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Human C-Reactive Protein Binds Activating Fcγ Receptors and Protects Myeloma Tumor Cells from Apoptosis

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SUMMARY

Elevated levels of C-reactive protein (CRP) are present in many disease situations including malignancies and may contribute to the pathogenesis of cardiovascular disorders. This study was undertaken in a myeloma setting to determine whether CRP affects tumor cell growth and survival. We show that CRP enhanced myeloma cell proliferation under stressed conditions and protected myeloma cells from chemotherapy drug-induced apoptosis in vitro and in vivo. CRP binds activating Fcγ receptors; activates PI3K/Akt, ERK, and NF-κB pathways; and inhibits caspase cascade activation induced by chemotherapy drugs. CRP also enhanced myeloma cell secretion of IL-6 and synergized with IL-6 to protect myeloma cells from chemotherapy drug-induced apoptosis. Thus, our results implicate CRP as a potential target for cancer treatment.

INTRODUCTION

C-reactive protein (CRP), the first acute-phase protein described and an ancient and highly conserved protein of the pentraxin family, has five identical subunits forming a planar ring that confers very high stability to the protein. In healthy young adults, the median concentration of CRP is 0.8 mg/l, but following an acute-phase stimulus, values may increase by 10,000-fold, from less than 50 μg/l to more than 500 mg/l (Pepys and Hirschfield, 2003; Stein et al., 2000). Plasma CRP is produced primarily in the liver and synthesized by hepatocytes in response to intermediary inflammatory cytokines such as IL-1 and IL-6. CRP has been shown to bind to a variety of ligands, including pneumococcal polysaccharides, membrane phospholipids, apoptotic cells, fibronectin, and ribonuclear particles (Stein et al., 2000). CRP also binds C1q and activates the classical complement cascade and binds Fcγ receptors (FcγRs) leading to indirect (via classical complement) and direct opsonization (via FcγRs) (Stein et al., 2000). Through these mechanisms, CRP can play a direct role in a wide range of inflammatory processes and contributes to innate host immunity.

CRP is a sensitive systemic marker of inflammation and tissue damage. Elevated levels of CRP are present in patients with infections, inflammatory diseases, necrosis such as myocardial infarction (Pepys, 1983), or malignancies including multiple myeloma (MM) (Bataille et al., 1992; Tienhaara et al., 1994), lymphoma (Legouffe et al., 1998; Pedersen and Bergmann, 2003), and carcinoma (Reichle et al., 2004). Accumulating evidence has strongly suggested that in cardiovascular disease CRP is not only a

SIGNIFICANCE

C-reactive protein (CRP) is a sensitive systemic marker of inflammation and tissue damage. Elevated levels of CRP are detected in many diseases, and CRP may contribute to the pathogenesis of cardiovascular disorders. These findings led to our hypothesis that CRP may have a functional role in tumor cells since elevated levels of CRP are present in cancer patients. This study shows that CRP enhanced myeloma cell proliferation under stressed conditions and protected myeloma cells from chemotherapy drug-, IL-6 withdrawal-, or serum deprivation-induced apoptosis in vitro and in vivo. Thus, our results provide strong evidence for the effect of CRP on tumor cell apoptosis and implicate CRP as a potential target for cancer treatment.

marker of inflammation but also contributes to pathogenesis of the disease (Venugopal et al., 2005). Evidence includes the results that CRP directly activated various vascular cells to secrete cytokines, enhanced their expression of adhesion molecules, increased monocyte/macrophage chemotaxis and adhesion, facilitated extracellular matrix remodeling, enhanced endothelial dysfunction, and activated coagulation (for review, see Berenson et al., 2006; Garcia et al., 2003). Furthermore, human CRP has been shown to increase myocardial and cerebral infarct size in rats subjected to coronary or cerebral artery ligation, respectively, and this drastic enhancement of infarct size by human CRP was completely abrogated by in vivo complement depletion of the rats using cobra venom factor (Gill et al., 2004; Griselli et al., 1999). These findings led to our hypothesis that CRP may also have a functional role in tumor cells since elevated levels of CRP are present in cancer patients (Bataille et al., 1992; Legouffe et al., 1998; Pedersen and Bergmann, 2003; Tienhaara et al., 1994). The present study was undertaken in a myeloma setting to determine whether CRP might affect tumor cell growth and survival. Our study identifies CRP as an important protector of myeloma cell apoptosis.

RESULTS

CRP Protects Myeloma Cells from Apoptosis

To examine the effects of CRP on myeloma cells, different concentrations of highly purified human natural CRP (nCRP; >98% purity by SDS-PAGE) or recombinant CRP (rCRP) from Calbiochem-Novabiochem (La Jolla, CA), dialyzed extensively against PBS to remove sodium azide, were added to cultures of myeloma cell lines ARP-1, MM.1S, U266, and MM1-144, and cell proliferation was examined 48 hr later by ^{3}H-thymidine incorporation assay. The purity of these proteins was verified by our own assay (see Figure S1A in the Supplemental Data available with this article online). The presence of CRP at concentrations as high as 50 μg/ml did not affect myeloma cell proliferation (Figure S1B and S1C). Similarly, CRP did not affect the survival of myeloma cells measured by Annexin-V binding (Figure S1D) and TUNEL (data not shown) assays in cells cultured for 48 hr. However, the addition of CRP to cultures of primary myeloma cells, which otherwise undergo spontaneous apoptosis ex vivo, significantly enhanced cell proliferation (Figure 1A) and reduced cell death, as shown by the results comparing myeloma cells from two patients ($p < 0.05$ to $p < 0.01$; Figure 1B) and the pooled results using cells from 8 other MM patients ($p < 0.05$ to $p < 0.01$; Figure 1C), although the degree of protection varied among patients. Myeloma cells likely did not produce CRP because no detectable CRP protein was found in their culture supernatants by ELISA. Because both nCRP and rCRP had almost identical effects on myeloma cells, nCRP was used in the following experiments.

As the previous data indicated that CRP affected cell proliferation and survival under stress conditions, we investigated the effects of CRP on stressed myeloma cell lines. First, we examined whether CRP protects myeloma cells from serum starvation-induced apoptosis. As shown in Figure 2A, a dose-dependent reduction of apoptosis was observed in the four myeloma cell lines in cultures deprived of serum with the addition of CRP. The protecting effects of CRP were detected at a concentration as low as 10 μg/ml, and at 50 μg/ml, CRP protected up to 70% of myeloma cells from apoptosis induced by serum starvation ($p < 0.05$ to $p < 0.01$). Increasing CRP concentrations to a maximum of 100 μg/ml did not show greater effects on the cells (data not shown). We selected this range of concentrations of CRP for these experiments because these concentrations are present in cancer patients (Fassas et al., 2005; Hara et al., 2000; Legouffe et al., 1998). Next, we tested two IL-6-dependent myeloma cell lines XG1 and ANBL-6 and observed the same protective effects of CRP on apoptosis induced by IL-6 withdrawal ($p < 0.05$ to $p < 0.01$; Figure 2B). More importantly, CRP also protected, with the similar efficacy, myeloma cells from dexamethasone- ($p < 0.05$ to $p < 0.01$; Figure 2C) and melphalan-induced apoptosis of myeloma cell lines ($p < 0.05$ to $p < 0.01$; Figure 2D). We also examined the effects of CRP on primary myeloma cells in a coculture with osteoclasts since we have shown that osteoclasts were able to sustain myeloma cell survival ex vitro (Yaccoby et al., 2004; Yang et al., 2006). Primary myeloma cells from patients were isolated and cultured for 48 hr in the presence of melphalan, with or without addition of CRP. As shown in Figure 2E, significantly fewer apoptotic myeloma cells were recovered from cultures with the addition of melphalan and CRP ($p < 0.05$, compared with melphalan-treated cultures). Similar results were also obtained in cocultures of primary myeloma cells with bone marrow stromal cells (BMSCs; data not shown). As CRP products contained low levels of endotoxin (LPS: 0.002–0.005 EU/μg) detected by the Limulus assay, experiments were performed to exclude the possibility that endotoxin mediated the effects. As shown in Figure 2F, addition of LPS to the cultures or use of boiled CRP failed to protect myeloma cells from dexamethasone-induced apoptosis. Likewise, human serum amyloid P component, which is a closely related pentraxin protein circulating in human plasma, had no such effects. Thus, the results clearly demonstrate the ability of CRP, but not other contaminants or proteins, to protect myeloma cells from apoptosis in vitro.

