Recognition and functional activation of the human IgA receptor (FcαRI) by C-reactive protein

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Edited* by Jeffrey V. Ravetch, The Rockfeller University, New York, NY, and approved February 11, 2011 (received for review December 8, 2010)

C-reactive protein (CRP) is an important biomarker for inflammatory diseases. However, its role in inflammation beyond complement-mediated pathogen clearance remains poorly defined. We identified the major IgA receptor, FcαRI, as a ligand for pentraxins. CRP recognized FcαRI both in solution and on cells, and the pentraxin binding site on the receptor appears distinct from that recognized by IgA. Further competitive binding and mutational analysis showed that FcαRI bound to the effector face of CRP in a region overlapping with complement C1q and Fcγ receptor (FcγR) binding sites. CRP cross-linking of FcαRI resulted in extracellular signal-regulated kinase (ERK) phosphorylation, cytokine production, and degranulation in FcαRI-transfected RBL cells. In neutrophils, CRP induced FcαRI surface expression, phagocytosis, and TNF-α secretion. The ability of CRP to activate FcαRI defines a function for pentraxins in inflammatory responses involving neutrophils and macrophages. It also highlights the innate aspect of otherwise humoral immunity-associated antibody receptors.

serum amyloid P component | CD89 | acute phase protein

C-reactive protein (CRP), a member of the pentraxin family, is a major acute-phase protein in humans and is a clinical marker of infection (1). Interest in the biological activities of CRP has increased dramatically in recent years because of its association with inflammatory diseases such as atherosclerosis and autoimmune diseases such as systemic lupus erythematosus. Other pentraxins include serum amyloid P component (SAP), pentraxin 3 (PTX3), neuronal pentraxin 1 (NPTX1) and neuronal pentraxin 2 (NPTX2). They are innate pattern-recognition molecules targeted to various microbial and self determinants including polysaccharides, phosphocholine, and phosphoethanolamine on the surface of microorganisms, apoptotic or necrotic cells, and nuclear autoantigens. CRP and SAP are produced in the liver in response to inflammatory cytokines such as IL-6 and IL-1. Although the role of CRP in pathogen clearance through complement activation has been established (2), the participation of pentraxins in activating cellular immune functions is poorly understood because of a lack of knowledge of their cellular receptors. CRP and SAP have been shown to bind and activate Fcγ receptors (FcγR) on monocytes and macrophages (1, 3–6). In addition, CRP suppressed immune complex-mediated nephrotoxic nephritis in a mouse model (7). Despite their distinct folds, both antibody and pentraxins bind FcγR in a 1:1 stoichiometry, obligating pathogen opsonization or immune complex formation as the mechanism for receptor clustering and activation (6, 8, 9). In addition, they share an overlapping binding site on FcγR, predicting a mutually exclusive FcγR association between antibodies and pentraxins.

Human macrophages and neutrophils express a major receptor for IgA, Fcα receptor I (FcαRI)/CD89, which activates through the common Fc receptor (FcR) γ-chain. FcαRI activation by IgA immune complexes leads to phagocytosis, antigen presentation, and the release of cytokines, superoxide, and other inflammatory mediators (10). Despite sharing the common γ-chain for signaling, IgA and IgG antibodies recognize their own receptors and do not cross-react. The structural recognition of IgA by FcαRI is distinct from that of IgG by Fcγ receptors (FcγR) (8, 11). Nevertheless, the ability of pentraxins to bind FcγR with broad specificities and the functional similarity between FcγR and FcαRI prompted us to investigate whether pentraxins recognize the receptor for IgA. Here we identify FcαRI as a receptor for pentraxins. The establishment of specific interactions of pentraxins with FcR provides insight into the mechanism by which these soluble pattern-recognition molecules activate macrophages and neutrophils. The finding also reveals a role for FcαRI in the innate immune response.

Results

Pentraxins Recognize FcαRI in Solution. CRP and SAP first were shown to bind FcγRI-transfected cells and activate phagocytosis through FcγRI and FcγRIIa (4, 5). More recently, a systematic solution binding study revealed a broader recognition between pentraxins and all isoforms of FcγR (6). This broader recognition between pentraxins and FcγR is supported by their closely related structures, in that CRP and SAP share identical structural folds and form similar pentamers. Similarly, FcγR consist of homologous tandem Ig-like domains with IgG binding sites located in the two structurally similar membrane proximal domains (8, 12, 13). The permissive pentraxin–FcγR recognition led us to investigate further pentraxin recognition of other FcR, including an IgA receptor, FcαRI, and an IgE receptor, FcεRI. Both FcαRI and FcεRI consist of two tandem Ig-like domains. Functionally, FcαRI and FcεRI share a common signaling γ-chain with FcγR and participate in antibody-mediated inflammation, phagocytosis, and cytokine release. To examine whether pentraxins interact with these FcR, recombinant FcαRI and FcεRI were immobilized on CM5 BIAcore sensorchips together with FcγRIIa (CD32A) as a control. The binding with various dilutions of CRP, SAP, or PTX3 as the analytes showed that CRP and SAP, but not PTX3, bound to immobilized FcαRI, with affinities of 2.8 ± 0.2 and 3.2 ± 0.2 μM, respectively (Fig. 1), similar to their binding to FcγR (6). The kinetic rate constants for CRP and SAP binding to FcαRI are quite different. Although CRP binding to FcαRI (K_a = 3.1 ±1.4 × 10^5 $M^{-1}{\cdot}s^{-1}$; K_d = 0.35 ± 0.02 s^{-1}) resembles the pentraxin and IgG binding to the low-affinity FcγR (6), the SAP binding to FcαRI (K_a = 1.5 ± 0.5 × 10^4 $M^{-1}{\cdot}s^{-1}$; K_d = 0.031 ± 0.008 s^{-1}) displays slower kinetic association and dissociation rates. The molecular basis for the observed differential rate constants between CRP and SAP binding to FcαRI is not clear. FcαRI is located genetically on human chromosome 19 in a region close to the leukocyte receptor complex (LRC) that encodes killer cell Ig-like receptor (KIR), immunoglobulin-like transcript/leukocyte Ig-like receptors (ILT/LILR), and natural killer cell p46 (NKp46) (14,

