive immunity during infection. The effect of CRP on the Ab response to *S. pneumoniae* during infection has not been reported. However, CRP selectively inhibited the anti-PC response to heat-killed *S. pneumoniae* by epitope blocking (31). Mice transgenic for human CRP also were resistant to infection with *Salmonella* (18, 24). In that study, Ab responses were determined 14 days after infection with an avirulent *Salmonella* strain and the IgG2a Ab response to *Salmonella* was significantly higher in CRP-transgenic mice.

The current study used a DC transfer model to evaluate the effect of CRP on the uptake and presentation of pneumococcal Ags. DC coordinate signals from the innate immune response to provide appropriate Ag presentation and costimulation to T cells. Studies in which DC were incubated with *S. pneumoniae* in vitro and then used to immunize recipients helped to define the requirements for protein- and polysaccharide-specific Ab responses to intact *S. pneumoniae* (32–34). Transfer of DC pulsed in vitro with killed *S. pneumoniae* elicited primary Ab responses against both protein and polysaccharide Ags in recipients. Mice immunized with *S. pneumoniae*-pulsed DC also had increased secondary Ab responses following challenge with free bacteria (32).

We hypothesized that CRP binding to *S. pneumoniae* would target the bacteria to Fc$\gamma$R on DC and increase the effectiveness of Ag presentation. Targeting Ag to Fc$\gamma$R increases Ab responses to soluble Ags and cytotoxic T cell responses to tumor cells (35, 36). We examined the uptake of CRP-opsonized killed *S. pneumoniae* R36a by DC and its effect on DC maturation markers and cytokine synthesis. We further compared the ability of DC pulsed with CRP-opsonized or nonopsonized R36a to transfer protective immunity. Finally, we examined the Ab response to *S. pneumoniae* in mice after immunization by DC transfer. The results show that transfer of DC pulsed in vitro with CRP-opsonized killed R36a provides protection against challenge with virulent serotype 3 *S. pneumoniae* (Pn3) 4 wk after immunization. The transfer of DC pulsed with CRP-opsonized R36a also produced greater secondary and memory IgG responses following challenge with free R36a than transfer of DC pulsed with unopsonized *S. pneumoniae*. The increases in Ab responses and protection from infection were not seen in DC from FcR $\gamma$-chain$^{-/-}$ mice, which lack the ability to signal through the activating Fc$\gamma$R (Fc$\gamma$RI, Fc$\gamma$RIII, and Fc$\gamma$RIV) (14, 37). Thus, we found that CRP targeting of Ag to Fc$\gamma$R on DC enhanced Ab responses and protection from bacterial infection. The ability of CRP to interact with Fc$\gamma$R provides an early innate pathway for enhancing the subsequent development of specific Ab and protective immunity.

## Materials and Methods

### *Reagents*

Human CRP was purified from pleural fluid by affinity chromatography on PC-Sepharose, gel filtration, and Fast protein liquid chromatography ion exchange on MonoQ as described previously (38). 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 tested for endotoxin by a quantitative chromogenic Limulus amebocyte lysate assay (Cambrex). If needed, endotoxin was removed on an Etox Acticlean column (Sterogene) so that final CRP preparations contained <0.3 ng endotoxin/mg protein.

Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* as a $His_6$-tagged fusion protein and purified by Ni-NTA affinity chromatography as previously described (39). PC-BSA was prepared at a PC:BSA ratio of 15:1 as described elsewhere (40).

### *Mice*

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). FcR $\gamma$-chain$^{-/-}$ mice on a B6 × 129 background (B6; 129 P2-Fcer1g$^{tm1Rav}$/J) were purchased from The Jackson Laboratory and bred at the Department of Veterans Affairs Animal Facility. FcR $\gamma$-chain$^{-/-}$ mice on a C57BL/6 background (B6.129 P2-Fcer1g$^{tm1Rav}$ N12) and (B6 × 129)$F_1$ mice were purchased from Taconic Farms. Mice were used between 8 and 14 wk of age. All results shown except those in Fig. 5 used DC derived from FcR $\gamma$-chain$^{-/-}$ mice on the B6 × 129 background. All DC transfer experiments used wild-type recipients. All experimental procedures in which mice were used were approved by the Institutional Review Board of the Department of Veterans Affairs Medical Center.

### *Bacteria and CRP opsonization*

Pn3 and R36a (a rough unencapsulated variant of type 2 *S. pneumoniae*) were purchased from the American Type Culture Collection. Pn3 were passaged through mice to maintain virulence and stored at −80°C in 10% glycerol and 5% DMSO. Before use, colonies from overnight blood agar plates were inoculated into Todd-Hewitt broth with 0.5% yeast extract and grown to mid-log phase. The concentration of bacteria 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 to kill the bacteria and destroy pneumolysin activity. R36a was stored at −80°C in sterile PBS. For CRP opsonization, R36a were preincubated for 30 min at 37°C with 100 $\mu$g/ml CRP in HBSS with calcium. Binding of CRP to R36a is saturated at this concentration with 20 $\mu$g of CRP bound/$10^8$ CFU R36a (41). Where indicated, PC was added to the preincubation mixture at a concentration of 0.1 mM to inhibit CRP binding to R36a.

### *DC culture*

Jaws II is an immortalized DC line derived from bone marrow cultures of C57BL/6 mice deficient in p53. Jaws II cells have surface markers characteristic of myeloid DC and can be activated to secrete cytokines and present Ag (42). Jaws II cells were purchased from the American Type Culture Collection and grown as recommended in Alpha Minimum Essential Medium (Invitrogen Life Technologies) supplemented with 4 mM L-glutamine, 20% non-heat inactivated FCS, 20 $\mu$g/ml gentamicin, and 10 ng/ml GM-CSF (PeproTech).

Bone marrow-derived DC (BMDC) were isolated and grown as previously described by Inaba et al. (43), with slight modifications. Briefly, bone marrow cells were depleted of T and B lymphocytes using mouse pan T and mouse pan B immunomagnetic Dynabeads (Dynal Biotech). Cells were grown in 24-well culture plates at $10^6$ cells/ml/well in IMDM with 50 $\mu$g/ml gentamicin, 10% heat-inactivated FCS, 4 mM L-glutamine, 50 $\mu$M 2-ME, and 10 ng/ml murine GM-CSF (PeproTech). After 4 days in culture, 10 ng/ml murine IL-4 (PeproTech) was added. After 6–8 days of culture, nonadherent and semiadherent cells were collected and combined. The cells were washed twice in complete IMDM, resuspended at $10^6$ cells/ml, and any remaining macrophages were removed by plastic adherence for 1 h at 37°C (32). BMDC prepared in this way were ~90% CD11c positive and had DC morphology.