CRP Binds FcγR on Myeloma Cells

CRP shares several functional similarities with IgG, including complement activation and binding to FcγR (Stein et al., 2000). It is well documented that CRP binds to FcγRI (CD64) (Crowell et al., 1991; Marnell et al., 1995), FcγRII (CD32) (Bharadwaj et al., 1999; Stein et al., 2000), and probably FcγRIII (CD16) (Khreiss et al., 2002). To examine whether FcγRs serve as cell surface receptors for CRP on myeloma cells, we first determined whether and which of these receptors are expressed by myeloma cells (monoclonal antibodies [mAbs]; FcγRI, clone 10.1; FcγRII, clone 7.3; FcγRIII, clone 3G8; and isotype controls were purchased from BD PharMingen, San Diego, CA). As shown in Figure 3A, all of these FcγRs were detected on the

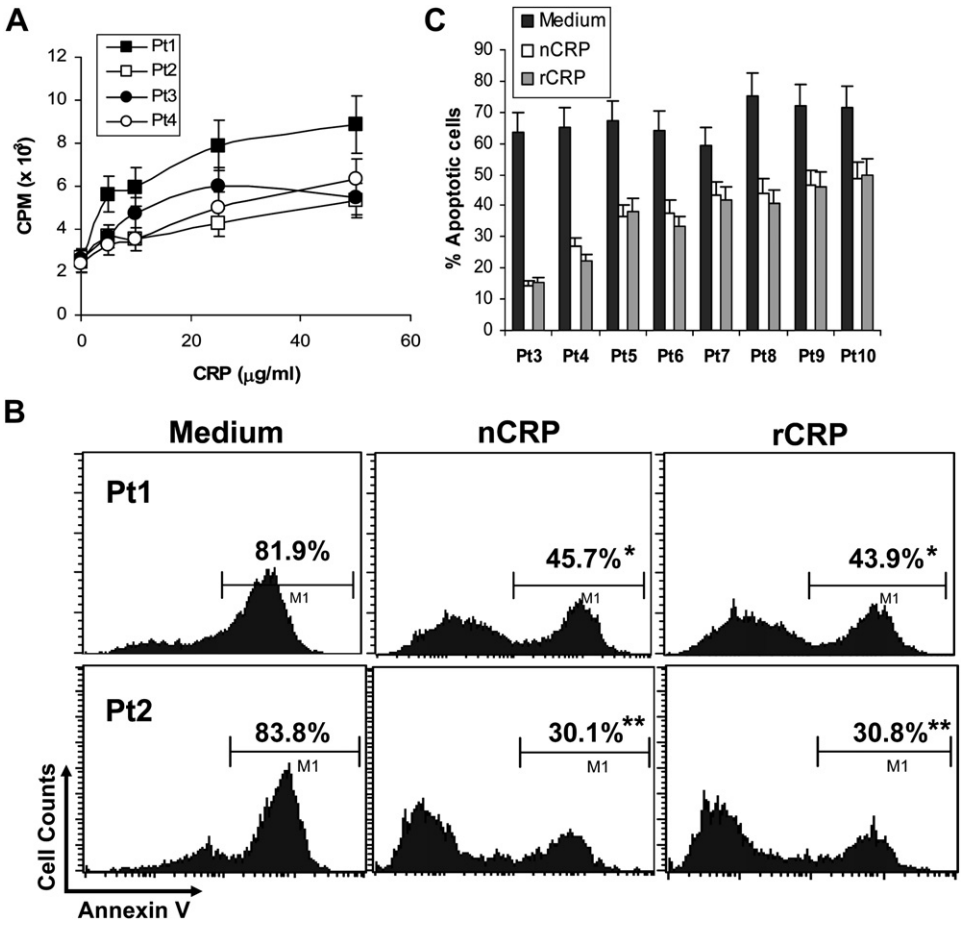

Figure 1. CRP Enhances the Proliferation and Reduces Apoptosis of Primary Myeloma Cells Ex Vivo

(A) Proliferation, determined by ^{3}H-thymidine incorporation assay, of freshly isolated myeloma cells from 4 MM patients in 48 hr cultures with the addition of various concentrations of human natural CRP.

(B) Representative histograms showing the percentages of apoptotic myeloma cells from 2 patients in 48 hr cultures with or without (medium) the addition of CRP (25 μg/ml).

(C) Percentages of apoptotic myeloma cells from other 8 MM patients in 48 hr cultures with or without (medium) the addition of CRP (25 μg/ml). nCRP, natural CRP; rCRP, recombinant CRP. *$p < 0.05$; **$p < 0.01$. Error bars, SEM.

surface of the myeloma cells. These receptors were also detected, with similar densities, on other myeloma cell lines including ARK, MM.1R, RPMI-8266, and CAG, and on primary myeloma cells from eight patients examined (data not shown). Next, immunoprecipitation with mAbs against CRP (R&D Systems, Minneapolis, MN) or mAbs against FcγRs was used to examine whether and which FcγRs bound to CRP on myeloma cells. As shown in Figure 3B, immunoprecipitation with mAbs against FcγRII, but not FcγRI or FcγRIII or isotype controls (data not shown), produced a strong band of CRP protein visualized by anti-CRP antibody, whereas all the three mAbs, but not isotype controls (data not shown), immunoprecipitated their corresponding receptors. Likewise, mAbs against CRP also precipitated FcγRII but not FcγRI or FcγRIII (Figure 3C). To further confirm the binding of CRP to FcγRs, myeloma cells were incubated with CRP and various concentrations of the F(ab′)2 fragments of the FcγR mAbs (Fitzgerald Industries International, Inc., Concord, MA) or IgG controls, followed by extensive washing and incubation with FITC-conjugated anti-CRP antibody. As shown in Figure 3D, mAb 7.3 (FcγRII), but not other mAbs (data not shown) or mouse IgG, inhibited CRP binding to myeloma cells in a dose-dependent manner. Furthermore, we showed that the F(ab′)2 fragments of blocking mAbs against CRP or FcγRII (mAb 7.3), but not FcγRI, FcγRIII, or IgG controls (data not shown), completely abrogated the protective effects of CRP on dexamethasone-induced apoptosis of myeloma cells (Figure 3E). To examine the clinical relevance of CRP, we stained bone marrow biopsies of myeloma patients. As shown in Figure 3F, bone marrow myeloma cells from all five randomly selected, newly diagnosed patients were strongly stained for surface CRP, whereas normal control marrow biopsies were not stained. These findings indicate that circulating, liver-derived CRP is indeed accumulating on the surface of

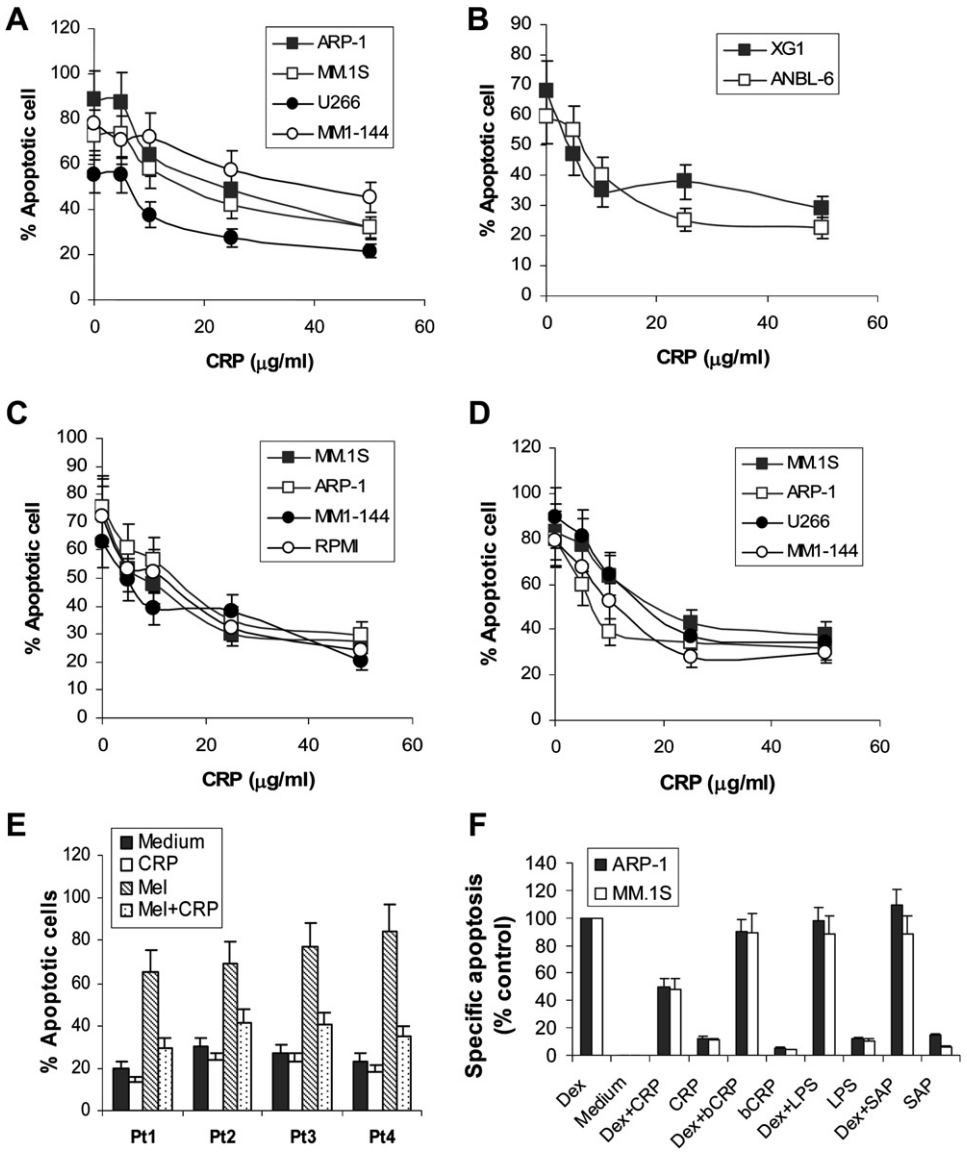

Figure 2. CRP Protects Established Myeloma Cell Lines and Primary Myeloma Cells from Induced Apoptosis In Vitro