Author contributions: J.L., K.D.M., L.L.M., and R.W. performed research; and C.M., T.W.D.C., and P.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018369108/-/DCSupplemental.

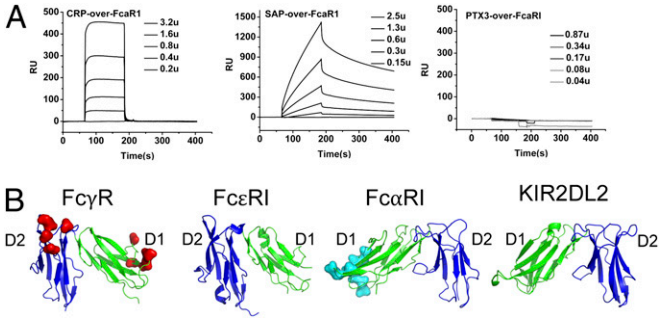

Fig. 1. CRP and SAP bind to FcαRI in solution. (*A*) The binding between serial dilutions of CRP, SAP, or PTX3 in micromolar concentrations (u) and immobilized FcαRI on a CM5 sensor chip. (*B*) The D1 (green) and D2 (blue) domains of FcγRIIa (3D5O), FcεRI (1F2Q), FcαRI (1OW0), and KIR2DL2 (2DL2) are shown in respective D2 orientations. The CRP binding sites on FcγRIIa and the IgA binding site on FcαRI are shown as surface patches.

15). FcαRI is most homologous to NKp46 and KIR, sharing 30–35% in sequence identity. Structurally, FcαRI also resembles KIR and NKp46, with a similar juxtaposition in its two Ig-like domains, which is opposite those in FcγR and FcεRI (Fig. 1*B*). However, CRP failed to bind immobilized FcεRI, KIR, and NKp46 (Fig. S1), suggesting that the pentraxin recognition of FcαRI is specific. The ability of pentraxins to recognize both FcαRI and FcγR further extends the functional similarity between them.

CRP Recognizes FcαRI on Transfected RBL Cells. To determine whether the observed solution binding between pentraxins and FcαRI also occurred on cell surfaces, we investigated CRP binding to RBL cells, a rat basophilic leukemia cell line, stably transfected with a Gly-248 variant of FcαRI (referred to as "G248 cells") (16). The expression of FcαRI on G248 cells can be detected readily by an anti-FcαRI antibody, MIP8a, (Fig. 2). G248 cells and untransfected RBL cells were incubated with CRP, followed by a FITC-labeled anti-CRP antibody (2C10) for FACS analysis (Fig. 2*C*). Greater CRP binding was observed to G248 cells than to untransfected RBL cells, although significant binding to the untransfected cells was observed also, probably because of the binding of CRP to rat FcγR on RBL cells (17, 18). More importantly, the binding of CRP to G248 cells was reduced to the level of RBL cells in the presence of MIP8a but not in the presence of its isotype control. The binding of CRP to G248 cells was dose dependent with an apparent K_d of 0.3 μM (Fig. 2*D*). These results are in agreement with the data obtained by surface plasmon resonance (SPR) measurements and showed that CRP recognizes FcαRI specifically both in solution and on transfected cells.

Pentraxin-Binding Site on FcαRI Is Distinct from That of IgA. The crystal structure of FcαRI in complex with the Fc region of IgA showed that IgA recognizes the N-terminal Ig-like domain of FcαRI (D1) (Fig. 1*B*) (11). This recognition is very different from that of IgG binding to FcγR, which involves both the N- and C-terminal domains (D1 and D2) of the receptor (8, 9). The stoichiometry of these interactions is also different, with IgG binding to FcγR at 1:1, and IgA binding to FcαRI at 1:2. The IgG binding site on FcγR partially overlaps with that of the pentraxins, so that pentraxins compete with IgG for FcγR binding (6). To determine whether pentraxins and IgA share a common binding site on FcαRI, a solution binding competition experiment between CRP and IgA was carried out using soluble FcαRI as analyte on an IgA-immobilized sensor chip. If CRP shares a common binding site with IgA, the addition of CRP to the analyte would be expected to block the receptor binding to immobilized IgA. However, the addition of CRP to FcαRI enhanced the receptor binding to IgA (Fig. 3*A*). The enhanced binding response probably is caused by the binding of the higher molecular mass of the CRP–FcαRI complex to IgA, suggesting that CRP and IgA bind to distinct regions of FcαRI. Using the FcαRI-transfected RBL cells (G248 cells), we observed that although MIP8a blocked both IgA and CRP binding to the transfected RBL cells (Figs. 2*B* and 3*C*), a second anti-FcαRI mAb (A59), which binds to the D2 domain of FcαRI away from the IgA binding site, partially inhibited CRP but not IgA binding to the FcαRI-transfected cells (Fig. 3 *B* and *C*). Similarly, preincubation with IgA failed to block CRP binding to FcαRI on the transfected G248 cells (Fig. 3*B*). Conversely, unlabeled CRP at a concentration of 1.3 μM failed to inhibit IgA (Cy3-labeled) binding to G248 cells at 0.03 or 0.125 μM of IgA (Fig. 3*C*). These results are consistent with CRP and IgA binding to distinct regions of FcαRI and raise the possibility that CRP and IgA could interact simultaneously with FcαRI and potentially costimulate cells.

