Transcriptional Suppression of Interleukin-12 Gene Expression following Phagocytosis of Apoptotic Cells

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Summary

Phagocytosis of apoptotic cells usually results in an anti-inflammatory state with inhibition of proinflammatory cytokines such as IL-12. How apoptotic cell-derived signals regulate IL-12 gene expression is not understood. We demonstrate that cell-cell contact with apoptotic cells is sufficient to induce profound inhibition of IL-12 production by activated macrophages. Phosphatidylserine could mimic the inhibitory effect. The inhibition does not involve autocrine or paracrine actions of IL-10 and TGF-β. We report the identification, purification, and cloning of a novel zinc finger nuclear factor, named GC binding protein (GC-BP), that is induced following phagocytosis of apoptotic cells by macrophages or by treatment with phosphatidylserine. GC-BP selectively inhibits IL-12 p35 gene transcription by binding to its promoter in vitro and in vivo, thus decreasing IL-12 production. Blocking GC-BP by RNA interference restores IL-12 p35 transcription and IL-12 p70 synthesis. Finally, GC-BP itself undergoes functionally significant tyrosine dephosphorylation in response to apoptotic cells.

Introduction

The elimination of apoptotic cells and cell bodies by phagocytes represents an evolutionarily conserved means to prevent exposure of surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents (Savill et al., 1993). Resolution of inflammation depends not only on the effective removal of apoptotic cells but also on active suppression of inflammatory mediator production. Aberrations in either mechanism are associated with chronic inflammatory conditions and autoimmune disorders (Haslett et al., 1994). Animal studies have begun to identify some of the important molecules in the clearance of potentially antigenic material from the circulation, such as DNase I (Napirei et al., 2000), serum amyloid P component (SAP) (Bickerstaff et al., 1999), C1q (Mitchell et al., 2002), and C-reactive protein (CRP) (Du Clos et al., 1994). In human systemic lupus erythematosis (SLE), impaired phagocytosis of apoptotic material by macrophages has been reported (Herrmann et al., 1998). The impaired clearance of apoptotic cells, resulting in an accumulation of late-apo-

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ptotic and secondary necrotic cells including oligosomes, might lead to an activation of autoreactive T cells (Voll et al., 1997). The process of removing dead cells is carried out by a wide variety of cell types and involves multiple receptors, such as scavenger receptors, oxidized low-density lipoprotein receptors, CD14, CD68, CD36, vitronectin receptor, complement receptors, and phosphatidylserine receptor (PSR), that interact with their ligands, such as phosphatidylserine (PS) expressed on apoptotic cells (Savill and Fadok, 2000).

Cytokines play significant roles in the etiology and pathology of many autoimmune diseases. The uptake of apoptotic cells by phagocytes is thought to suppress autoimmune responses through the release of IL-10, TGF-β, PAF, and PGE2 and inhibition of TNF-α, GM-CSF, IL-12, IL-1β, and IL-18 production (Voll et al., 1997). Produced primarily by macrophages and dendritic cells (DCs), IL-12 is a heterodimeric cytokine that links the cellular and humoral branches of the host immune defense apparatus (Trinchieri, 1995). The genes encoding the two heterologous chains of IL-12, p40 and p35, are located on different human and mouse chromosomes. IL-12 is an important player in T cell-mediated autoimmunity (Trembleau et al., 1995). Specifically, IL-12 administration exacerbates autoimmune phenomena by inducing the differentiation of Th1 autoreactive cells (Leonard et al., 1997), whereas the lack of IL-12 p40 in genetically deficient mice or mice treated with anti-IL-12 antibody abrogated diseases in experimental models of autoimmunity such as insulin-dependent diabetes mellitus (IDDM) in NOD mice (Rothe et al., 1997), experimental allergic encephalomyelitis (EAE) (Leonard et al., 1995), experimental autoimmune uveitis (EAU) (Yokoi et al., 1997), and collagen-induced arthritis (CIA) (McIntyre et al., 1996). Aberrant levels of IL-12 are produced by macrophages isolated from young mice prone to lupus (MRL and NZB/W) (Liu and Beller, 2002). The diabetes-associated quantitative trait locus, *Idd4*, was found to be responsible for the IL-12 p40 overexpression in non-obese diabetic (NOD) mice (Simpson et al., 2003). In human patients with SLE, elevated levels of IL-12 (or IL-12 p40) and IL-18 are observed (Wong et al., 2000), and the higher serum IL-12 levels are correlated with fever, but not with renal diseases, in subjects (Spadaro et al., 2002).

In this work, we observed that phagocytosis of apoptotic cells by macrophages inhibited IL-12 p40 and p35 gene expression. We investigated the molecular mechanisms whereby the interaction between phagocytes and apoptotic cells impacts on the expression of IL-12 p40 and p35 genes.