### *Bacterial labeling and phagocytosis assay*

Bacteria were labeled with a lipophilic fluorescent label, chloromethylbenzamido derivative of 3,3′-dioctadecylindocarbocyanine (CM-DiI; Molecular Probes) to measure uptake by DC (32). R36a were washed in HBSS and incubated in the dark at $10^7$ CFU/ml with 5 mM CM-DiI for 10 min at 37°C and 30 min on ice. The bacteria were washed twice in HBSS containing calcium and incubated with 100 $\mu$g/ml CRP for 30 min at 37°C.

To measure uptake of CM-DiI-labeled R36a, day 6–8 BMDC were removed by gentle pipetting and washed in IMDM with 10% FCS, 4 mM L-glutamine, and 50 $\mu$M 2-ME in the absence of GM-CSF and IL-4. The cells were placed in 24-well plates at a concentration of 1 × $10^6$ cells/ml/well. CRP-opsonized R36a or R36a were added to the cells at a ratio of 800 bacteria to 1 DC. The plate was centrifuged at 1200 × *g* for 15 s and then

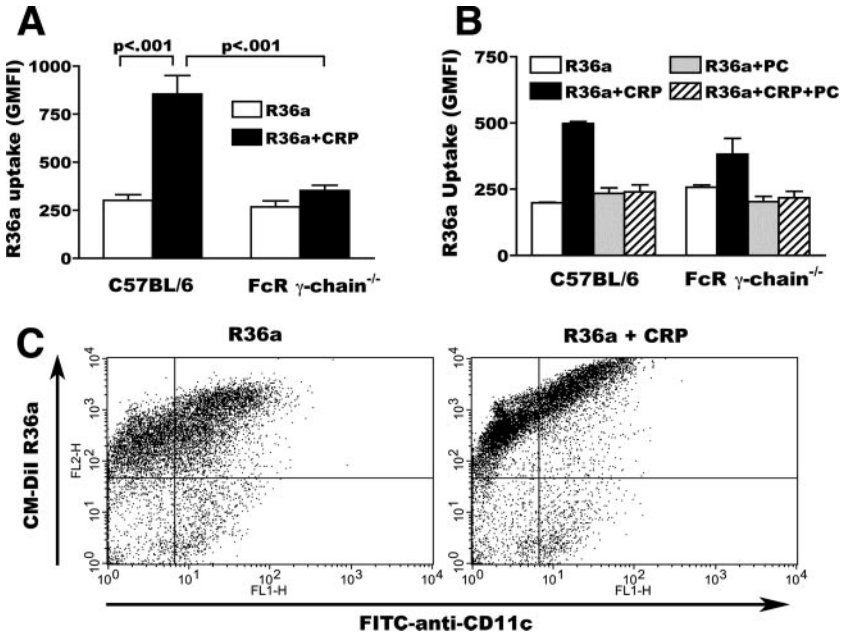


**FIGURE 1.** Increased uptake of CRP-opsonized R36a by wild-type, but not by FcR $\gamma$-chain$^{-/-}$ DC. *A*, R36a were labeled with CM-DiI, treated with 100 $\mu$g/ml CRP for 30 min, and added to BMDC at a ratio of 800:1 (R36a:DC). Cells were incubated for 1 h, then washed and stained with FITC anti-CD11c. Uptake of R36a was determined by two-color flow cytometry. Results are presented as the CM-DiI fluorescence of the CD11c$^+$ cells. Mean $\pm$ SEM of four experiments. *B*, The same experiment was done with the addition of $10^{-4}$ M PC where indicated. Mean $\pm$ SEM of two experiments. *C*, Representative two-color flow diagrams for C57BL/6 DC after incubation with R36a (*left*) or CRP-treated R36a (*right*).

incubated at 37°C in 10% $CO_2$ for 1 h. Excess free bacteria were removed by centrifugation at 1200 $\times$ *g* in cold PBS containing 0.05% $NaN_3$ and 0.1% BSA (PAB). The cells were stained with FITC-conjugated hamster anti-CD11c (clone HL3; BD Pharmingen) or an isotype control. The cells were washed in PAB and fixed in 2% paraformaldehyde in PBS. Uptake of R36a by DC was analyzed by two-color flow cytometry as the geometric mean of the FL2 fluorescence (due to CM-DiI-labeled R36a) associated with the CD11c$^+$ cells (identified by FL1 fluorescence).

### *Protocol for DC pulsing and transfer*

Day 6–8 BMDC were collected and macrophages were removed by adherence. BMDC were transferred into sterile Teflon beakers, allowed to settle for 30 min, and incubated with R36a or CRP-treated R36a. Jaws II cells were treated in the same way without the adherence step. For in vivo transfer, DC were pulsed with 800 bacteria per DC for 4.5 h in IMDM with 10% FCS, 4 mM L-glutamine, 50 $\mu$M 2-ME, 10 ng/ml GM-CSF, and 10 ng/ml IL-4. Cells were then extensively washed to remove noningested bacteria and the pulsed DC pellet was resuspended in PBS at 5 $\times$ $10^6$ cells/ml. Mice were injected with 0.2 ml of DC i.p. and boosted 28 days later with 0.2 ml of 5 $\times$ $10^6$ CFU/ml heat-killed R36a i.p.

### *ELISA for serum Abs to PC*

Immulon 2 plates (Thermo Labsystems) were coated with PC-BSA (5 $\mu$g/ml) in PBS overnight at 4°C and blocked with 0.1% gelatin in TBS-0.05%