FcαRI and FcγRIIa Bind to Similar Regions on CRP. The pentameric ring of pentraxins has two faces, a ligand-binding face that recognizes microbial ligands in a calcium-dependent manner and an effector face that interacts with complement C1q and FcγR. To determine whether FcαRI also binds to the effector face of the pentraxins, competitive CRP binding between C1q and FcαRI was carried out using BIAcore with immobilized recombinant FcαRI and FcγRIIa. CRP, when present at 2.7 μM in the analyte, displayed binding similar to that of immobilized FcαRI and FcγRIIa (Fig. 4*A*). In contrast, C1q did not bind either receptor. Because both C1q and FcγRIIa interact with the effector face of CRP, their binding to CRP is mutually exclusive. As expected, the CRP binding to FcγRIIa was partially reduced with the addition of 0.25 μM of C1q to the CRP-containing analyte and was eliminated when the concentration of C1q was increased to 1 μM (Fig. 4*A*). Similarly, the presence of 1 μM but not 0.25 μM of C1q blocked the CRP binding to immobilized FcαRI. Because C1q exists as a hexamer of trimer with each trimeric head capable of binding to one pentameric CRP (19), 0.25 and 1 μM of C1q are

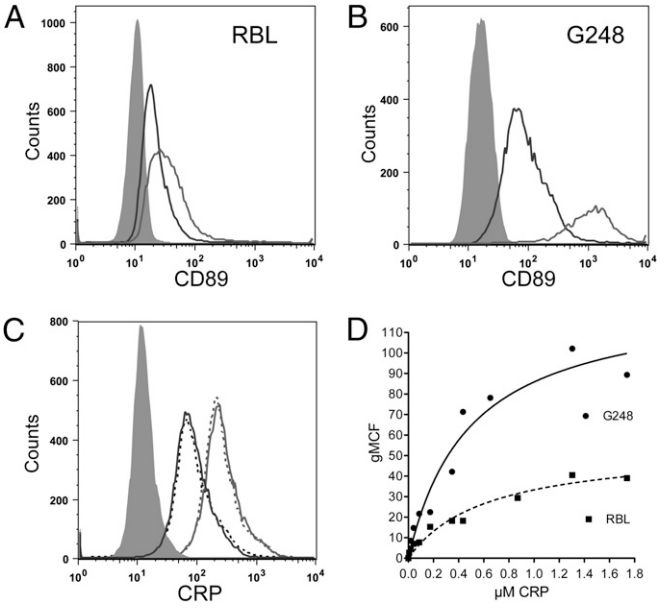

Fig. 2. CRP binds to FcαRI on transfected RBL cells. (*A* and *B*) Anti-FcαRI (MIP8a) (gray) or isotype control (black) staining of RBL cells (*A*) or FcαRI-transfected RBL (G248) cells (*B*). (*C*) CRP (150 μg/mL) bound to G248 cells (gray) better than to RBL cells (black). The CRP binding to G248 cells was blocked by MIP8a (heavy dashed line) but not by its isotype control (thin dashed line). Filled areas represent unstained cells. Horizontal axes show fluorescence intensities. (*D*) Dose-dependent CRP binding to G248 or RBL cells detected using FITC-2C10. Data are representative of at least three experiments.