Results

Differential Regulation of Cytokine Production during Phagocytosis of Apoptotic Cells

We found that in macrophages primed with IFN-γ followed by stimulation with LPS, IL-12 p40 and p70 production was strongly inhibited following the phagocyto-
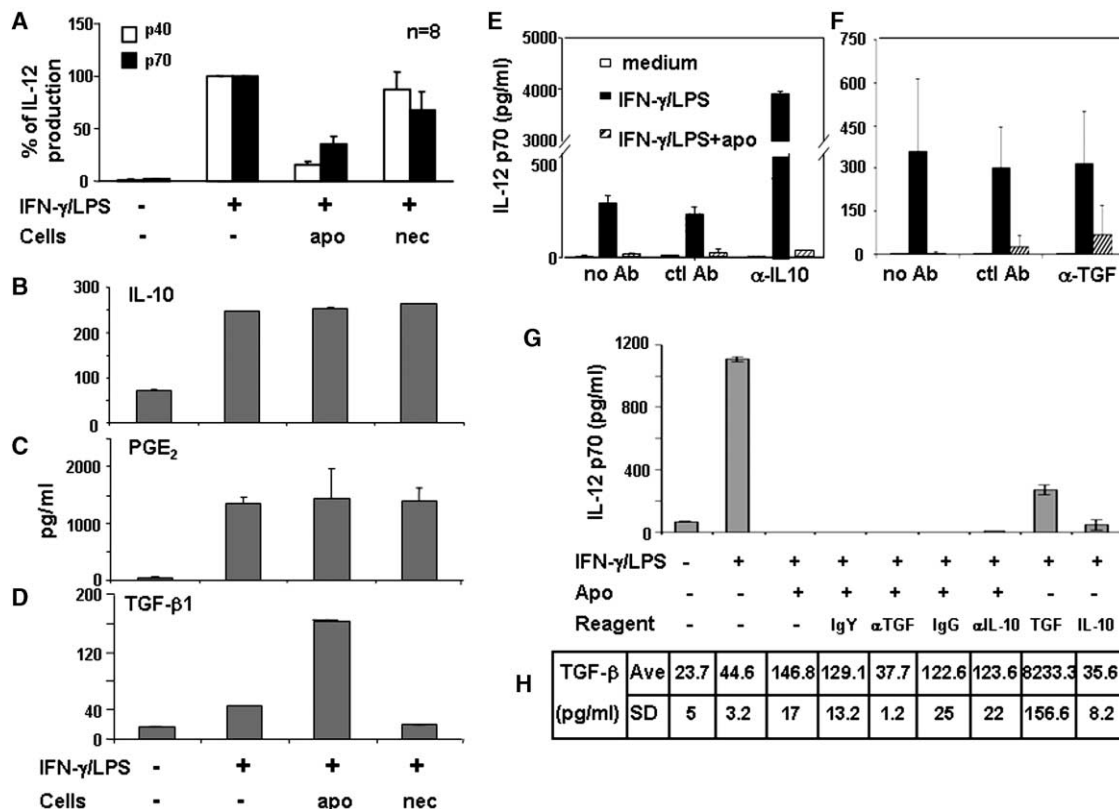
Figure 1. Apoptotic Cells Attenuate IL-12 Production by Activated Macrophages

(A–D) A total of 1×10^6 cells of human peripheral blood monocyte-derived macrophages were stimulated with IFN-γ and LPS. Apoptotic or necrotic cells (2:1 ratio of apoptotic or necrotic cells/macrophages) were added with LPS. Supernatants were harvested 16 hr post-LPS stimulation and were analyzed for the production of IL-12 p40 and (A) p70, (B) IL-10, (C) PGE$_2$, and (D) TGF-β1 by ELISA. The results of IL-12 p40 and p70 were normalized to the cytokine levels of IFN-γ/LPS-activated macrophages, which were set as 100%. The data represent the mean $\pm$ SD of eight individual experiments. The results of (B)–(D) are the averages of three individual experiments with SD.

(E and F) Neutralization of (E) IL-10 and (F) TGF-β1 in human macrophages. Anti-IL-10 mAb, anti-TGF-β1 mAb, or an isotype-matched control (ctl) IgG was added (10 μg/ml) to the macrophage cultures. IL-12 p70 concentrations were measured by ELISA.

(G) Neutralization of IL-10 and TGF-β in mouse macrophages. J774 cells were stimulated with IFN-γ and LPS and treated with apoptotic cells (added at the time of LPS addition). Anti-mIL-10 (1 μg/ml), anti-TGF-β (2 μg/ml), and control antibodies were added 1 hr before apoptotic cells. Recombinant TGF-β (10 ng/ml) and IL-10 (50 ng/ml) were added with LPS. mIL-12 p70 was measured from culture supernatant 20 hr following LPS stimulation.