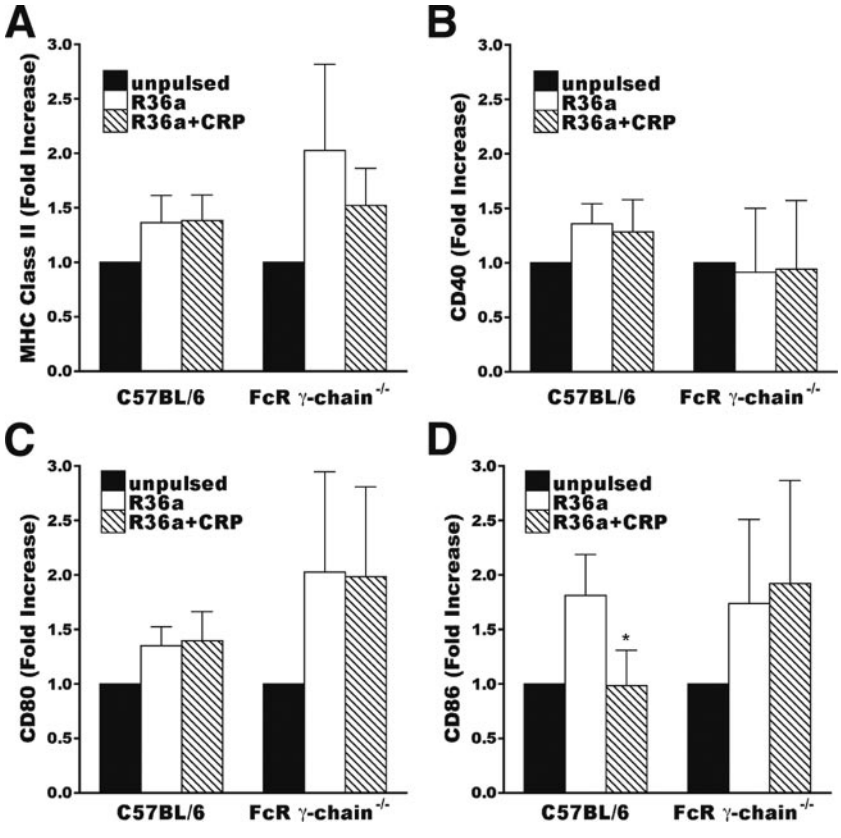


**FIGURE 2.** Effect of CRP and R36a on DC maturation markers. R36a were incubated with 100 $\mu$g/ml CRP for 30 min and added to BMDC at a ratio of 800:1 (R36a:DC). DC were stained for maturation markers after 20 h. The geometric mean fluorescence intensity for each marker relative to the isotype control was determined. Results from three experiments were normalized to the unpulsed DC control to allow presentation of combined data. The markers were MHC class II (*A*), CD40 (*B*), CD80 (*C*), and CD86 (*D*). Significant differences between R36a and R36a + CRP are indicated by $*$, $p < 0.05$.

Tween 20 at 37°C for 2 h. The plates were washed with TBS-Tween 20 and serum samples diluted in TBS-Tween 20 were added and incubated for 1.5 h at 37°C or overnight at 4°C. The plates were washed and biotin-conjugated goat anti-mouse IgG or IgM Ab (Caltag Laboratories) was added and incubated for 1.5 h at 37°C. Plates were washed and developed with streptavidin (SA)-HRP followed by substrate (McIlvain's buffer containing 1 mg/ml ABTS and 0.01% $H_2O_2$). The plates were then read at $A_{405}$ on a VERSAmax microplate reader (Molecular Devices) and analyzed with SOFTmax PRO software (Molecular Devices). Standard curves were established using a mouse IgG2a mAb to BSA (BSA-33; Sigma-Aldrich). Serum samples were analyzed at different dilutions and a dilution within the range of the assay was determined for each time point. All samples from a single experiment were analyzed at the same time. Results are expressed as the $A_{405}$ multiplied by the serum dilution to allow comparisons of multiple time points.

### *ELISA for serum Abs to PspA*

Immulon 4 plates (Thermo Labsystems) were coated with recombinant PspA (5 $\mu$g/ml) in PBS overnight at 4°C and blocked with 1% BSA in PBS overnight at 4°C. The plates were washed with PBS-0.05% Tween 20 and serum samples diluted in PBS-Tween 20 were added and incubated overnight at 4°C. Plates were then washed with PBS-Tween 20, followed by biotin-conjugated anti-IgG Abs for 1.5 h at 37°C. Plates were washed and developed with SA-HRP followed by substrate (tetramethylbenzidine and $H_2O_2$ in a buffered solution; BD Pharmingen). Plates were read at 450 nm. Data were analyzed and presented as described for the anti-PC ELISA, except that a pool of anti-PspA-positive sera was used to establish the standard curve.

### *Analysis of DC surface Ags by flow cytometry*

All steps were performed on ice. FcRs were blocked with 2.5 $\mu$g/ml/$10^6$ cells anti-CD16/32 mAb (clone 2.4G2; American Type Culture Collection) in PAB. Cells were stained by incubation for 30 min with biotinylated, FITC, or PE-conjugated mAbs (BD Pharmingen) specific for CD11c (clone HL3), I-$A^b$ (clone AF6-120.1), CD40 (clone 3/23), CD80 (16-10A1), and CD86 (clone GL1). Biotinylated Abs were detected with allophycocyanin-conjugated SA (allophycocyanin-SA). Irrelevant isotype- and species-matched mAbs were used as staining controls. Staining controls included single-color staining for PE, FITC, and allophycocyanin, unlabeled cells, and isotype- and species-matched mAbs. After staining, the cells were washed three times in PAB. Cells not requiring allophycocyanin-SA were fixed with 2% paraformaldehyde in PBS. Allophycocyanin-SA was then added and incubated for 30 min. The cells were washed three times in PAB and then fixed.

Cells were analyzed using a BD Biosciences FACSCalibur flow cytometer with CellQuest software. Data were collected from a minimum of 30,000 cells. All fluorescence data were collected on a log scale and results are reported as the change in geometric mean fluorescence intensity compared with the isotype control.

### *Cytokine ELISAs*

ELISA kits for mouse IL-10, IL-12, and TNF-$\alpha$ were purchased from BD Pharmingen and used according to the manufacturer's recommendations.

### *Infection with Pn3*

Pn3 were administered to anesthetized mice by intranasal instillation of 50 $\mu$l/mouse. Mice infected with virulent Pn3 were housed in microbial barrier cages in a laminar flow unit. Mice were monitored for signs of morbidity and euthanized when they were no longer ambulatory or were having severe difficulty breathing using guidelines approved by the Institutional Review Board of the Department of Veterans Affairs Medical Center. The challenge dose was $5 \times 10^6$ CFU/mouse, verified by serial dilution plating on blood agar plates.

### *Data analysis*

Graphical and statistical analyses were performed using GraphPad Prism version 4.0. The level of significance was considered to be $p < 0.05$. One-way ANOVA and Bonferroni's post test analysis were done to assess significant differences at various time points as well as to assess the overall curve differences between groups in the Ab response experiments. Survival curves were plotted according to the method of Kaplan and Meier and compared by the log-rank test (Mantel-Haenszel test). This analysis takes into account the time of death as well as the absolute numbers of mice surviving. Other methods of analysis used are shown in the figure legends.