Shown are the percentages of apoptotic cells detected by Annexin-V–binding assay in 48 hr cultures in the presence of various concentrations of CRP. Apoptosis was induced by (A) serum starvation (medium containing no serum) in four myeloma cell lines; (B) IL-6 withdrawal in two IL-6-dependent myeloma cell lines; (C) dexamethasone (10 μM) or (D) melphalan (5 μM) treatment in four myeloma cell lines; and (E) melphalan (5 μM) in primary myeloma cells isolated from 4 MM patients (Pt1–Pt4) in cocultures with osteoclasts with or without addition of CRP (25 μg/ml). Cocultures with medium alone served as control. Details are described in the Experimental Procedures. Similar results were also obtained in cocultures of the primary myeloma cells with BMSCs. (F) Effects of CRP (25 μg/ml), boiled CRP (bCRP, 100°C for 2 hr; 25 μg/ml), LPS (1 ng/ml; Sigma), and serum amyloid P component (SAP; 25 μg/ml; Sigma) on dexamethasone (10 μM)-induced apoptosis in myeloma cells ARP-1 and MM.1S. Higher concentrations of LPS (up to 50 ng/ml) or SAP (up to 100 μg/ml) were also used and showed no protective effects on cell apoptosis (data not shown). The same results were also obtained by TUNEL assay. Results of three independent experiments are shown. Error bars, SEM.

myeloma cells in vivo. Taken together, these results clearly show that CRP mediates its effect via binding to FcγRII on myeloma cells. These results were verified with both myeloma cell lines and primary myeloma cells from patients.

Human FcγRII contains either an ITAM (immunoreceptor tyrosine-based activation motif) or an ITIM (immunoreceptor tyrosine-based inhibitory motif) in its intracellular tail (Xiu et al., 2002), depending on the isoform. FcγRIIA and FcγRIIC are activating receptors because they contain an ITAM motif, whereas FcγRIIB is an inhibitory receptor due to its ITIM motif (Xiu et al., 2002). As CRP preferentially binds FcγRII, we wanted to know which of these receptors are responsible for CRP-mediated signaling in myeloma cells. Using western blot analysis with specific antibodies (FcγRIIA, N20; FcγRIIB, C-20; FcγRIIC, P-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), we

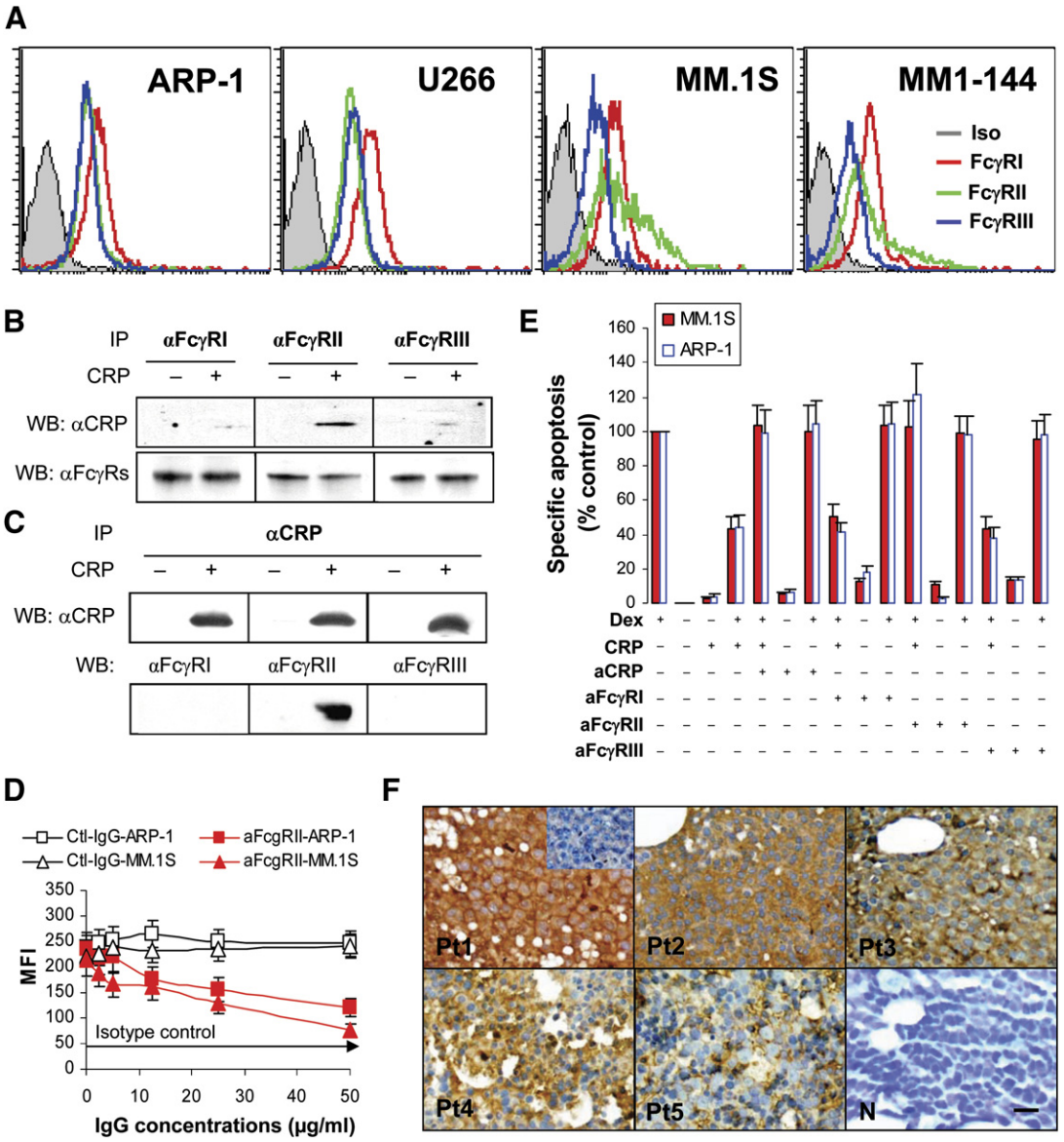

Figure 3. Expression of Surface FcγRs and Their Binding to CRP on Myeloma Cells

(A) Flow cytometry analysis showing the expression of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) on four myeloma cell lines.

(B and C) Immunoprecipitation (IP) using antibodies against FcγRs (B) or CRP (C) in myeloma cells treated with or without CRP (25 μg/ml). Blotting antibodies (WB) used were against CRP or FcγRs. Shown are results obtained with myeloma cell line ARP-1.

(D) Inhibitory effects of the F(ab′)2 fragments of anti-FcγRII mAb (clone 7.3) on CRP (25 μg/ml) binding to myeloma cells APR-1 and MM.1S. Myeloma cells were incubated with CRP (25 μg/ml) and various concentrations of the F(ab′)2 fragments of the FcγR mAbs or IgG controls, followed by extensive washing and incubation with FITC-conjugated anti-CRP antibody. F(ab′)2 fragments of mouse IgG served as control. Isotype control represents cells staining with FITC-conjugated anti-CRP antibody. MFI, mean fluorescence intensity. Same results were obtained with other cell lines and primary myeloma cells.

(E) Effects of CRP and FcγR blocking antibody F(ab′)2 fragments (20 μg/ml) on CRP (25 μg/ml)-mediated protection of dexamethasone (10 μM)-induced apoptosis in myeloma cells. Results of three independent experiments are shown.