(H) TGF-β1 levels in all cultures corresponding to those in (G) were measured by ELISA.

sis of apoptotic cells (Figure 1A). The production of the anti-inflammatory cytokine IL-10 (Figure 1B) and prostaglandin E$_2$ (PGE$_2$) (Figure 1C) was not significantly altered, whereas TGF-β1 production (Figure 1D) was greatly enhanced. To determine if autocrine IL-10 and TGF-β1 production was responsible for the suppressed IL-12 production, neutralizing antibodies to IL-10 and TGF-β1 were added to the macrophage culture in the presence or absence of apoptotic cells. Although the IL-10 antibody strongly enhanced IL-12 production by activated macrophages, it was not able to restore IL-12 production inhibited by apoptotic cells (Figure 1E). Use of a neutralizing antibody to TGF-β1 resulted in a heterogeneous response among the six donors tested, with a combined small increase in IL-12 p70 production inhibited by apoptotic cells (Figure 1F). To circumvent the problem associated with human subject variations, we performed the same study in the mouse macrophage cell line J774, which has been used extensively as a model system for IL-12-related studies (Murphy et al., 1995). As shown in Figure 1G, although addition of re-

combinant mTGF-β and IL-10 strongly suppressed IL-12 p70 production stimulated by IFN-γ and LPS, use of neutralizing antibodies to TGF-β and IL-10 had no impact on apoptotic cell-induced inhibition of IL-12. Neutralization of the endogenous TGF-β was complete, as shown in the ELISA data (Figure 1H). These results indicate that autocrine IL-10 and TGF-β production is not a major player in apoptotic cell-mediated inhibition of IL-12 synthesis.

Cell-Cell Contact, Not Phagocytosis, Is Sufficient for Inhibition of IL-12 Production

To determine if phagocytosis was required for the observed inhibition of IL-12 production by apoptotic cells, we used cytochalasin D (cytoD) to block actin polymerization and prevent phagocytosis without interfering with cell-cell contact. Figure 2A shows that the addition of cytoD at increasing concentrations 30 min before (-30 min, triangle) or at the time of addition of apoptotic cells (0 min, square) strongly inhibited the uptake of fluorescently labeled apoptotic cells (by $\sim$70%–87%).
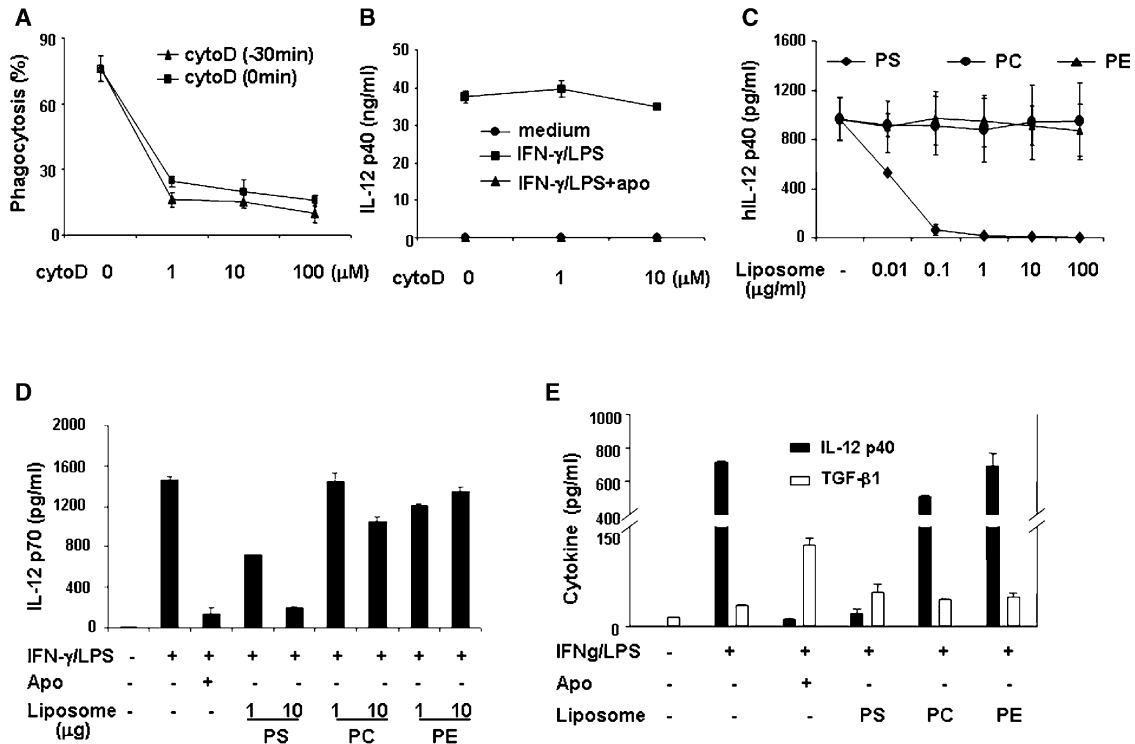
Figure 2. Role of Cell-Cell Contact and PS in Apoptotic Cell-Mediated Inhibition of IL-12 Production