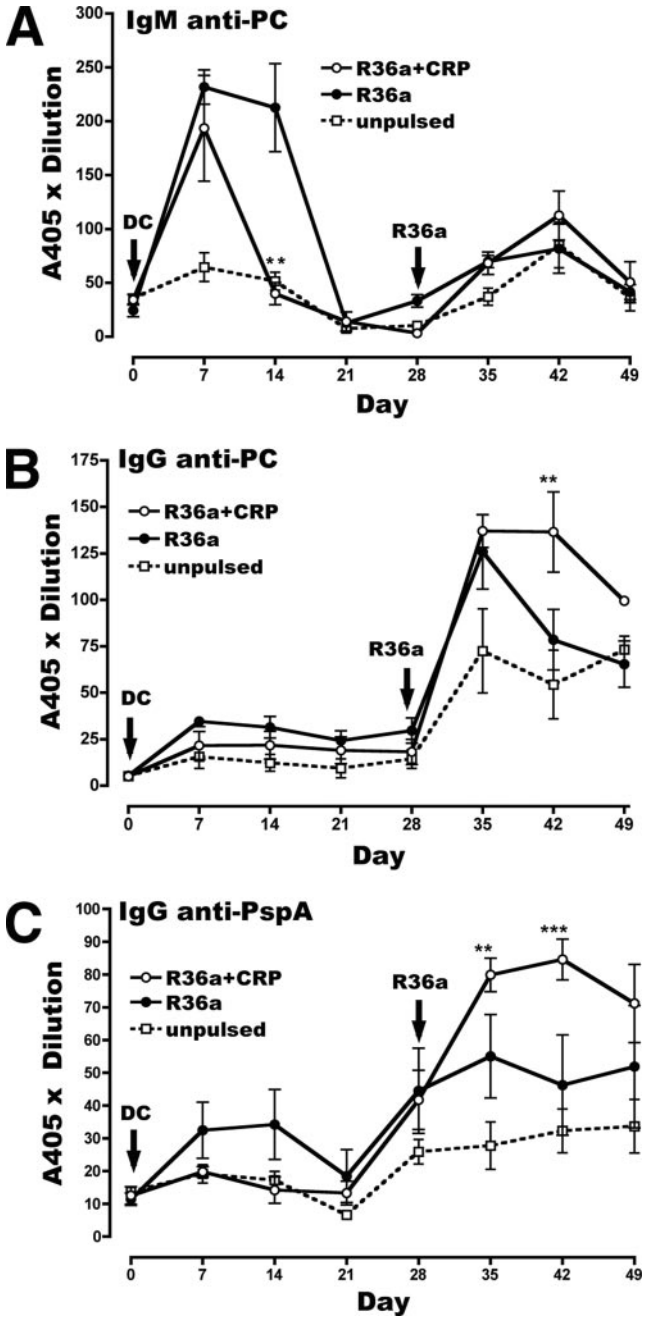


**FIGURE 3.** CRP opsonization increases IgG responses to PC and PspA in recipients of DC pulsed with R36a. BMDC from C57BL/6 mice were pulsed for 4.5 h with R36a or CRP-treated R36a and washed extensively to remove free bacteria. Recipient mice ($n = 5$/group) were injected with $10^6$ DC i.p. on day 0. On day 28 (arrow), all mice were injected i.p. with $5 \times 10^6$ free R36a. Serum was collected weekly for Ab determinations by ELISA. Results are expressed as the absorbance readings multiplied by the serum dilution. Data were analyzed by one-way ANOVA. Significant differences between R36a and R36a + CRP groups are indicated by **, $p < 0.01$; ***, $p < 0.0001$. Results are representative of two independent experiments using BMDC. *A*, IgM anti-PC response. *B*, IgG anti-PC response. *C*, IgG anti-PspA response.

## Results

### *CRP increases uptake of R36a by DC through FcγR*

DC pulsed with a nonencapsulated strain of *S. pneumoniae* R36a have been used to induce Ab responses to shared pneumococcal Ags, PC, and PspA in mice (32). We postulated that CRP opsonization would

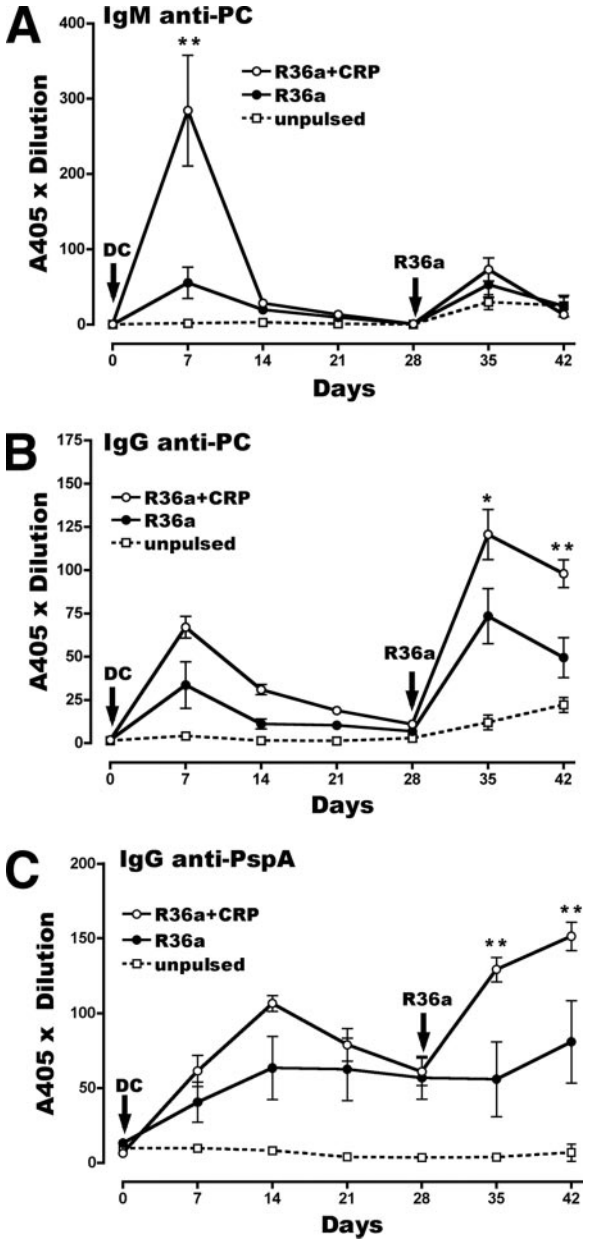


**FIGURE 4.** CRP opsonization increases IgG responses to PC and PspA in recipients of Jaws II DC pulsed with R36a. The Jaws II DC line was pulsed for 4.5 h with R36a or CRP-treated R36a and washed extensively to remove free bacteria. C57BL/6 recipient mice ($n = 5$/group) were injected with $10^6$ Jaws II DC i.p. on day 0. On day 28 (arrow), all mice were injected i.p. with $5 \times 10^6$ free R36a. Serum was collected weekly for Ab determinations by ELISA. Results are expressed as the absorbance readings multiplied by the serum dilution. Data were analyzed by one-way ANOVA. Significant differences between R36a and R36a + CRP groups are indicated by **, $p < 0.01$; ***, $p < 0.0001$. Results are representative of two experiments using the Jaws II C57BL/6 DC cell line transferred into C57BL/6 recipients. *A*, IgM anti-PC response. *B*, IgG anti-PC response. *C*, IgG anti-PspA response.