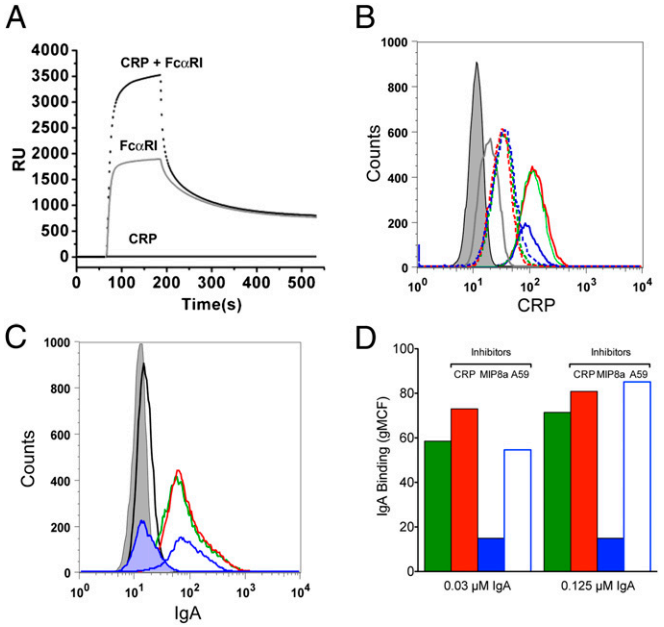

Fig. 3. CRP and IgA bind at nonidentical sites on FcαRI. (*A*) The solution binding response for CRP (2.9 μM), recombinant FcαRI (4.7 μM), or their combination onto an IgA immobilized CM5 sensor chip. CRP alone did not bind IgA. (*B*) The binding of CRP alone (red) in the presence of IgA (1,000 μg/mL) (green), or mAb A59 (blue) to G248 cells (solid lines) and RBL cells (dashed lines). Unstained G248 cells (gray line) and RBL cells (shaded gray) are shown. Horizontal axes of the histograms show fluorescence intensities. (*C*) Cy3-IgA binding to G248 cells alone (green) or in the presence of 150 μg/mL CRP (red), blocking MIP8a (blue shaded), and nonblocking anti-FcαRI mAb A59 (blue line). Unstained G248 cells (black line) and Cy3-IgA–stained RBL cells (shaded gray) are shown. (*D*) Bar graph shows the geometric mean channel fluorescence for Cy3–IgA binding.

expected to titrate a maximum of 1.5 and 6 μM of CRP, respectively. This prediction is consistent with the observed partial or no inhibition of CRP (2.7 μM) binding to FcR at the lower concentration of C1q and the complete blockage of CRP binding at the higher concentration of C1q. The stoichiometric inhibition of CRP binding to FcαRI by C1q suggests that FcαRI also interacts with the effector face of CRP. Thus, CRP recognition of C1q, FcαRI, and FcγR are mutually exclusive.

To determine whether FcγR and FcαRI recognize similar sites on pentraxins, we examined the receptor binding of CRP mutants that previously had been identified as defective in FcγR binding. His-38, Thr-173, and Leu-176 form part of the putative FcR binding site on CRP, and mutations of each one reduced both FcγR and C1q binding significantly (6, 20). When these CRP mutants were assayed for FcαRI binding using BIAcore, H38A and L176A bound to the receptor similarly to wild-type CRP, but T173A showed increased FcαRI binding compared with the wild type (Fig. 4*B*). These mutational data suggest that although both FcαRI and FcγR recognize the same face of CRP, the specific interface residues are likely to differ. This notion is not surprising, because FcαRI adopts a 3D domain arrangement opposite that of FcγR (Fig. 1*B*). The T173A mutant of CRP will provide a useful reagent to look at differential effects of the two receptor classes.

Based on the assumption that FcαRI and FcγR bind to a similar site on pentraxins, a docking model for FcαRI binding to CRP was generated using the crystal structure of the SAP–FcγRIIa complex (Fig. 4*C*). Despite its opposite domain orientation, FcαRI could be docked onto CRP based on the FcγRIIa-complexed SAP structure because of the pentameric symmetry of pentraxins. The model shows that it is possible for FcαRI to interact with pentraxins in a diagonal orientation similar to FcγRIIa in the SAP-complexed structure. However, unlike FcγRIIa, which contacts the A and C subunits of SAP, the opposite D1–D2 hinge angle of FcαRI results in the receptor contacting the A and D subunits of pentraxins (Fig. 4C). The structural model of the CRP–FcαRI complex shows Thr-173 and Leu-176 but not His-38 as the immediate receptor-contacting residues (Table S1), consistent with the mutant binding data showing that the binding of Thr-173 but not His-38 was affected as compared with the wild-type CRP.

CRP Cross-Linking of FcαRI Leads to the Activation of Cellular Functions. As do FcγR and FcεRI, FcαRI associates with the common FcR γ-chain and signals through the γ-chain immunoreceptor tyrosine-based activation motif (ITAM) (10). Cross-linking of FcαRI leads to activation of several kinases including spleen tyrosine kinase (Syk) and ERK (21). We have shown previously that pentraxin recognition of FcγR results in phagocytosis and cytokine secretion by monocytes and macrophages (1, 5, 6, 22, 23). To investigate whether CRP recognition of FcαRI results in receptor activation, we examined both ERK phosphorylation and degranulation in FcαRI-transfected RBL cells (9.4 cells) upon CRP cross-linking. Because RBL cells express FcεRI, which associates with and can compete with FcαRI for the FcR γ-chain, and FcαRI is known to exist in a γ-chain–free form (10), we obtained an FcαRI-transfected RBL cell line, referred to as "RBL 9.4," that expresses a chimeric FcαRI with the cytosolic domain of the receptor replaced by that of the FcR γ-chain (24). ERK phosphorylation was readily detectable in these RBL 9.4 cells upon cross-linking of the receptor either by using MIP8a or by binding IgA followed by anti-IgA cross-linking (Fig. 5*A*). More importantly, the binding of CRP to FcαRI-expressing RBL 9.4 cells followed by cross-linking with anti-CRP antibody (2C10) induced higher levels of ERK phosphorylation by both Western blot and FACS analysis than the same treatment of untransfected RBL cells (Fig. 5 *A* and *B*). CRP-induced ERK phosphorylation was detectable up to 15 min after the cross-linking. RBL cells express the high-affinity IgE receptor, FcεRI, and the FcR γ-chain, and cross-linking of FcεRI by IgE leads to potent degranulation as measured by the release of β-hexosaminidase. This γ-chain–dependent degranulation also was observed in G248 cells upon antibody cross-linking of FcαRI (16). Importantly, significant re-