(A) Inhibition of phagocytosis. RAW264.7 cells were plated at 2×10^6 cells/well in 6-well plates and were stimulated with IFN-γ and LPS. Various concentrations of cytoD solubilized in DMSO were added to macrophages either 30 min before ($-$30 min) or at the same time as LPS (0 min). Control cells received DMSO only. TAMRA-labeled apoptotic Jurkat cells were added with LPS in a 2:1 ratio. After 1 hr of incubation, unbound apoptotic cells were washed off (three times with PBS), and the phagocytes were harvested by using Trypsin/EDTA. Phagocytosis was measured by FACS analysis.

(B) IL-12 p40 production in the presence of cytoD. RAW264.7 cells were stimulated with IFN-γ and LPS for 16 hr in the presence (triangle) or absence (square) of apoptotic Jurkat cells and 1 or 10 μM cytoD (added to all cultures 30 min before the addition of apoptotic cells). Cell-free supernatant was harvested and assayed for IL-12 p40 production by ELISA. Data represent the mean $\pm$ SD of three individual experiments.

(C) Inhibition of IL-12 p40 production by PS. RAW264.7 cells were stimulated with IFN-γ and LPS alone or together with various concentrations of liposomes containing PS, PC, or PE. The next day, supernatant was harvested and IL-12 p40 expression was measured by ELISA. Data represent the mean $\pm$ SE of four independent experiments.

(D) Inhibition of IL-12 p70 by PS. Human monocyte-derived macrophages were stimulated with IFN-γ and LPS alone or together with 1 or 10 μg liposomes containing PS, PC, or PE. The next day, supernatant was harvested and IL-12 p70 expression was measured by ELISA.

(E) A total of 1×10^6 RAW264.7 cells were treated with IFN-γ and LPS alone or together with apoptotic cells or 10 μg of various liposomes containing PS, PC, or PE. The next day, supernatant was harvested. TGF-β1 and IL-12 p40 were measured by sandwich ELISA. The results are the mean $\pm$ SD of two independent experiments.

Figure 2B shows that at concentrations of 1 and 10 μM cytoD, which suppressed phagocytosis by $\sim$80% (Figure 2A), IL-12 p40 production by the IFN-γ/LPS-activated mouse monocytic cell line, RAW264.7, remained completely inhibited following the ingestion of apoptotic cells. This finding indicates that phagocytosis of apoptotic cells is not required to induce the inhibition of IL-12 p40 production.

PS is a lipid that is exposed on dying cells as an early sign of apoptotic cell death and serves as a critical recognition molecule on apoptotic cells for clearance by phagocytes. PS is considered to be a signaling molecule through its interaction with certain phagocytic receptors such as the PS receptor (PSR) (Fadok et al., 2000), which is expressed on the surface of activated macrophages. Treatment of RAW264.7 macrophages with various concentrations of PS-containing liposomes, but neither phosphatidylcholine (PC) nor phosphatidylethanolamine (PE) liposomes, induced a profound inhibition of IL-12 p40 synthesis (Figure 2C). A similar effect was seen on IL-12 p70 production in primary human macrophages (Figure 2D), suggesting that the repressive effect of apoptotic cells on IL-12 production could be mediated through the interaction of PS with its receptor(s) on macrophages.

We also determined the effects of the three liposomal phospholipids on TGF-β1 production in RAW264.7 cells (Figure 2E). Although PS could induce modest levels of TGF-β1 in IFN-γ/LPS-activated macrophages ($\sim$60% increase over the control), it did not correlate with the $\sim$97% decrease in IL-12 p40 output. This is consistent with the data presented in Figure 1G, suggesting no major role of the endogenous TGF-β in the regulation of IL-12 production by apoptotic cells.