(F) Staining for CRP in bone marrow biopsies of five randomly selected MM patients (Pt1–Pt5) and one representative normal control (N) out of four examined by immunohistochemistry with mAb against CRP and conjugated secondary antibody. The insert on the panel for Pt1 shows a representative control staining with conjugated secondary antibody without the primary anti-CRP mAb. Scale bar, 20 μM. Error bars, SEM.

found that myeloma cells commonly express FcγRIIA and FcγRIIC, whereas normal B cells, 1 out of 10 myeloma cell lines, and 4 out of 9 primary myeloma cells express FcγRIIB (Figure 4A). Surface expression of these FcγRIIs was confirmed by staining the cells with PE- or FITC-conjugated mAbs (clone IV.3 for FcγRIIA; FLI8.26 for FcγRIIA/IIB, StemCell Technologies, Tukwila, WA; and 7.3 for FcγRIIs, data not shown). As FcγRIIB was absent from

most of the myeloma cell lines and primary myeloma cells, it is likely that the activating FcγRIIA and/or FcγRIIC are the major receptors for CRP. Indeed, immunoprecipitation using antibodies against either FcγRIIA, FcγRIIC, or CRP, but not IgG controls (data not shown) revealed that, on APR-1 cells that express both FcγRIIA and FcγRIIC, CRP was associated with both of these receptors (Figure 4B). On MM1-144 cells that express FcγRIIB and FcγRIIC, CRP was predominantly associated with FcγRIIC but not FcγIIB (Figure 4C). These results were verified with patient-derived primary myeloma cells on which both FcγRIIA/C and FcγIIB were expressed.

We also examined the functional role of FcγRII in CRP-mediated protection of myeloma cell apoptosis. First, we used the F(ab′)2 fragments of mAb specific for FcγRIIA (IV.3), a blocking antibody specific for both FcγRIIA and FcγRIIC (C-17, Santa Cruz Biotechnology) or an antibody specific for FcγRIIB (AF1330, R&D Systems) to disrupt the interaction of CRP with the receptors on ARP-1 (expressing FcγRIIA and FcγRIIC), MM.1S (expressing FcγRIIC), and MM1-144 (expressing FcγRIIB and FcγRIIC). As shown in Figure 4D, addition of FcγRIIA/C-antibody F(ab′)2 fragments abrogated CRP-mediated protection of myeloma cell apoptosis induced by dexamethasone in all three cell lines tested, whereas addition of the F(ab′)2 fragments of FcγRIIB-specific antibody or IgG controls (data not shown) to MM1-144 had no effect. F(ab′)2 fragments of IV.3 only partially abrogated CRP-mediated protection of ARP-1 and had no effect on MM.1S or MM1-144 cells. Next, small interfering RNAs (siRNAs) for FcγRII genes were synthesized and used to knock down the receptor expression in myeloma cells. Receptor-specific siRNAs successfully and significantly (70%–80%) knocked down the expression of FcγRIIC in MM.1S (Figure 4E), FcγRIIA, and/or FcγRIIC in ARP-1 (Figure 4F), and FcγRIIB and/or FcγRIIC in MM1-144 (Figure 4G), respectively, on day 3 after transfection without affecting the expression level of FcγRI or other irrelevant FcγRII in the cells, which remained at low levels for up to 7 days (data not shown). Mock transfection or control siRNA had no effects. As shown in Figures 4E–4G, knockdown of FcγRIIC on MM.1S, both but not FcγRIIA or FcγRIIC individually on ARP-1, or FcγRIIC but not FcγRIIB on MM1-144 cells completely abrogated CRP-mediated protection of apoptosis induced by dexamethasone. As expected, all of these treatments killed a small percentage of cells (Caplen and Mousses, 2003). These results confirmed our findings that FcγRIIA and FcγRIIC, but not FcγRIIB, appear to be the major receptors for CRP and indicate that both FcγRIIA and FcγRIIC, when co-expressed, could bind to CRP and generate signaling to the cells.

Having identified the receptors for CRP, we sought to elucidate cell-signaling pathways responsible for CRP-mediated protection of myeloma cell apoptosis. We focused on the PI3K/Akt, mitogen-activated protein (MAP) kinase, and NF-κB pathways, as these are the downstream kinases of ITAM (Xiu et al., 2002) and are essential for cell growth and survival (Franke et al., 1997). First, we examined the impact of CRP on these pathways in myeloma cells without induction of apoptosis. As shown in Figure 5A, binding of CRP increased the protein levels of phosphorylated Akt (pAkt) and pERK and activated NF-κB in the cells, which was evident by increased IκBα phosphorylation and NF-κB subunit p65 translocation into nuclei (detected as nuclear p65; np65). The level of nonphosphorylated Akt and ERK and total NF-κB p65 (tp65) remained unchanged. In the presence of antibody against FcγRII, but not control IgG (data not shown), the activation of Akt, ERK, and NF-κB (pIκBα) was inhibited (Figure 5B). On the other hand, CRP downregulated the expression of phosphorylated p38 (pp38) and had no effect on JNK (Figure 5A). To verify these results, we used PI3K-specific inhibitors LY294002 and wortmannin, MEK-specific inhibitor PD98059, an IκBα-specific inhibitor, and an NF-κB inhibitor, which blocks NF-κB translocation into nuclei (all purchased from Calbiochem-Novabiochem). As shown in Figure 5C, these inhibitors indeed inhibited CRP-induced Akt and ERK phosphorylation and NF-κB subunit p65 translocation into nuclei, respectively. Furthermore, inhibitors to PI3K (LY294002 and wortmannin) and MEK (PD98059) completely abrogated the protective effects of CRP on dexamethasone-induced apoptosis in myeloma cells, whereas treatment of the cells with the inhibitors alone did not affect their viability (Figure 5D). Interestingly, the IκBα and NF-κB inhibitors only partially abrogated CRP-mediated protection of myeloma cell apoptosis. Altogether, these results indicate that CRP protects myeloma cells from apoptosis via binding to surface FcγRII, activating the PI3K/Akt, ERK, and NF-κB pathways, and inactivating the p38 pathway.

To investigate the effects of CRP on downstream caspase cascades, activation and cleavage of caspase-9, -8, -3, and subsequent cleavage of poly (ADP-ribose) polymerase (PARP) in myeloma cells treated with or without (medium control) dexamethasone in the presence or absence of CRP were detected by western blotting with specific antibodies. As shown in Figure 6A, activation and processing of caspase-9, but not caspase-8, and cleavage of caspase-3 and PARP were observed in myeloma cells treated with dexamethasone. CRP significantly reduced dexamethasone-induced activation and processing of caspases (-9, -3) and PARP in the cells (protein quantification data are shown in Figure 6B). We also examined the involvement of mitochondria-associated pro- and antiapoptosis proteins, because the intrinsic apoptosis pathway was activated in dexamethasone-treated cells. As shown in Figure 6C, dexamethasone treatment increased the expression of proapoptotic proteins Bad and Bax and decreased that of phosphorylated Bad (pBad). Furthermore, dexamethasone also downregulated antiapoptotic protein Bcl-xL and upregulated phosphorylated Bcl-2 (pBcl-2), all of which favor the induction of apoptosis via increased permeability of mitochondria (Srivastava et al., 1998; Zha et al., 1996). CRP partially but significantly abrogated dexamethasone-induced upregulation of pBcl-2 and Bax and restored the expression of pBad and Bcl-xL (Figure 6D), resulting in protection of the cells from apoptosis.