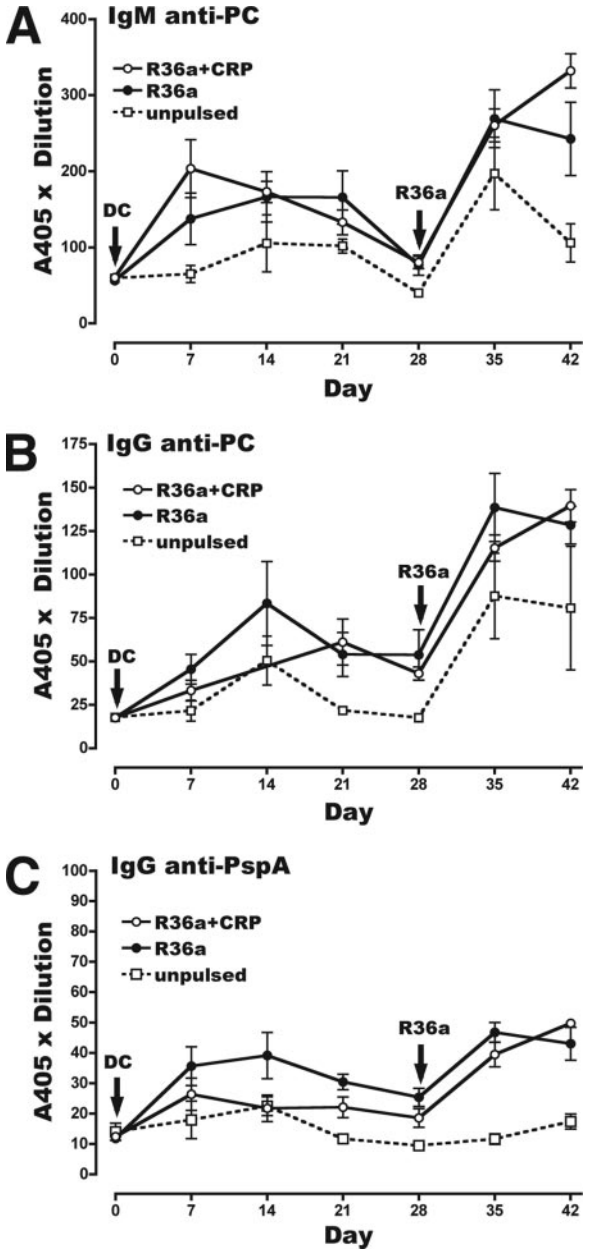


**FIGURE 5.** CRP opsonization has no effect on Ab responses to PC and PspA in C57BL/6 recipients of FcR γ-chain$^{-/-}$ (N12) DC pulsed with R36a. BMDC from C57BL/6 FcR γ-chain$^{-/-}$ mice were pulsed for 4.5 h with R36a or CRP-treated R36a and washed extensively to remove free bacteria. C57BL/6 recipient mice ($n = 5$/group) were injected with $10^6$ BMDC i.p. on day 0. On day 28 (arrow), all mice were injected i.p. with $5 \times 10^6$ free R36a. Serum was collected weekly for Ab determinations by ELISA. Results are expressed as the absorbance readings multiplied by the serum dilution. Data were analyzed by one-way ANOVA. No significant differences were seen between the R36a and R36a + CRP groups. Results are from one experiment using C57BL/6 FcR γ-chain$^{-/-}$ DC transferred into C57BL/6 recipients. Similar results were seen in two additional experiments using B6 × 129 FcR γ-chain$^{-/-}$ DC transferred into B6 × 129 recipients. *A*, IgM anti-PC response; *B*, IgG anti-PC response; and *C*, IgG anti-PspA response.

increase the ability of pulsed DC to induce Ab responses in recipients by promoting uptake of Ag through FcγR. Initial studies were performed in vitro to determine whether CRP opsonization would increase the uptake of R36a by DC and whether uptake required FcγR. This was tested first in a 1-h phagocytosis assay in the absence of GM-CSF and IL-4. R36a were labeled with CM-DiI and uptake by CD11c$^+$ BMDC was measured by two-color flow cytometry (Fig. 1). Preincubation of R36a with CRP increased uptake more than 3-fold using C57BL/6 BMDC (Fig. 1). CRP had no significant effect on the uptake of R36a by BMDC from FcR γ-chain$^{-/-}$ mice. We have previously shown that CRP binds to the γ-chain-associated receptor FcγRI (15) and that the FcR γ-chain is required for CRP-dependent