Apoptotic Cells Preferentially Inhibit IL-12 p35 and IL-12 p40 mRNA Expression
To further explore the molecular basis of the inhibition of IL-12 production by phagocytosis of apoptotic cells, we analyzed the mRNA expression of cytokines in acti-
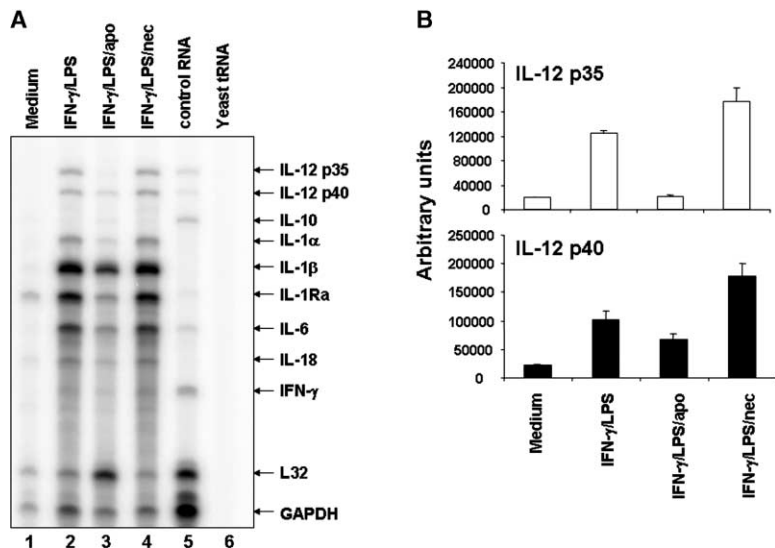
Figure 3. Phagocytosis of Apoptotic Cells Inhibits IL-12 p35 and p40 mRNA Expression

(A) Primary human macrophages were stimulated with IFN-γ and LPS in the presence or absence of apoptotic or necrotic T lymphocytes. Total RNA was harvested at 4 hr post-LPS stimulation and subjected to RNase protection analysis (RPA). Lane 1, unstimulated cells; lane 2, IFN-γ- and LPS-stimulated cells; lane 3, activated cells that phagocytosed apoptotic cells; lane 4, activated cells that ingested necrotic cells; lane 5, positive control RNA; lane 6, negative control RNA (yeast tRNA). This shows a representative of three independent experiments.

(B) Quantification of inhibition of IL-12 p35 and p40 mRNA. The degree of inhibition of IL-12 p35 and p40 mRNA expression by apoptotic cells in three independent RPAs was quantified following normalization to GAPDH expression by using the ImageQuant software and is shown with mean ± SE.

vated human macrophages in the presence or absence of apoptotic or necrotic cells (Figure 3A). In three independent experiments, the levels of IL-12 p35 and p40 mRNAs were reduced by ∼82% and ∼32%, respectively (Figure 3B), in addition to the modest inhibition of several other proinflammatory cytokines such as IL-1α, IL-1β, and IL-6. These results indicate a more selective inhibition of IL-12 p35 at the mRNA level.

IL-12 p35 Transcription Is Inhibited following Phagocytosis of Apoptotic Cells

The selective inhibition of IL-12 p35 mRNA expression by apoptotic cells prompted us to focus on the transcriptional mechanisms involved in the inhibition of the IL-12 p35 gene expression. We used a well-established transient transfection system in the mouse macrophage cell line RAW264.7 and a human IL-12 p35 promoter-luciferase reporter (Grazia Cappiello et al., 2001). We found that phagocytosis of apoptotic cells, but not necrotic cells, induced a profound inhibition of the full-length (−1082 to +61) IL-12 p35 promoter activity (Figure 4A, group #1). Deletion of a 65 bp region in the 3′ end of the p35 promoter (group #2, −1082/−4) rendered it totally resistant to the inhibitory effects of apoptotic cells. However, two 5′ deletion constructs (groups #3 and #4) responded to apoptotic cells with the activity of the full-length construct, thus suggesting that an apoptotic cell response element is located downstream of −4. To further elucidate the precise location of the apoptotic cell response element, we investigated the region between −4 and +61. By computer-assisted sequence analysis, three putative transcription factor binding sites were identified within this region of the IL-12 p35 promoter: GCF (Kageyama and Pastan, 1989) (+13 to +19), CTCF (Klenova et al., 1998) (+4 to +8), and ZEST (Park et al., 1998) (+29 to +34).

To further delineate the precise response element, mutagenesis by base substitutions was performed to disrupt these three sites. As shown in Figure 4B, mutations of the entire 7 bp GCF site (Construct B), but not the CTCF and ZEST mutants (data not shown), resulted

in a total loss of response to apoptotic cells. Additional analyses of the region between +13 and +21 revealed that the GC dinucleotide at +17/+18 (Construct D) was most critical to the response of the p35 promoter to apoptotic cells, as substitutions of this motif resulted in a complete loss of response to apoptotic cells, while mutations surrounding the GC motif (Constructs C and E) did not impact the response to apoptotic cells.