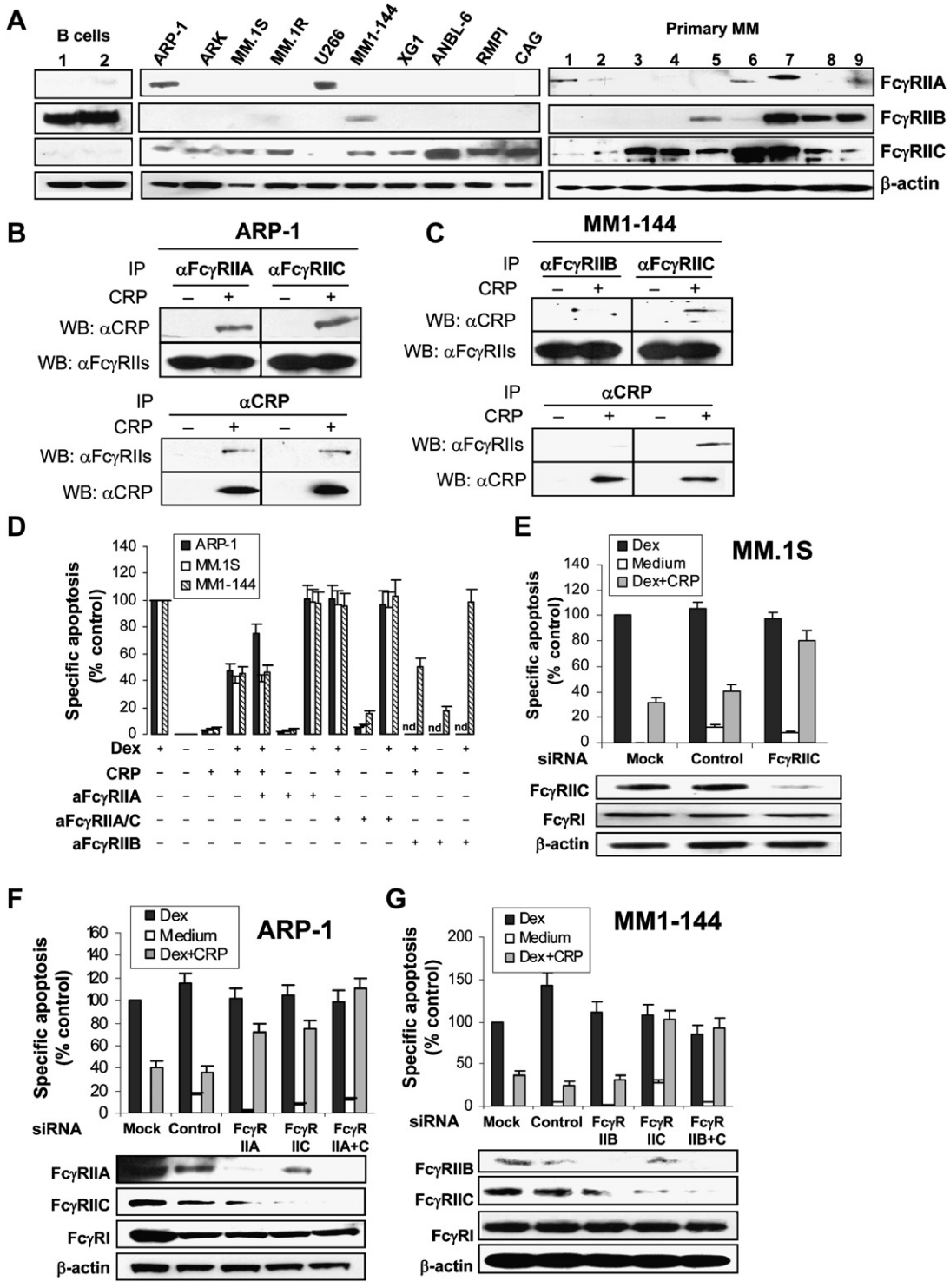

Figure 4. Expression and Role of Surface FcγRIIs on CRP-Mediated Protection of Myeloma Cell Apoptosis

(A) Western blot analysis showing protein expression of FcγRIIA, FcγRIIB, and FcγRIIC by normal blood B cells from two healthy blood donors, 10 myeloma cell lines, and purified primary myeloma cells from 9 MM patients. Immunoprecipitation (IP) using (B) antibodies against FcγRIIA or FcγRIIC (upper panel) and antibody against CRP (lower panel) in ARP-1 cells, or (C) antibodies against FcγRIIB or FcγRIIC (upper panel) and antibody against CRP (lower panel) in MM1-144 cells treated with or without CRP (25 μg/ml). Blotting antibodies (WB) used were against CRP or FcγRIIs. Similar results were obtained from other myeloma cell lines and primary myeloma cells. Results of five independent experiments are shown. (D) Effects of FcγRIIA, FcγRIIA/C, or FcγRIIB blocking antibody F(ab′)2 fragments (20 μg/ml) on CRP (25 μg/ml)-mediated protection of dexamethasone (10 μM)-induced apoptosis in myeloma cell lines. As ARP-1 and MM.1S do not express FcγRIIB, no experiments were performed with FcγRIIB blocking antibody on

CRP Synergizes with IL-6 in Protecting Myeloma Cells from Apoptosis

IL-6 is an important survival cytokine for myeloma (Kawano et al., 1988), and previous studies demonstrated that IL-6 protected myeloma cells from dexamethasone-induced apoptosis (Hardin et al., 1994). Therefore we examined the relationship of CRP with IL-6 in myeloma cell survival. By ELISA we measured IL-6 secretion by myeloma cells treated with or without dexamethasone and/or CRP. As shown in Figure S2A, myeloma cells such as cell lines MM.1S and ARP-1 constitutively secrete a low amount of IL-6 (<20 pg/ml), which could be elevated by treatment with dexamethasone alone or in combination with CRP ($p < 0.05$). CRP alone did not upregulate secretion of IL-6. Next we added exogenous IL-6 (5 ng/ml) or IL-6 in combination with IL-6 and IL-6 receptor neutralizing antibodies to cell cultures to examine the effects of IL-6 and IL-6 neutralization and blockade on IL-6- and/or CRP-mediated protection of myeloma cell apoptosis. As shown in Figure S2B, although the blocking antibodies, but not IgG controls (data not shown), completely abrogated the effect of IL-6, they had no effect on CRP-mediated protection of myeloma cell apoptosis. Moreover, the combination of CRP and IL-6 protected a greater number of myeloma cells from dexamethasone-induced apoptosis than either CRP or IL-6 alone. We also compared the effects of CRP, IL-6, and BMSCs on dexamethasone-induced apoptosis of primary myeloma cells and showed that CRP-mediated protection is comparable to protection mediated by IL-6 or BMSCs (Figure S2C). These results indicate that IL-6 was not responsible for CRP-mediated protection of myeloma cell apoptosis. Under stress conditions, CRP enhances myeloma cell secretion of IL-6, which in turn synergizes with or is additive to CRP in protecting myeloma cells from apoptosis.

In Vivo Effects of CRP on Established Myeloma

To investigate the in vivo effects of CRP on myeloma cell apoptosis, we inoculated severe combined immunodeficient (SCID) mice subcutaneously with myeloma cell lines ARP-1 or MM.1S and monitored tumor development. When palpable tumors ($\geq$10 mm in diameter) developed, mice were treated with intraperitoneal injections of either dexamethasone (1 mg per mouse daily for 6 consecutive days), or melphalan (0.5 mg per mouse daily for 6 consecutive days). The doses and treatment schedules were modeled after typical treatment regimens for myeloma patients (Alexanian et al., 1992; Barlogie et al., 1986); prolonged treatments with the drugs would not be possible due to their toxicities. In some mice, CRP (200 μg per mouse) was injected around subcutaneous tumors prior to each chemotherapy treatment. Results showed that, compared with controls (PBS-treated mice), treatment with dexamethasone (Figures 7A and 7C) or melphalan (Figures 7B and 7D) significantly reduced tumor burdens and retarded tumor growth, measured as tumor volumes (Figure 7) and levels of circulating M-proteins (Figure S3) secreted by the myeloma cells. However, injection of CRP protected tumors and undermined the therapeutic effects of these chemotherapy drugs, evident by the significantly greater tumor burdens in CRP- and dexamethasone- (Figures 7A and 7C; $p < 0.01$), or CRP- and melphalan-treated SCID mice (Figures 7B and 7D; $p < 0.01$ and $p < 0.05$) and in CRP- and melphalan-treated SCID-hu mice (Figure 7E; $p < 0.01$) compared with mice treated with the drugs alone. Treatment with CRP alone did not affect tumor growth in SCID or SCID-hu mice. Altogether, these results indicate that CRP could effectively protect against myeloma cell apoptosis in vitro and in vivo.

To confirm whether CRP utilizes the same mechanisms of apoptosis protection in tumor cells in vivo, tumor-bearing mice were sacrificed after treatment, and tumors were removed for immunohistochemical analysis. As shown in Figure 8A, injections of CRP led to binding of CRP to the tumor cells and upregulation and activation of pAkt and pERK in myeloma-bearing mice. Moreover, injection of CRP prior to each chemotherapy treatment resulted in inhibited cleavage of caspase-9 and caspase-3 and reduced numbers of apoptotic myeloma cells induced by dexamethasone (Figure 8B). Hence, these results validated the mechanisms of CRP-induced signaling and antiapoptosis in tumor cells.

DISCUSSION

The aim of this study was to examine whether human CRP could affect tumor cell growth and survival. We modeled this in myeloma and discovered that addition of CRP to cultures at levels seen in patients with MM or other tumors promoted myeloma cell proliferation under stressed conditions and protected myeloma cells from chemotherapy drug-, IL-6 withdrawal-, or serum deprivation-induced apoptosis in vitro. The protective effect was verified in vivo in myeloma SCID and SCID-hu mouse models. These phenomena may be clinically relevant since CRP was found accumulate on the surface of bone marrow myeloma cells from patients with MM. Although myeloma cells expressed all three types of FcγR, we identified FcγRII, more specifically FcγRIIA and FcγRIIC, as the primary receptors for CRP on the tumor cells. Our results demonstrated

these two cell lines. nd, not done. Knockdown of surface FcγRIIs by FcγRII-specific siRNA and its effect on CRP-mediated protection of dexamethasone-induced apoptosis in (E) MM.1S (expressing FcγRIIC), (F) ARP-1 (expressing FcγRIIA and FcγRIIC), or (G) MM1-144 (expressing FcγRIIB and FcγRIIC) cells. Controls included mock control, transfection of the cells with control siRNA (control), and detection of FcγRI protein expression. The upper panels represent percentages of specific apoptosis and lower panels represent protein expression detected by western blot analysis. In these experiments, cells were transfected with 400 nM siRNA and, 72 hr later, washed and incubated with or without dexamethasone (10 μM) and/or CRP (25 μg/ml) for 48 hr. Apoptosis was detected by Annexin-V-binding assay. Representative results of three independent experiments are shown. Error bars, SEM.