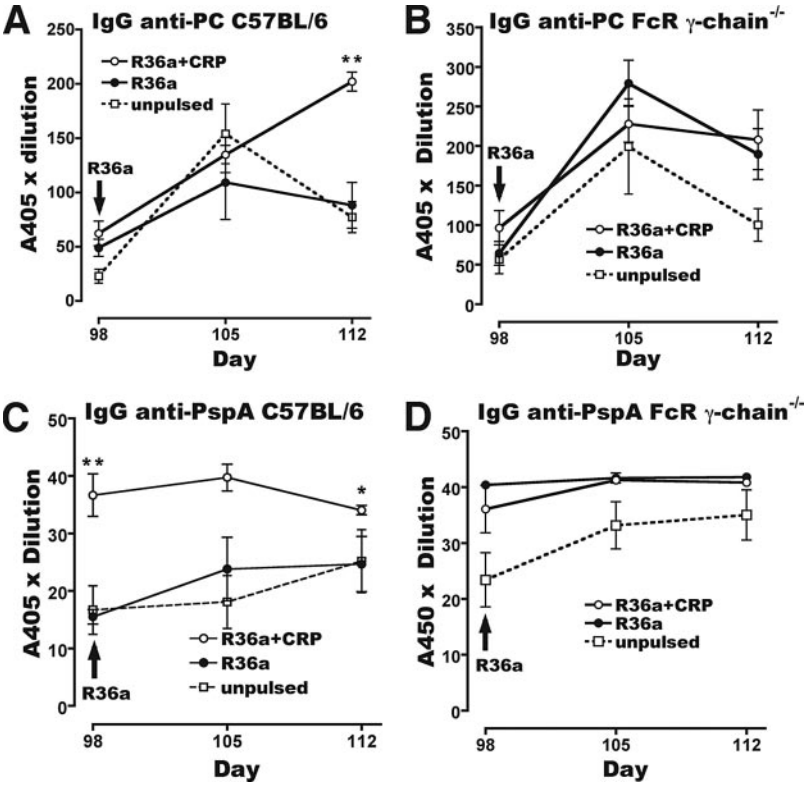


**FIGURE 6.** CRP opsonization increases late IgG responses to PC and PspA in C57BL/6 recipients of DC pulsed with R36a. Ab responses to PC and PspA in C57BL/6 recipients of C57BL/6 DC or B6 × 129 recipients of B6 × 129 FcR γ-chain$^{-/-}$ DC pulsed with R36a or CRP-R36a. DC were pulsed for 4.5 h with R36a or CRP-treated R36a and washed extensively to remove free bacteria. Recipient mice ($n$ = 5/group) were injected with $10^6$ DC i.p. on day 0. On day 28 (data not shown) and day 98 (arrow), all mice were injected i.p. with $5 \times 10^6$ free R36a. Serum was collected weekly for Ab determinations. Results are expressed as the absorbance readings multiplied by the serum dilution. Data were analyzed by one-way ANOVA. Significant differences were seen between the R36a and R36a + CRP groups in the recipients of wild-type (*A* and *C*), but not DC from FcR γ-chain$^{-/-}$ mice (*B* and *D*). *, $p < 0.05$; **, $p < 0.01$. *A* and *B*, IgG anti-PC response; *C* and *D*, IgG anti-PspA response.

phagocytosis of zymosan by mouse macrophages (44). The CRP-dependent increase in DC uptake of R36a was also eliminated by adding PC to the preincubation mixture to inhibit CRP binding to the bacteria (Fig. 1*B*). CRP-treated R36a were not agglutinated compared with control R36a as determined by flow cytometry and absorbance at 600 nm (data not shown).

For immunization experiments, DC were pulsed with R36a for 4.5 h in the presence of GM-CSF and IL-4. At 4.5 H, bacterial uptake is nearly saturated at this ratio of bacteria to DC (32). Therefore, we also tested the effect of CRP on R36a uptake by DC under these conditions. There was a 2-fold increase in uptake of CRP-treated R36a by C57BL/6 BMDC after a 4.5-h incubation at a ratio of 800 bacteria:1 DC ($p < 0.01$; data not shown). This increased uptake was also FcγR dependent, as CRP did not affect R36a uptake by BMDC from FcR γ-chain$^{-/-}$ mice in the 4.5-h assay (data not shown).

### *Effect of CRP opsonization on the expression of DC maturation markers in response to R36a*

Uptake of Ag by DC in vivo and stimulation through TLR or other innate receptors stimulates their migration from the periphery to lymph nodes and their maturation into efficient APC. Maturation of DC is characterized by increased expression of MHC and co-stimulatory molecules that facilitate Ag-specific interactions with lymphocytes. DC maturation may be stimulated by recognition of microbial determinants through innate recognition receptors, including TLR, and by cytokines. To determine the effect of CRP opsonization on the DC response to R36a, DC were cultured in medium alone or with R36a, CRP-opsonized R36a, or 10 μg/ml LPS as a positive control. Expression of MHC class II, CD40, CD80, and CD86 was measured by flow cytometry after 4.5, 12, or 20 h of culture. Expression of DC maturation markers was increased by LPS, R36a, or CRP-opsonized R36a at all three incubation times. The data from the 20-h incubation with medium alone, R36a, and CRP-opsonized R36a are shown (Fig. 2). The level of expression of MHC class II, CD40, and CD80 was increased to a similar degree by R36a or CRP-opsonized R36a (Fig. 2, *A–C*). When results of three independent experiments were analyzed, there were no significant differences in the expression of MHC class II, CD80, or CD40 by DC incubated with CRP-opsonized R36a compared with R36a. A consistent difference in expression was seen for CD86 (Fig. 2*D*). CD86 expression was increased by R36a to a much greater extent than by CRP-opsonized R36a. This difference was significant ($p < 0.03$) when results from three independent experiments were analyzed using the paired $t$ test. No CRP-dependent difference in CD86 expression was found when DC from FcR γ-chain$^{-/-}$ mice were used (Fig. 2*D*).

### *CRP opsonization does not affect cytokine secretion by DC stimulated with R36a*

DC secretion of cytokines regulates adaptive immune responses. We therefore tested the effect of incubation of DC with R36a or CRP-opsonized R36a on secretion of three cytokines, TNF-α, IL-10, and IL-12, that influence Ab responses to *S. pneumoniae* (45). DC incubated with R36a or R36a and CRP synthesized all three cytokines after 20 h in culture. Despite the ability of CRP to increase the uptake of R36a, it had no significant effect on cytokine secretion compared with incubation with R36a alone (data not shown). CRP also had no effect on cytokine secretion by DC from FcR γ-chain$^{-/-}$ mice stimulated with R36a (data not shown).

### *CRP opsonization increases Ab responses to R36a-pulsed DC*

Injection of DC pulsed with intact *S. pneumoniae* elicits Ab responses to PC and PspA and primes recipients for increased Ab responses to subsequent injection of free bacteria (32). We hypothesized that opsonizing R36a with CRP would increase the effectiveness of this pulsed DC vaccine by increasing uptake of bacteria and targeting Ag through FcγR. BMDC from C57BL/6 mice were pulsed with R36a or CRP-opsonized R36a and injected into recipients. Control mice were injected with unpulsed BMDC. Ab

responses to the cell wall-associated hapten, PC, and the protein Ag PspA were measured weekly. Mice were injected with free R36a 4 wk after DC immunization to evaluate secondary Ab responses. The results are shown in Fig. 3. The primary response to R36a-pulsed DC was IgM anti-PC. Primary IgG Ab responses to PC and PspA were low as previously reported. The only significant difference in the primary Ab response to CRP-opsonized R36a was a more rapid decline in the IgM anti-PC response (Fig. 3*A*). However, CRP opsonization resulted in significant differences in the secondary IgG responses to both PC and PspA following injection of free R36a on day 28 (Fig. 3, *B C*). IgG Abs to both Ags were significantly increased in mice that received the CRP-opsonized vaccine at day 0. The experiment was repeated with similar results.