A Nuclear Factor, GC-BP, Binds to the IL-12 p35 Promoter

By a combination of DNA affinity binding/purification and mass spectrometry, we purified and identified a novel, by virtue of its activity, zinc finger transcription factor that specifically bound to the GCF element upon phagocytosis of apoptotic cells, but not necrotic cells, or upon PS treatment of RAW264.7 macrophages (Figure 5A, pointed to by an arrow). Comparison of amino acid sequences obtained with sequences in the database revealed that the protein was identical to the clone of hypothetical protein FLJ13479. We named this factor GC-BP for its ability to bind to the GC element, GCF.

The 619 amino acid sequence of GC-BP predicts strongly that it is a zinc finger-containing protein and transcriptional repressor. PSORT II predicts with a very high probability and reliability (>95%) that it is a nuclear protein with 16 putative C2H2-type zinc finger motifs that could interact with DNA (Figure 5B). It has a calculated molecular weight of 68.3 kDa. The *GC-BP* gene appears to be unique in the mouse genome and is located on chromosome 7. GC-BP, when expressed in recombinant form in a transcription/translation-coupled reaction in rabbit reticulocyte lysates, specifically bound to the IL-12 p35 promoter GCF site and forms three complexes (marked as I, II, III in Figure 5C, lane 2). The binding activities could be completely inhibited by an excess of unlabeled wild-type GCF sequence (W, lane 3), but far less efficiently by the mutant GCF sequence (M, lane 4). The complexes, especially II and III, formed with GC-BP could be inhibited by the use of an anti-GC-BP polyclonal Ab that we generated (see the Experi-
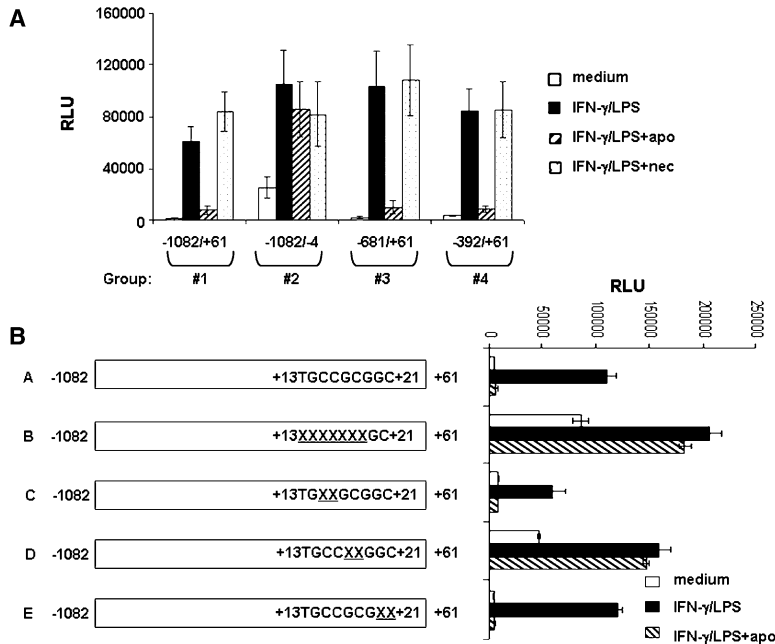
Figure 4. Localization of the Apoptotic Cell Response Element(s) in the IL-12 p35 Promoter

The wild-type human IL-12 p35 promoter (1082/+61) or deletion/substitution mutant constructs were linked to the firefly luciferase reporter gene and transiently transfected into RAW264.7 cells. Cells were treated with IFN-γ together with an exposure to apoptotic Jurkat cells for 16 hr, followed by stimulation of LPS for 7 hr. Luciferase activities of all constructs under all conditions were measured. Results represent the mean ± SD of three to four individual experiments.
(A) Response of the IL-12 p35 deletion constructs.
(B) Response of the IL-12 p35 base substitution constructs. The nucleotides at +13 to +21 are depicted in the constructs. The underlined crosses (X) indicate base substitutions by transversion.

mental Procedures) (lane 6), but not by the control antiserum (lane 5). These data demonstrate the specificity of the GC-BP/GCF interactions and confirm the ability of recombinant GC-BP, like its endogenous counterpart, to interact with the same sequence element.

To determine if GC-BP could interact with the endogenous, chromosomal *IL-12 p35* gene, we performed chromatin immunoprecipitation (ChIP) assay in RAW264.7 cells by using the polyclonal anti-GC-BP antibody and a pair of PCR primers that directed the detection to the region harboring the GCF. As shown in Figure 5D, GC-BP-specific binding was strongly and specifically detected in apoptotic cell-treated (lane 11), but not in untreated (lanes 9 and 10) or necrotic cell-treated, macrophages (lane 12), nor in control antibody-treated samples (lanes 5–8). This result demonstrates unequivocally that the native GC-BP could recognize its cognate binding site on the *IL-12 p35* gene promoter in response to apoptotic cells.