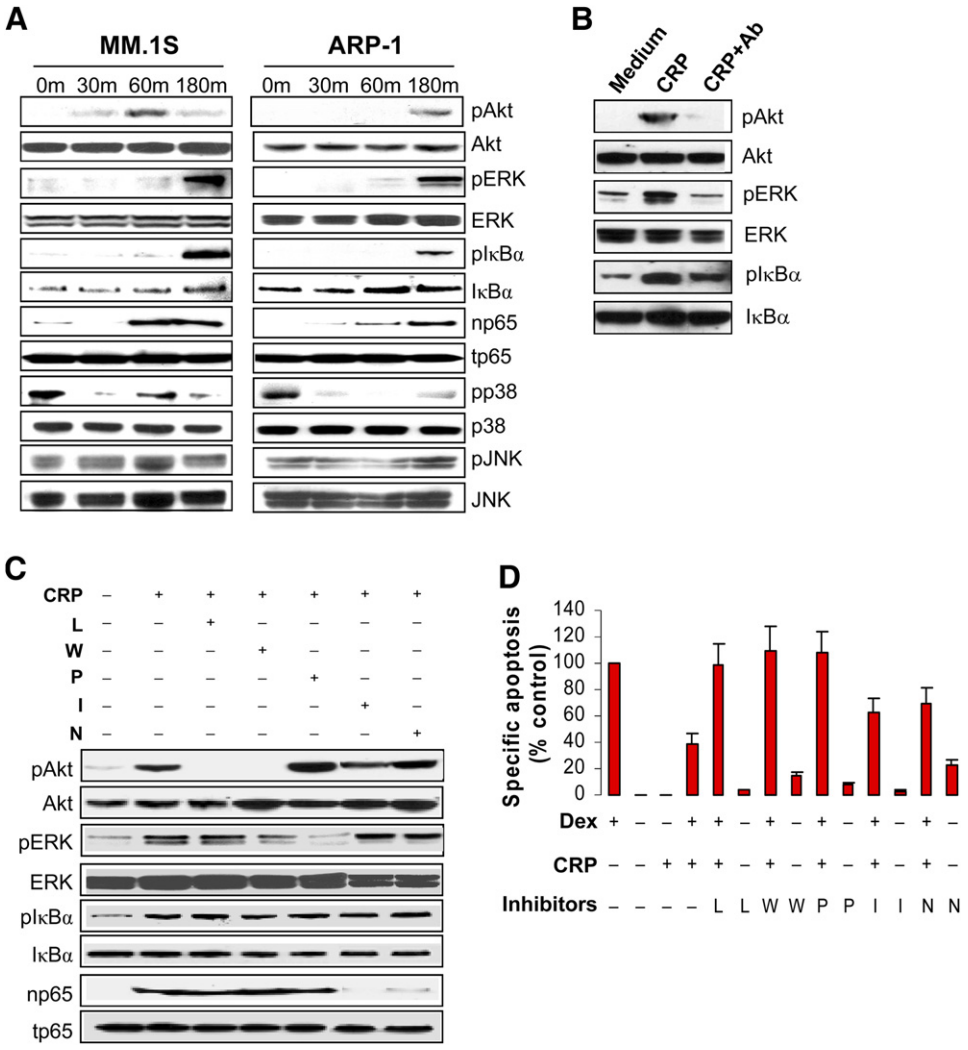

Figure 5. Elucidation of CRP-Induced Signaling Pathways in Myeloma Cells

Western blot analysis showing (A) protein levels of phosphorylated (p) and nonphosphorylated Akt, ERK, IκBα, p38, JNK, and the nuclei (np65) or total (tp65) NF-κB p65 in CRP-treated myeloma cell lines MM.1S and ARP-1 cells for various times. M, minutes. (B) FcγRII antibody inhibited CRP-mediated activation of Akt, ERK, and NF-κB (pIκBα). ARP-1 cells were treated with CRP in the presence of F(ab′)2 fragments (20 μg/ml) of antibody against FcγRII (mAb 7.3) or control IgG (data not shown) for 180 min, and cell lysates were prepared for analysis. Effects of kinase inhibitors on (C) CRP-mediated protein expression of the kinases and (D) CRP-mediated protection of dexamethasone-induced apoptosis in myeloma cells (MM.1S). LY249002 (L; 50 μM) and wortmannin (W; 1 μM) are PI3K-specific inhibitors, PD98059 (P; 50 μM) is an inhibitor for MEK1/2, I (15 μM) is an inhibitor for IκBα phosphorylation, and N (18 μM) is an inhibitor for NF-κB translocation into nuclei. The concentrations of the inhibitors were chosen based on the manufacturer's recommendations and our preliminary tests. In these experiments, myeloma cells (ARP-1) were cultured with the inhibitors at the final concentrations indicated with or without dexamethasone and/or CRP for 180 min (all kinases were activated) and cell lysates were prepared for western blot analysis (C), or for 48 hr and harvested for apoptosis (Annexin V-binding) assay (D). Representative experiment of three performed are shown. Similar results are obtained with other myeloma cell lines and primary myeloma cells. Error bars, SEM.

that CRP activated PI3K/Akt, ERK, and NF-κB in treated cells via binding to these receptors, which led to inhibited activation of caspase cascades induced by chemotherapy drugs such as dexamethasone and undermined the therapeutic efficacy of chemotherapy in the myeloma mouse models (Figure S4). Thus, our study demonstrates that CRP plays an active role in regulating tumor cell growth and survival.

FcγRs are expressed by various types of cells of the immune system and have a central role in controlling immune responses after interaction with antigen-antibody complexes (Xiu et al., 2002). The activation cascade through ITAM-associated FcγRs results in cellular activation, while ITIM-associated FcγRs, mainly FcγRIIB in humans, mediate the inhibition of the ITAM-induced activation cascade (Xiu et al., 2002) and generate an apoptotic signal in B cells (Ashman et al., 1996; Ono et al., 1997). CRP shares several functional properties with IgG and binds to FcγRs. However, CRP binds primarily to the low-affinity IgG FcγRIIA (Bharadwaj et al., 1999; Stein et al., 2000) and to some

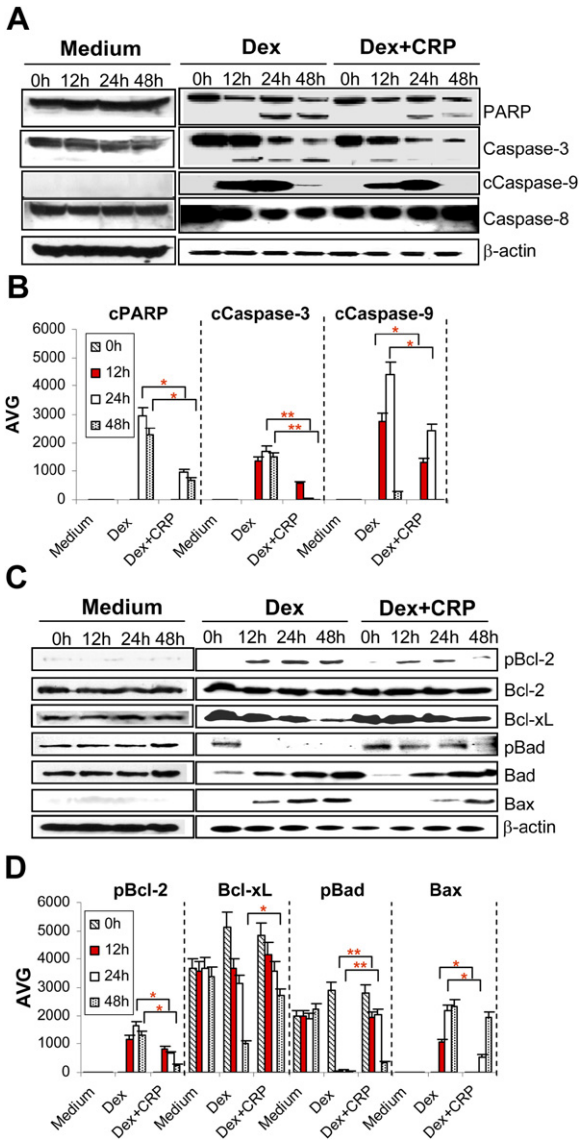

Figure 6. Elucidation of CRP-Induced Antiapoptotic Pathways in Myeloma Cells

Western blot analysis showing protein levels of (A) pro- and cleaved caspases and PARP and (C) mitochondria-related pro- and antiapoptotic proteins in myeloma cells MM.1S treated with or without (medium control) dexamethasone (10 μM) in the absence or presence of CRP (25 μg/ml). Also shown are densitometric data (AVG) of (B) pro- and cleaved caspases and PARP and (D) mitochondria-related pro- and antiapoptotic proteins in the myeloma cells. The protein levels of β-actin served as loading control. h, hour(s). Representative experiment of four performed are shown. Similar results are obtained with other myeloma cell lines and primary myeloma cells. *$p < 0.05$; **$p < 0.01$. Error bars, SEM.

extent to the high-affinity IgG FcγRI (Crowell et al., 1991; Marnell et al., 1995). These findings are consistent with our results that FcγRIIs serve as the major receptors for CRP on myeloma cells. Moreover, our study also provides data demonstrating that CRP appears to predominantly bind FcγRIIA and FcγRIIC. No binding of CRP to the inhibitory FcγRIIB was detected. These observations are especially interesting because they provide a plausible explanation for our observed phenomena that CRP uniformly protected myeloma cells from apoptosis even though some myeloma cells such as MM1-144 and a few primary myeloma cells expressed FcγRIIB. Based on the results, it is reasonable to expect that CRP mainly triggers an activating and antiapoptotic signaling in myeloma cells via binding the activating FcγRII.