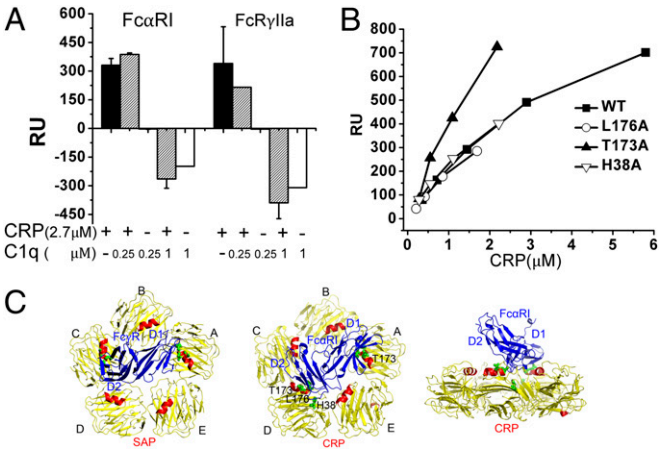

Fig. 4. FcαRI recognizes the CRP effector face. (*A*) Competitive solution binding between C1q and immobilized FcαRI or FcγRIIa to CRP using BIAcore. Recombinant FcαRI and FcγRIIa were immobilized individually on CM5 chips. The analytes were CRP (2.7 μM) in the presence or absence of 0.25 or 1 μM C1q. C1q alone resulted in close to zero response at 0.25 μM and negative responses at 1 μM, probably because of higher binding to the dextran sulfate surface in the reference cell. (*B*) The equilibrium binding responses of wild-type and mutant CRP to immobilized FcαRI. (Sensorgrams are shown in Fig. S2.) (*C*) The structure of the SAP–FcγRIIa complex (3D5O) and the docked CRP–FcαRI model in two orthogonal views. The mutation sites used in *B* are shown by green sticks. The putative interface residues in the CRP–FcαRI model are listed in Table S1.

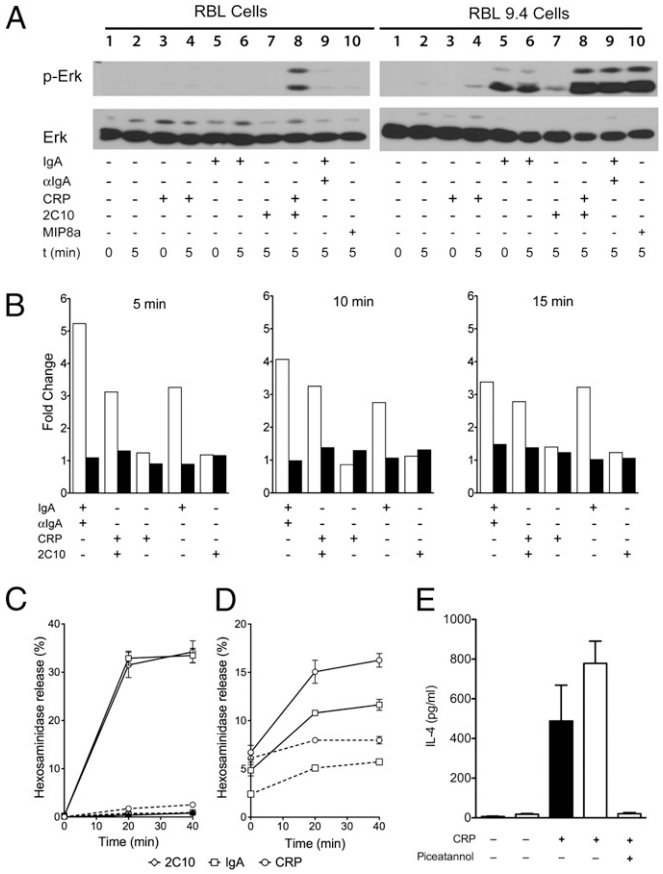

Fig. 5. CRP induces ERK phosphorylation, degranulation, and cytokine secretion in FcαRI-transfected RBL cells. (*A*) FcαRI-transfected 9.4 cells or untransfected RBL cells were preincubated with CRP or IgA (200 μg/mL), then incubated with 2C10, anti-IgA, or MIP8a at time point 0. Cells were lysed at time point 0 and at 5 min, and phospho-ERK was detected by Western blot. Blots were stripped and reprobed for total ERK. Results are representative of three experiments. (*B*) RBL 9.4 cells (open bars) or untransfected RBL cells (solid bars) were treated as in *A*, and ERK phosphorylation was determined at 5, 10, and 15 min by flow cytometry. (*C*) FcαRI-transfected 9.4 cells (solid lines) or RBL cells (dashed lines) were preincubated with CRP (circle) or IgA (square), then cross-linked with 2C10 or anti-IgA. No significant β-hexosaminidase release was detected in 9.4 cells treated with 2C10 alone (diamond). β-Hexosaminidase release was measured and expressed as the percentage of total activity. Mean ± SEM of triplicate wells from one experiment are shown. (*D*) β-Hexosaminidase release was measured as in *C* except on FcαRI-transfected G248 cells with streptavidin cross-linked biotin-CRP. (*E*) IL-4 secretion in G248 cells (open bars) or untransfected RBL cells (solid bars) treated with streptavidin cross-linked with CRP or without piceatannol.

lease of β-hexosaminidase was observed in both RBL 9.4 cells and G248 cells but not in untransfected cells upon CRP cross-linking (Fig. 5 *C* and *D*). The level of degranulation induced by CRP was comparable to that induced by IgA. In addition to degranulation, activated mast cells also produce IL-4 (25, 26). Because RBL is a mast cell line, we tested the level of IL-4 secretion upon cross-linking of FcαRI by CRP in G248 cells. A significant amount of IL-4 was detected upon CRP cross-linking of G248 cells. Further, the cytokine production was inhibited by piceatannol, a known Syk inhibitor of FcR γ-chain signaling in mast cells (27, 28) (Fig. 5*E*). These results suggest that CRP cross-linking activates an FcR γ-chain signaling pathway through FcαRI.