Overexpression of GC-BP Results in Selective Inhibition of IL-12 p35 Gene Expression

To determine if GC-BP could repress IL-12 p35 transcription, the IL-12 p35 promoter-reporter was cotransfected into RAW264.7 cells with a GC-BP expression vector. Expression of GC-BP dose dependently inhibited IL-12 p35 transcription (Figure 6A), while the control vector PCR3.1 had little effect (Figure 6B). The inhibitory effect by GC-BP, like that of apoptotic cells, was lost when the −1082/−4 construct was used (Figure 6C), indicating that GC-BP targets this region of the IL-12 p35 promoter. Furthermore, GC-BP expression in J774 cells (mouse macrophage) resulted in a selective and strong inhibition of IL-12 p35 mRNA induction by IFN-γ and LPS, only marginal inhibition of the p40 mRNA, and had no effect on IL-10 (data not shown).

To demonstrate the effect of GC-BP on IL-12 gene expression in primary cells, we generated bone marrow-derived macrophages (BMDMs) from mice and transduced them with a retrovirus expressing the enhanced green fluorescent protein (EGFP) or GC-BP plus EGFP (bi-cistronic). The overall transduction efficiency was ∼50% as judged by EGFP expression. GC-BP protein overexpression in transduced cells was confirmed by Western blot using the polyclonal GC-BP antibody (Figure 6D). The transduced cells were subjected to cell sorting by FACS to select for EGFP positivity, which yielded the EGFP high and low populations. Then, IL-12 p40 and p70 production was measured by ELISA. Due to the lack of an IL-12 p35-specific ELISA, and the fact that IL-12 p70 is composed of p40 and p35 molecules in a 1:1 molar ratio, IL-12 p70 production was used as an indirect indicator of IL-12 p35 synthesis. There was an ∼65% inhibition of IL-12 p40 in GC-BP-transduced BMDMs that were EGFP high and a slight inhibition in the EGFP-low population (Figure 6E). GC-BP expression completely abrogated IL-12 p70 in the EGFP-high macrophages, while it had little effect in EGF-low cells (Figure 6F).

Blocking GC-BP Expression by RNAi Results in Restoration of IL-12 p35 Transcription

To further assess the role of the endogenous GC-BP in IL-12 inhibition by phagocytosis of apoptotic cells, we applied the RNA interference (RNAi) technology to reduce endogenous GC-BP expression. We introduced a pool of three synthetic GC-BP-specific small interference RNAs (siRNA) into IFN-γ/LPS-activated J774 cells, and we showed that these siRNAs could efficiently and specifically block the expression of GC-BP protein in a dose-dependent manner; cyclin D1 expression, however, was not affected under these conditions (Figure 6G).

Consistently, blocking GC-BP expression in J744 cells by GC-BP-specific siRNAs resulted in a significant restoration of IL-12 p35 mRNA expression inhibited by apoptotic cells (compare column 9 to column 7, Figure 6H),
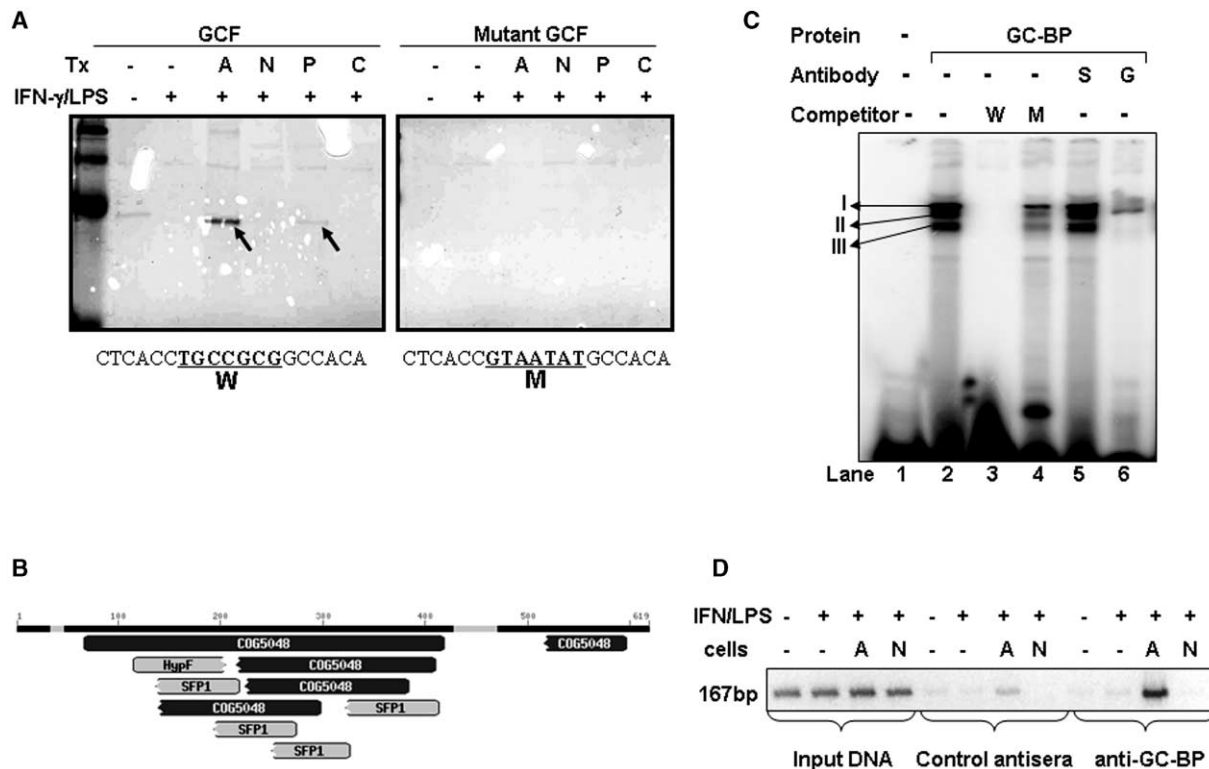
A