CRP is an acute-phase protein secreted by hepatocytes in response to inflammatory cytokines such as IL-1 and IL-6 (Agrawal et al., 2001; Mazer and Rabbani, 2004). Interestingly, higher levels of these cytokines are associated with MM (Sirohi and Powles, 2004) and other tumors (Gause et al., 1994; Kato et al., 1996) and may be responsible for the elevated production and secretion of CRP in the patients (Legouffe et al., 1998). As IL-6 is a survival factor for myeloma tumor cells (Kawano et al., 1988), we examined the relationship of IL-6 and CRP in protecting myeloma cells from apoptosis. Our results showed that although CRP and dexamethasone upregulated myeloma cell secretion of IL-6, IL-6 was not responsible for CRP-mediated protection. However, IL-6 and CRP exerted an additive effect in protecting myeloma cells from dexamethasone-induced apoptosis. Thus, it is plausible that the vicious circle of myeloma and its protective microenvironment (Sirohi and Powles, 2004) involves IL-6, CRP, myeloma cells, and stromal cells. Myeloma and stromal cells secrete IL-1 and IL-6, which induce hepatocytes to produce and secrete CRP. CRP protects myeloma cells from apoptosis induced by insults or chemotherapy drugs and stimulates myeloma cells to secrete more IL-6, which in turn provides additional protection to myeloma from apoptosis and stimulates hepatocytes to secrete more CRP. Thus, CRP could be a therapeutic target for breaking the vicious circle of myeloma to improve the therapeutic efficacy of currently available treatments.

EXPERIMENTAL PROCEDURES

Cell Lines, Primary Tumor Cells, and Reagents

Myeloma cell lines ARP-1 and ARK were established at the Arkansas Cancer Research Center from bone marrow aspirates of patients with MM (Hardin et al., 1994). MM.1S and MM.1R were kindly provided by Dr. Steven Rosen from Northwestern University, Chicago, IL. Other myeloma cell lines were purchased from ATCC (Rockville, MD). Primary myeloma cells were isolated from bone marrow aspirates obtained from patients during a routine clinic visit. $CD138^+$ myeloma cells were isolated by magnetic bead sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The study was approved by the Institutional Review Board at The University of Texas M.D. Anderson Cancer Center. Dexamethasone and melphalan were purchased from Sigma (St. Louis, MO). IL-6 and IL-6 receptor blocking antibodies and recombinant IL-6 were purchased from R&D Systems. F(ab′)2 Preparation Kit (Pierce, Rockford, IL) was used to prepare F(ab′)2 fragments of antibodies when they were commercially available as purified IgG.

Cell Proliferation Assay

Myeloma cells (1×10^5/ml) were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing different concentrations of CRP for 48 hr, and cell proliferation was determined

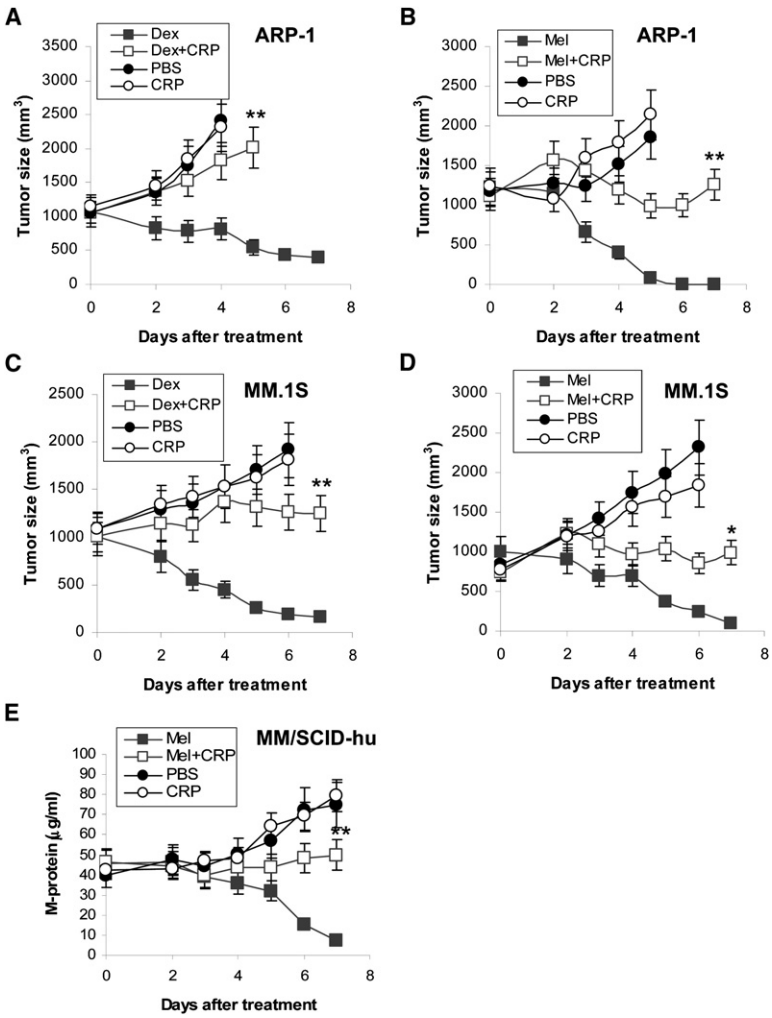

Figure 7. In Vivo Protective Effects of CRP on Established Myeloma in SCID or SCID-hu Mouse Models

Mice were xenografted subcutaneously with ARP-1 (A and B) or MM.1S (C and D), and tumor burdens were monitored as tumor volumes. When palpable tumors (≥ 10 mm in diameter) developed, mice were treated with intraperitoneal injections of either dexamethasone (1 mg per mouse daily for 6 consecutive days; [A] and [C]) or melphalan (0.5 mg per mouse daily for 6 consecutive days; [B] and [D]), or PBS. In some mice, CRP (200 μg per mouse) was injected subcutaneously (around tumors) prior to each chemotherapy treatment. Results from one representative experiment with 5 mice per group of three performed are shown. (E) In SCID-hu mice (3 per group), primary myeloma cells from patients (n = 3) were directly injected to implanted human bones, and tumor growth was monitored as levels of circulating human M-protein or its light chain. Myeloma-bearing SCID-hu mice received the same treatment as SCID mice. *$p < 0.05$; **$p < 0.01$. Error bars, SEM.

by ^{3}H-thymidine incorporation assay. Experiments were performed in triplicate.

Flow Cytometry Analysis

Expression of FcγRs was determined by direct immunofluorescence using FITC- or PE-conjugated antibodies against the different FcγRs. After staining, cells were resuspended in PBS and analyzed by a FACScan flow cytometer (Becton Dickinson).

Apoptosis Assays

Cells were incubated with or without the addition of various concentrations of CRP for different times. The fraction of apoptotic cells was determined by staining cells, suspended in Annexin-V binding buffer (PharMingen), with FITC-conjugated Annexin-V and PI according to the manufacturer's instructions. After 15 min of incubation at room temperature, samples were analyzed by flow cytometry. Apoptotic cells were determined as Annexin-V-positive cells. In some experiments, specific apoptosis (% of control) was used for data presentation, calculated as follows: [(% of apoptotic cells after a treatment − % of apoptotic cells in medium)/(% of apoptotic cells after dexamethasone treatment − % of apoptotic cells in medium)] × 100% (Solier et al., 2002).

Cell apoptosis induced by CRP was also analyzed by TUNEL assay. A flow cytometry-based cell death detection kit (APO-BrdU) was purchased from BD PharMingen, and experiments were performed according to the manufacturer's protocol. In situ TUNEL assay was performed on tumor sections to detect apoptosis in tumors isolated from SCID mice, according to the manufacturer's instructions.

ELISA for IL-6

Supernatants from cell cultures with or without the addition of dexamethasone (10 μM) and/or CRP (25 μg/ml) were collected after culturing for 48 hr, and the amounts of IL-6 in the supernatants were quantified using a commercially available ELISA kit (R&D Systems).