CRP Induces Neutrophil Surface Expression of FcαRI, Phagocytosis, and TNF-α Production. IgA cross-linking of FcαRI induced the receptor surface redistribution into lipid raft-like domains in FcαRI-transfected A20 cells (29). In neutrophils, FcαRI is mobilized rapidly from intracellular granules to the surface by chemokines and other mediators (30). We examined the effect of CRP binding on FcαRI surface expression on neutrophils using confocal microscopy. Labeled RBC coated with pneumococcal C polysaccharide (PnC) as CRP ligands were incubated with neutrophils, and the expression distribution of FcαRI was measured with mAb A59 and AF488-labeled secondary antibody. Interestingly, FcαRI was diffusely distributed on neutrophils, with a significant amount residing in the intracellular compartment (Fig. 6*A*). Binding of CRP-opsonized sheep red blood cells (SRBC) resulted in a sharp, thin layer of the receptor entirely distributed on the surface of treated neutrophils with no detectable intracellular localization of the receptor (Fig. 6*B*). This result suggests that CRP binding induces the surface expression of FcαRI. We next examined the role of CRP binding to FcαRI in phagocytosis of bacteria by neutrophils. *Streptococcus pneumoniae* serotype 27 (Pn27) was used because it expresses the CRP ligand phosphocholine in its capsule as well as its cell wall. FITC-conjugated Pn27 were opsonized with CRP and incubated with neutrophils. Phagocytosis was determined from the FITC intensity associated with neutrophils after quenching extracellular fluorescence. Phagocytosis of Pn27 was increased with CRP opsonization, and preincubation of the neutrophils with the anti-FcαRI (MIP8a) significantly inhibited the phagocytosis of CRP-opsonized Pn27 (Fig. 6*C*). Pretreatment with A59 or an IgG_1 isotype control did not inhibit the phagocytosis. Activated neutrophils produce type 1 inflammatory cytokines, including TNF-α (31, 32). We then examined whether CRP activation of FcαRI induces TNF-α production. The result showed that cross-linking CRP with an anti-CRP antibody (2C10) induced the neutrophils to secrete TNF-α, and the TNF-α production upon CRP or IgA cross-linking was blocked by an Fab fragment of the anti-FcαRI

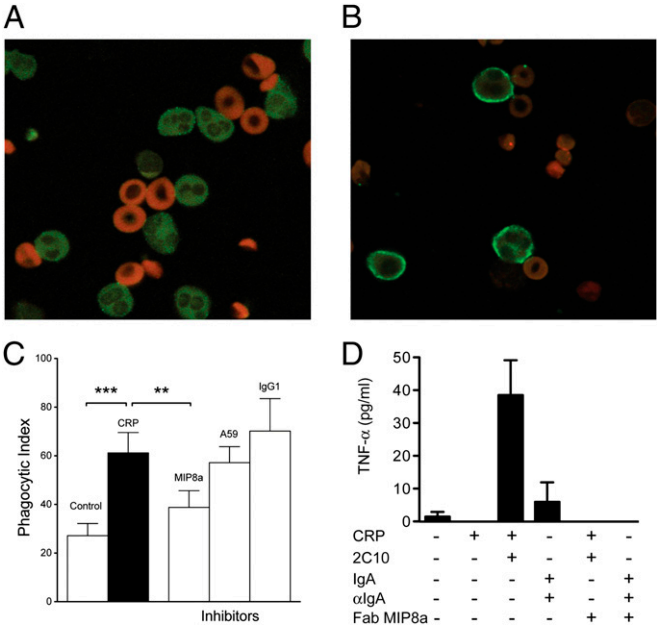

Fig. 6. CRP induces neutrophil FcαRI surface expression, phagocytosis, and TNF-α secretion. (*A* and *B*) Confocal images of neutrophils stained with anti-FcαRI (A59) and AF488 goat anti-mouse (green). Neutrophils were incubated for 30 min at room temperature with PKH26 (red)-labeled (*A*) or CRP-opsonized (*B*) PnC-SRBC. (*C*) Uptake of CRP-opsonized FITC-*S. pneumoniae* by neutrophils, expressed as phagocytic index (bacteria per 100 neutrophils), with or without inhibitors. Data are mean ± SEM of four experiments. ***$P < 0.001$; **$P < 0.01$. (*D*) CRP or IgA (200 μg/mL) cross-linking induced TNF-α release in human neutrophils. The TNF-α secretion upon either CRP or IgA treatment was inhibited by the Fab fragment of MIP8a.

antibody (MIP8a), (Fig. 6*D*). These data suggest that CRP can activate neutrophils effectively through FcαRI.

Discussion

That pentraxins recognize both FcαRI and FcγR is counterintuitive because the two receptors have opposite D1/D2 domain structural arrangements. In addition, IgA and IgG bind their receptors in distinctly different modes, and the two isotypes of antibodies do not cross-react. The ability of pentraxins to recognize both families of FcR probably results from their pentameric structure, which makes it possible to contact the same secondary structure elements from the two receptors with opposite domain arrangements using symmetrical but different pentraxin subunits. However, pentraxin recognition of FcαRI is not entirely the result of its permissive ligand binding, because pentraxins failed to bind FcεRI despite its closer sequence and structural homology than FcαRI to FcγR. Likewise, pentraxins did not recognize other "FcαRI-like" receptors, such as KIR and NKp46. In addition to binding, we showed that CRP cross-linking of FcαRI led to the activation of ERK, degranulation, and cytokine production in FcαRI-transfected cells, as well as to the induction of cell-surface FcαRI expression, phagocytosis of bacteria, and TNF-α release in neutrophils.