To confirm that Ag presentation occurred through DC, we performed the same experiment using a BMDC cell line, Jaws II, pulsed with R36a or CRP-opsonized R36a (Fig. 4). The results were similar with significantly enhanced secondary IgG responses to both PC and PspA in recipients of DC pulsed with CRP-opsonized R36a (Fig. 4, *B* and *C*). CRP also enhanced the primary IgM anti-PC response in this experiment (Fig. 4*A*). It is unlikely that CRP bound to uningested R36a has this effect, because CRP specifically inhibits anti-PC responses to free *S. pneumoniae* (31). In addition immunization using this vaccine model is dependent on using viable DC that express costimulatory molecules (32).

### *The effect of CRP opsonization on Ab responses to R36a-pulsed DC requires Fc$\gamma$R*

To investigate the requirement for Fc$\gamma$R in this model, BMDC from FcR $\gamma$-chain$^{-/-}$ mice were used in the vaccine. In the experiment shown in Fig. 5, DC were obtained from FcR $\gamma$-chain$^{-/-}$ mice on a C57BL/6 background (N12) and transferred into wild-type C57BL/6 recipients. The DC vaccine was effective in this model, but there were no significant effects of CRP opsonization. The same results were found in two additional experiments using B6 $\times$ 129 FcR $\gamma$-chain$^{-/-}$ DC transferred into (B6 $\times$ 129)$F_1$ recipients (data not shown).

### *CRP opsonization increases memory responses to R36a*

The results of the immunization experiments indicated that DC pulsed with CRP-opsonized R36a produced greater secondary IgG responses to both PC and PspA (Fig. 3). The data suggested that this vaccine might be particularly effective in generating memory to these Ags. To test this, we rested mice for 10 wk after secondary immunization, then challenged them with $5 \times 10^6$ R36a on day 98 after the initial DC immunization (Fig. 6). There was an IgG anti-PC response to this challenge in all three groups of mice. However, the responses in the mice immunized with unpulsed DC and R36a-pulsed DC were equivalent, whereas the responses in mice immunized with CRP-opsonized R36a DC were higher and prolonged (Fig. 6*A*). The IgG response to PspA was elevated in this group of mice even before the R36a injection on day 98 and remained significantly higher than in mice that originally received unpulsed or R36a-pulsed DC (Fig. 6*C*). Ab responses to PC and PspA were similar in all three groups of mice that initially received FcR $\gamma$-chain$^{-/-}$ DC (Fig. 6, *B* and *D*).

### *Immunization with DC pulsed with CRP-opsonized R36a protects against* S. pneumoniae *infection*

We next evaluated the effectiveness of the R36a-pulsed DC vaccine in *S. pneumoniae* pulmonary infection. Mice were immunized as before using DC pulsed with R36a or CRP-opsonized R36a. Unpulsed DC were injected into the control group of mice. After 4 wk with no additional injection of bacteria, the mice were challenged intranasally with Pn3 under anesthesia. Approximately

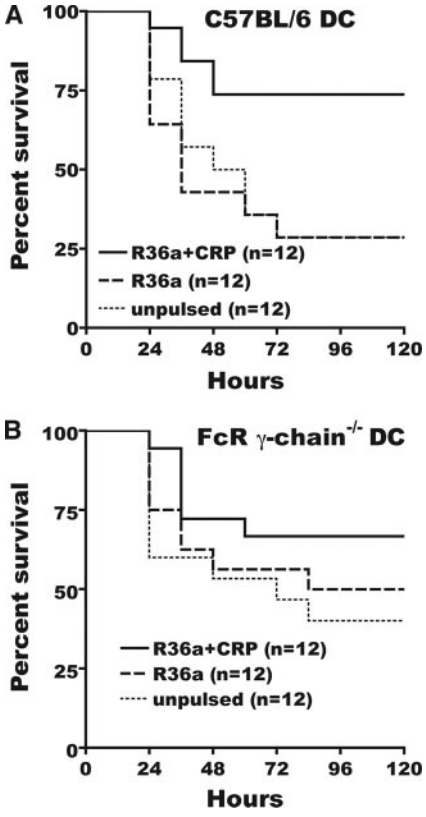


**FIGURE 7.** CRP opsonization increases survival in recipients of DC pulsed with R36a challenged with Pn3. BMDC were pulsed for 4.5 h with R36a or CRP-treated R36a and washed extensively to remove free bacteria. Recipient mice were injected with $10^6$ DC i.p. on day 0. On day 28, all mice were infected intranasally under anesthesia with $5 \times 10^6$ Pn3. *A*, Survival of C57BL/6 mice immunized with C57BL/6 DC that were unpulsed or pulsed with R36a or CRP-opsonized R36a. *B*, B6 $\times$ 129 mice immunized with BMDC from FcR $\gamma$-chain$^{-/-}$ mice that were unpulsed or pulsed with R36a or CRP-opsonized R36a. Two experiments were combined ($n = 12$/group). For the C57BL/6 mice, $p < 0.05$ for immunization with CRP-treated R36a vs either other group. For the recipients of FcR $\gamma$-chain$^{-/-}$ DC, there were no significant differences in survival among the three groups.

75% of mice in the control group and the group of mice immunized with the R36a-pulsed DC vaccine succumbed to the infection. However, mice immunized with DC that had been pulsed with CRP-opsonized R36a showed a significant increase in survival with only ~25% mortality (Fig. 7*A*). The results are combined from two independent experiments using six mice in a group. The same experiment was repeated with FcR $\gamma$-chain$^{-/-}$ mice. In these mice, survival was equivalent in all three groups, indicating that vaccination with R36a-pulsed DC was not effective despite CRP opsonization.

## Discussion

DC are highly responsive to innate signals, including those provided by TLR recognition of bacterial components. Other components of the innate immune system, including CRP, natural Abs to PC, complement, and specific ICAM-3-grabbing nonintegrin-related 1 (SIGN-R1) provide early protection in *S. pneumoniae* infections (46–48). The current studies focused on the effect of CRP recognition of *S. pneumoniae* on the subsequent development of adaptive immunity. CRP, in contrast to IgM natural Abs and complement, provides an additional mode of signaling by its interaction with Fc$\gamma$R on DC. Targeting soluble and tumor Ags to Fc$\gamma$R

on DC generally enhances Ag processing and increases immunity (35, 36, 49). Previous experiments have shown that DC pulsed with heat-killed *S. pneumoniae* and injected into mice induce anti-polysaccharide and anti-protein Ab responses (32). We used this model to show that CRP binding to *S. pneumoniae* before incubation with DC directs the bacteria to Fc$\gamma$R and thereby increases Ab responses both to *S. pneumoniae*-pulsed DC and to subsequent immunization with free bacteria.