Generation of Osteoclasts and BMSCs, and Coculture with Myeloma Cells

The method for generating osteoclasts has been described previously (Yaccoby et al., 2004). PBMCs were cultured in 24-well plates in α-MEM medium supplemented with 10% fetal calf serum, antibiotics, RANK ligand (50 ng/ml), human M-CSF (25 ng/ml), and 10 nM dexamethasone for 10 to 14 days to generate tartrate-resistant acid phosphatase (TRAP)$^{+}$ and vitronectin receptor^{+} osteoclasts. Before coculture, osteoclasts were washed, and freshly isolated myeloma plasma cells from patients (0.5×10^{6} cells/ml) were added to the wells and cocultured with osteoclasts, with or without the addition of melphalan and/or CRP. BMSCs were generated from bone marrow mononuclear cells from patients with myeloma as described previously (Uchiyama et al., 1993). After an adherent cell monolayer formed, these cells were used for coculture with myeloma cells. At the end of the studies, myeloma cells were detached from osteoclasts or BMSCs and subjected to analysis for apoptosis.

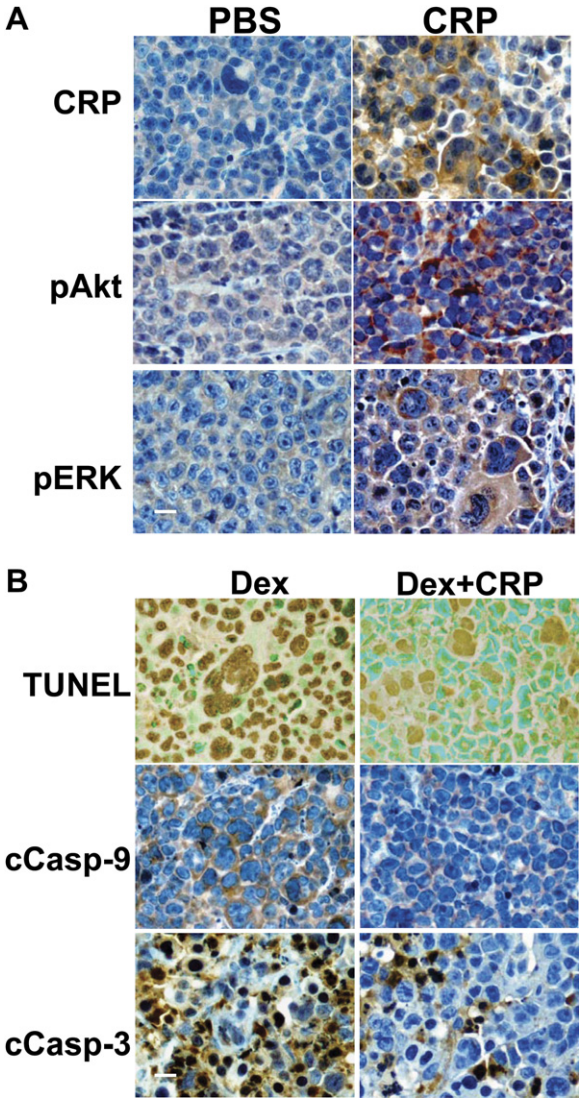

Figure 8. In Vivo Validation of Signaling and Apoptosis Mechanisms

(A) Myeloma (ARP-1)-bearing mice were injected subcutaneously (around tumors) with CRP (200 μg per mouse) or PBS and sacrificed 24 hr later. Tumors were removed for immunohistochemical staining for CRP, phosphorylated Akt (pAkt) and pERK;

(B) Myeloma (ARP-1)-bearing mice were injected intraperitoneally with dexamethasone (1 mg per mouse). Prior to each chemotherapy, CRP (200 μg per mouse) was injected subcutaneously (around tumors) to some mice (Dex + CRP). Mice were sacrificed 24 hr later after the second chemotherapy. Tumors were removed for immunohistochemical staining for apoptotic cells detected by in situ TUNEL assay, cleaved caspase-9 (cCasp-9), and caspase-3 (cCasp-3). Representative results of three experiments are shown. Scale bar, 20 μM.

Immunohistochemistry and Immunofluorescence Analyses

Formalin-fixed, paraffin-embedded sections of bone marrow biopsies from myeloma patients or tumors from SCID mice were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Malignant plasma cells were identified by morphologic assessment. Binding of CRP to myeloma cells, expression of pAkt and pERK, and cleaved caspase-3 and -9 were detected using specific antibodies. Detection of signal was achieved using secondary biotinylated antibodies and streptavidin/horseradish peroxidase. Chromagen 3,3-diaminobenzidine/H_2O_2 (DAKO) was used and slides were counterstained with hematoxylin. All slides were observed with light microscopy, and images were captured with a SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

Western Blotting

Cells were cultured with or without CRP (25 μg/ml) for different times, harvested, washed, and lysed with lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN3, 1% Triton X-100, 1% NP-40, 1 × protease inhibitor cocktail). Cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies against caspase-3, -7, -8, -9, PARP, Bcl-2, Bcl-XL, Bad, Bax, Akt, or ERK1/2, p38, JNK, FcγRIIA, FcγRIIB, and FcγRIIC (Cell Signaling Technology, Inc., Beverly. MA, and Santa Cruz Biotechnology). The membrane was stripped and reprobed with anti-β-actin antibody (Sigma) to ensure equal protein loading. Secondary antibodies conjugated to horseradish peroxidase were used for detection and followed by enhanced chemiluminescence (Pierce Biotechnology) and autoradiography. For protein quantification, blots were scanned and analyzed by spot densitometry, and results are expressed as average value of pixels enclosed (AVG). AVG is calculated as the sum of all the pixel values after background correction divided by area.

Immunoprecipitation

Myeloma cells were incubated with CRP (25 μg/ml) for 30 min, washed, and lysed in 1 ml RIPA buffer (10 mM Tris-HCL buffer, pH 7.5, 1% NP-40, 0.25% deoxycholate wt/vol, 2 mM EDTA, 10 mM orthovanadate). Cell lysates were precipitated with goat antibodies against CRP (R&D Systems) or FcγRs (Santa Cruz Biotechnology) and protein G-Sepharose, which had been preabsorbed with purified goat IgG, in a 50% wt/vol slurry. Immunoprecipitated proteins were washed in RIPA buffer, subjected to SDS-PAGE, and immunoblotted with specific antibodies against CRP or FcγRs.

RNA Interference

siRNAs corresponding to human FcγRIIs were purchased from Dharmacon (Lafayette, CO). Cells were harvested, plated on a 24-well plate at a concentration of 2×10^5 cells per well and 24 hr later, transiently transfected with specific siRNAs or nonspecific/control siRNA using the Oligofectamine transfection reagent (Mirus, Madison, WI) according to the manufacturer's instructions. Three days after the transfection, cells were harvested to examine FcγR protein expression or used for experiments.

Animal Studies

CB.17 SCID mice were purchased from Harlan (Indianapolis, IN). All mice were maintained in American Association of Laboratory Animal Care-accredited facilities, and studies were approved by the Institutional Animal Care and University of Texas M.D. Anderson Cancer Center. Six- to eight-week-old female CB.17 SCID mice were subcutaneously inoculated in the right flank with 1×10^6 ARP-1 or MM.1S cells in 50 μL RPMI-1640 medium. Three to five weeks later when palpable tumors (≥ 10 mm in diameter) developed, mice (n = 5 for all groups, the same experiments were repeated three times) were intraperitoneally injected with dexamethasone (1 mg per mouse) or melphalan (0.5 mg per mouse) daily for 6 consecutive days. To examine the effects of CRP on tumor cells, some mice also received subcutaneous (around tumors) injections of CRP (200 μg per mouse) prior to each treatment with dexamethasone or melphalan. Control mice were injected intraperitoneally with equal volumes of PBS. Tumor size was measured every day in two dimensions using a caliper, and tumor volume (mm^3) was calculated as $4\pi/3 \times$ (tumor width/2)2 × (tumor length/2) (LeBlanc et al., 2002). Serum was also collected from mice daily during the treatment and tested for myeloma-secreted M-proteins (human immunoglobulins) or their light chains by ELISA. Mice were humanely

sacrificed when moribund or when subcutaneous tumor reached 15 mm in diameter.

The SCID-hu mouse model was established in CB.17 SCID mice as previously described (Yaccoby et al., 1998). Freshly isolated primary myeloma cells (1×10^6/mouse) from patients were directly injected to implanted human bones and once myeloma was established (circulating human M-proteins or their light chains reaching ≥ 40 μg/ml), treatment with dexamethasone began, which was the same as for SCID mice. Serum was collected from mice daily during the treatment and tested for myeloma-secreted M-proteins (human immunoglobulins) or their light chains by ELISA.

Statistical Analysis

All data are shown as means ± SEM. The Student's t test was used to compare various experimental groups; significance was set at p less than 0.05.

Supplemental Data

The Supplemental Data include four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/3/252/DC1/.

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