FcαRI is expressed primarily on cells of the myeloid lineage, including monocytes, macrophages, neutrophils, and eosinophils (10). Similar to FcγR, the expression of FcαRI is up-regulated by LPS, TNF-α, and other proinflammatory stimulators (33) but is down-regulated by polymeric IgA (34). The regulation of FcR expression by inflammatory mediators coincides with increased serum levels of CRP during the acute-phase response, suggesting their potential involvement in pentraxin-mediated innate immunity, especially early in infection before effective antibody responses. The expression of FcαRI on the surface of neutrophils was increased rapidly in response to chemoattractants, and this increase was shown to be caused by its release from intracellular storage granules (30). Similarly, we found CRP treatment induces redistribution of the receptor to the cell membrane, potentially contributing to the activation of the receptor on macrophages and neutrophils during infection. Because cells expressing FcαRI often express FcγR, it remains to be seen if pentraxins can coengage FcαRI and FcγR and whether such coengagement activates their functions synergistically. Alternatively, it is not clear whether the structural difference between FcαRI and FcγR would result in a different functional outcome in pentraxin-mediated FcR activation, thus contributing to cell-type–dependent pathogen responses.

Macrophages and neutrophils are major innate inflammatory responders to infection. Their effector functions are initiated primarily through the activation of Toll-like receptors (TLR) by microbial and endogenous TLR ligands and of FcR by antibody immune complexes. The recent characterization of pentraxins as ligands for FcγR and currently for FcαRI adds another dimension, an FcR-mediated innate immune response, as a potential contribution to host defense against pathogens, parallel to the TLR pathway. TLR- and FcR-mediated innate immune responses have both similar and contrasting features. TLR and FcR often are coexpressed on myeloid immune cells. Both TLR and FcR expression are regulated by inflammation and infection. Although there are more TLR than FcR, the larger number of TLR presumably reflects their direct recognition of diverse microbial and pathogenic ligands, and TLR are activated directly in response to the increase in the concentration of these ligands. In contrast, FcR recognize a small number of conserved pentraxins and achieve ligand diversity through the pattern recognition of the pentraxin–ligand binding. The activation of FcR then would depend on the increased concentration of pentraxins during infection. It is possible that both microbial activation of TLR pathways and CRP-opsonized microbial pathogen activation of FcR pathways occur concurrently, resulting in synergistic and complementary innate immune responses, and that together they provide a powerful first line of host immune defense.

Materials and Methods

Reagents are listed in *SI Materials and Methods*.

BIAcore Binding Experiments. SPR studies were performed using a BIAcore 3000 (GE Healthcare) with BIAevaluation 4.1 software in 10 mM Hepes (pH 7.4), 0.15 M NaCl, 1.0 mM $CaCl_2$ at a flow rate of 50 μL/min. For affinity analysis, FcγRIIa and FcαRI were immobilized on carboxylated dextran CM5 sensor chips using primary amine coupling. Serial dilutions of SAP, CRP, and PTX3 from 7.2–0.04 μM were added. For C1q competition binding experiments, the analytes consisted of 2.72 μM CRP with 0.4 mg/mL C1q. To measure the competition between human IgA and CRP for FcαRI binding, a CM5 chip was coupled with IgA at levels of 6,000–9,000 resonance units (RU). The analytes consisted of 2.9 μM of CRP with or without 4.7 μM of refolded FcαRI. The dissociation constants were obtained by either steady-state or kinetic curve fittings.

Cell Surface Binding by FACS Analysis. An RBL cell line was stably transfected with a Gly-248 variant of human FcαRI (16). Human FcαRI exists in two common alleles (Gly and Ser) as a result of an SNP at amino acid 248 in the cytoplasmic domain of the receptor gene. The G248 variant of FcαRI produced a more robust proinflammatory cytokines than did the S248 variant in transfected cells as well as in human neutrophils. RBL cells and G248 cells were harvested with trypsin and washed in PBS containing 0.1% BSA and 0.05% sodium azide (PAB). Cells were incubated with CRP or Cy3-IgA for 30 min at 4 °C and washed twice with PAB. CRP binding was detected with an anti-CRP mAb (FITC-2C10). Data were acquired using a FACScan (BD Biosciences) or Accuri flow cytometer (AccuriCytometer, Inc.) and analyzed with FlowJo software (Tree Star, Inc.).

Homology Modeling of the FcαRI-CRP Complex. An initial complex between FcαRI (1OW0) and CRP (1GNH) was prepared by manual superposition of the corresponding components onto FcγRIIa and SAP in the SAP–FcγRIIa complex (3D5O). Docking was performed by tumbling FcαRI over CRP but was largely constrained to the contact interface in the SAP–FcγRIIa complex using the shape-only correlation in Hex5 with standard parameters. After clustering, the three lowest-energy orientations (−371.4 to −392.4 kJ/mol) were selected as the final model.