The results show that CRP increases the uptake of heat-killed intact *S. pneumoniae* R36a by DC. The increased uptake was dependent on FcR $\gamma$-chain expression, was inhibited by PC, and was not associated with agglutination of the bacteria. DC pulsed with CRP-opsonized R36a also produced higher Ab responses in recipients. It is unlikely that this increased Ab response was solely due to increased transfer of Ag, because primary Ab responses in recipients were for the most part not affected by CRP. In previous experiments using this DC transfer method of immunization, there was no detectable Ag on the surface of the transferred cells (32). We have previously demonstrated CRP inhibition of anti-PC, with no effect on anticapsular polysaccharide or anti-PspA responses when immunization is done with free *S. pneumoniae* (Ref. 31 and C. Mold unpublished data). Thus there is no indication that transfer of uningested *S. pneumoniae* contributes to the results.

Targeting Ag to Fc$\gamma$R on DC has been shown to increase Ab responses to soluble Ags and to tumor cells. In previous studies, immune complexes induced DC maturation to a greater extent than Ag alone (49). However, Ags were used that did not stimulate DC maturation on their own. In our model, components of the bacteria provide strong TLR stimuli that increase expression of MHC class II, CD40, and costimulatory molecules CD80 and CD86. The only consistent difference between the DC response to *S. pneumoniae* and CRP-opsonized *S. pneumoniae* was lower CD86 expression when CRP was present. CD80 and CD86 are related molecules expressed on APCs that share counter receptors, CD28 and CTLA-4, on T lymphocytes. CD80 or CD86 binding to CD28 provides T cell activation signals, whereas binding to CTLA-4 restricts T cell activation and is important in terminating T cell responses. The relative roles of CD80 and CD86 in T cell activation and regulation are unclear. Pentcheva-Hoang et al. (50) reported that CD28 accumulation at the immunological synapse was favored by CD86 expression and CTLA-4 accumulation was favored by CD80 expression. These results and others suggest that CD86 expression should favor T cell activation. However, recent work using human peripheral blood T cell responses to allogeneic DC found that CD86 expression blocked T cell proliferation by stimulating regulatory T cells (51). Thus, relative expression levels of CD80 and CD86 are likely important in T cell responses to Ag, but their precise roles are not known. It is also possible that the lower expression level of CD86 on DC stimulated with CRP and Ag indicates a delayed maturation of these cells, which may promote survival following transfer and enhanced immune responses in the recipients. CD86 expression is a later event during DC maturation than expression of MHC class II, CD40, and CD80. CRP treatment of human monocyte-derived DC has been reported to delay their maturation in vitro (52).

Cytokine production by DC during Ag presentation directs the subsequent adaptive immune response by influencing the differentiation of Th cells. Immune complex binding to Fc$\gamma$R has been shown to increase IL-10 and decrease IL-12 secretion by DC responding to soluble Ag (53). We have previously reported that CRP increases IL-10 production in mouse macrophage cultures and in vivo (17). However, in those experiments, costimulation with CRP and LPS was required for IL-10 synthesis. CRP increases secretion of TNF-$\alpha$ and IL-1$\beta$ by human PBMC stimulated with lower numbers of R36a (28). However, CRP opsonization of R36a under the conditions used to transfer immunity did not have a major effect on DC secretion of TNF-$\alpha$, IL-10, or IL-12. Any effect of CRP may have been obscured by the strong stimulation of pattern recognition receptors by components of the bacteria.

The effects of CRP opsonization on Ab responses were most pronounced when IgG secondary and memory responses were measured. Initial exposure of DC to Ag in the presence of CRP produced a long-lasting effect on recipients, an effect that was still evident 4 mo after the initial immunization. Mice initially immunized with CRP-opsonized bacteria by DC transfer produced enhanced IgG Abs to both PC and PspA following injection of free bacteria. These increased IgG responses were seen using either BMDC or the DC cell line Jaws II to present Ag. However, when DC from FcR $\gamma$-chain$^{-/-}$ mice were used, CRP opsonization had no effect. Because IgG Abs provide the most protection against *S. pneumoniae* infection, the results suggested that CRP opsonization would increase the effectiveness of a DC-based vaccine.

This was tested directly using mice infected intranasally with virulent Pn3 4 wk after immunization with pulsed DC. The vaccine prepared with CRP-opsonized *S. pneumoniae* was protective against this challenge, despite containing no capsular polysaccharide Ag. The DC vaccine prepared with bacteria alone was no more protective than unpulsed DC, and the vaccine containing CRP-opsonized *S. pneumoniae* was not effective in FcR $\gamma$-chain$^{-/-}$ mice. Although the results are consistent with the enhanced Ab responses that were found, the challenge was given at a time when Ab levels had waned and there were no significant differences in serum Ab levels among the groups of vaccine recipients. There is a discrepancy between the protection from infection and priming for Ab responses in the group immunized with R36a-pulsed DC. Compared with the control group, these mice showed increased Ab responses but no protection from infection. Thus, the protection provided by the transferred DC is either not directly attributable to anti-pneumococcal Ab, is due to differences in Abs at other sites such as the respiratory tract, or reflects a very rapid Ab response following the intranasal inoculation. A rapid IgM anti-PC Ab response that correlated with resistance to infection and was dependent on SIGN-R1 expression was observed 24 h following intranasal infection with Pn3 (47). It is possible that previous immunization with CRP-opsonized *S. pneumoniae* enhanced this very early response or increased the mucosal Ab response.

The possible role of T cells in the protective effect of CRP has not been examined to date. It has been determined that T cells, in the absence of Abs can protect mice from fatal bacteremia after intranasal immunization and challenge (54). In addition, T cells, and not Abs, are required for elimination of the colonization of the nasopharynx by *S. pneumoniae* (55, 56). Alternatively, the protective effect of CRP in this model may be related to its anti-inflammatory activities, which have been described in several models of acute inflammation and autoimmune disease (57). The ability of DC to regulate inflammatory as well as immune responses is an area of current interest, and we are actively investigating the ability of CRP to generate such regulatory DC.

## Disclosures

The authors have no financial conflict of interest.

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