ERK Phosphorylation Assay. RBL or 9.4 RBL cells were harvested with trypsin and then washed in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM Glucose, 10 mM Hepes, 0.5% BSA, pH 7.4) and resuspended at 5.0×10^6 cells/mL One hundred-microliter aliquots of cells were incubated with buffer or CRP (200 μg/mL) for 1 h at 37 °C. Cells were incubated with 2C10 (40 μg/mL) or buffer. For flow cytometry analysis, cells were fixed with 2% formaldehyde, followed by 90% methanol and washed with PBS plus 4% FBS. Cells were stained with P-p44/42 MAPK (T202/Y204) (1:100) rabbit Ab (Cell Signaling Technologies) for 15 min, washed twice, and then stained with a secondary Alexa Fluor 488 $F(ab')_2$ goat anti-rabbit IgG (1:500) (Invitrogen) for 15 min. For Western blotting, RBL or RBL 9.4 cells were seeded at 1.5×10^6 cells in 60-mm dishes overnight in complete medium. After treatment, cells were washed with ice-cold HBSS and then lysed with HBSS containing 1% Triton X-100 with protease and phosphatase inhibitors (Thermo Scientific). Lysates were incubated for 20 min on ice, centrifuged at $20{,}000 \times g$ for 25 min, separated by 10% SDS/PAGE, and transferred to pvdf membranes. Membranes were probed with P-p44/42 MAPK (T202/Y204) rabbit Ab and then probed with anti-rabbit IgG HRP (Cell Signaling Technologies). Membranes were stripped with Restore (Thermo Scientific) and probed for total ERK using p44/42 MAPK.

Degranulation and IL-4 Production Assays. RBL cells or transfected RBL cells (G248 or 9.4) were cultured overnight in 48-well plates and then washed in Tyrode's buffer. Some cells were incubated with 200 μg/mL IgA or CRP for 1 h at 37 °C. Buffer was removed, and buffer or 40 μg/mL of $F(ab')_2$ anti-IgA or 2C10 was added, and cells were incubated at 37 °C. For G248 cells, 50 μg/mL of CRP aggregates (AggCRP) were added at time point 0, and activity was measured over time. Supernatants were collected, and β-hexosaminidase activity was measured with respect to total release determined by lysis with 1% Triton X-100. Activity was measured by incubation with substrate, 1.4 mg/mL 4-nitrophenyl-*N*-acetyl β-D-glucosaminide in 75 mM sodium citrate, pH 4.5, for 1 h at 37 °C. Reactions were stopped by addition of 0.2 M glycine, pH 10.7, and activity was calculated from the A405 (% release = 100 × supernatant A405/A405 of detergent lysed cells). To assay for IL-4 production, G248 or untransfected RBL cells were seeded into 96-well plates at a density of 2×10^4 and were preincubated with or without biotin-labeled CRP

(100 μg/mL) and/or piceatannol (25 μg/mL) (Sigma) for 30 min, followed by streptavidin (20 μg/mL) (Sigma) cross-linking of CRP. After 20 h incubation at 37 °C, the supernatant was assayed for rat IL-4 production using ELISA (R&D Systems, Inc.) according to manufacturer's instructions. Data shown are mean ± SEM of triplicate wells from one representative experiment.

Confocal Microscopy. Human neutrophils were incubated in chamber slides (Thermo Scientific) for 2 h. PnC-SRBC were incubated with 150 μg/mL of CRP for 45 min at 37 °C, washed, and added to polymorphonuclear leukocytes (PMN) at an 8:1 ratio for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. Slides were treated with Image-iT (Invitrogen). Cells were stained with anti-FcαRI antibody A59, washed, stained with a goat anti-mouse antibody labeled with Alexa Fluor 488 (Invitrogen), washed, and mounted in ProLong Gold Antifade (Invitrogen). Images were acquired using a Zeiss LSM 510 inverted laser scanning microscope.

Neutrophil Phagocytosis and Cytokine Secretion Assays. Neutrophils were purified by Ficoll-Hypaque centrifugation and resuspended at 2×10^6 cells/mL in RPMI-1640 plus 10% FCS. *S. pneumoniae* serotype 27 (Pn27) (ATCC) was grown to log phase, washed in PBS, heat killed, and FITC conjugated. Neutrophils were combined with FITC-Pn27 and 100 μg/mL CRP, centrifuged briefly, and incubated for 30 min at 37 °C. Phagocytosis was measured by mean FITC-fluorescence on gated neutrophils after washing and adding Trypan blue to quench uningested bacteria and expressed in phagocytic index as number of Pn27 ingested/100 neutrophils. For cytokine secretion, neutrophils were treated for 1 h with CRP or IgA (200 μg/mL) with or without the Fab fragment of MIP8a (15 μg/mL). Medium was removed, 2C10 or anti-IgA (40 μg/mL) was added, and cells were incubated overnight at 37 °C. Supernatants were analyzed for TNF-α using an R&D Systems ELISA kit.

ACKNOWLEDGMENTS. We thank Dr. Jeffrey Edberg for providing the FcαRI-transfected G248 RBL cell line, Dr. Renato Monteiro for providing 9.4 RBL cells, and Dr. Barbara Bottazzi for providing the recombinant PTX3. Images were generated in the Cancer Center Fluorescence Microscope Shared Resource, University of New Mexico. This work was supported by intramural research funding from the National Institute of Allergy and Infectious Diseases, by National Research Service Award F31AI080178 (to K.D.M.), by National Institutes of Health Grant R21 AI085414, and by a Merit Review Award from the Department of Veterans Affairs.

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