B

C

D

Figure 5. Identification of GC Binding Protein, GC-BP

(A) A DNA affinity binding assay was performed with GCF oligo as described in the Experimental Procedures. DNA bound proteins were eluted and separated by SDS-PAGE gel. The fractionated proteins were visualized by Commassie blue staining. A, apoptotic cells; N, necrotic cells; P, PS; C, PC. The wild-type and mutant GCF sequences are displayed with the core sequence in bold and underlined.

(B) Conserved domains in GC-BP. The GC-BP sequence was subjected to a search through the NCBI Conserved Domain Database by using RPS-Blast. Three types of conserved domains are identified: zinc finger (COG5048); HypF, hydrogenase maturation factor (posttranslational modification, protein turnover, chaperones) (COG0068); SFP1, putative transcriptional repressor regulating G2/M transition (transcription/cell division and chromosome partitioning) (COG5189).

(C) Recombinant GC-BP binding to the IL-12 p35 promoter. Recombinant GC-BP was synthesized in the transcription/translation (pT$_N$T)-coupled reaction in rabbit reticulocyte lysates and used in an EMSA with the GCF probe or the mutant GCF. The control was lysates derived from the pT$_N$T system expressing the firefly luciferase. In competition, 50× excesses (molar ratio) of unlabeled GCF oligo (W) or mutant GCF (M) oligo were used. An anti-GC-BP polyclonal antiserum (G) and control antiserum (S) were used to demonstrate the specificity of the binding. The same volume of lysates (2 μl) was applied in all lanes.

(D) Chromatin immunoprecipitation (ChIP) assay. RAW264.7 cells were stimulated and fed with apoptotic cells or necrotic cells as described in (A). ChIP analysis was performed with a polyclonal antiserum against murine GC-BP (lanes 9–12) or the control preimmunization bleed (lanes 5–8). Input DNA (lanes 1–4) was isolated and analyzed the same way, with omission of the antibody treatment.

while the siRNAs had a slightly enhancing effect on basal mRNA expression (columns 2 and 3) and no effect on IFN-γ/LPS-induced IL-12 p35 mRNA expression (column 6) in the absence of apoptotic cells.

The GC-BP-specific siRNAs caused a dramatic restoration of IL-12 p70 production that was totally inhibited following phagocytosis of apoptotic cells (compare column 9 to column 7, Figure 6I) while having little effect on the IL-12 p40 production (data not shown). Note that IL-12 p70 is composed of p35 and p40 in a 1:1 molar ratio. Although p40 production was not restored, the residual amount of p40 was still considerably higher than the amount of p70 produced. The use of siRNA in the absence of apoptotic cells with or without LPS did not have any specific impact on IL-12 p70 (columns 2 and 3 and 5 and 6, Figures 6I).

These results confirm that apoptotic cell-induced GC-BP is a selective inhibitor of IL-12 p35 transcription.

Regulation of GC-BP Expression and Tyrosine Phosphorylation

To investigate the mode of activation of GC-BP in response to apoptotic cells, we first examined its mRNA expression, which was found to be constitutively present in macrophage cells regardless of the activation state of the cell in the presence or absence of apoptotic cells (data not shown). Western blot analysis of GC-BP in RAW264.7 cells showed that there was no significant difference in the level of GC-BP protein in resting versus activated cells, in the nucleus versus the cytoplasm, or in the absence or presence of apoptotic cells added at two different time points with respect to cell activation by LPS (Figure 7A). These data indicate that GC-BP mRNA or protein expression as well as nuclear localization are not regulated during phagocytosis of apoptotic cells, and data suggest that its regulation may involve posttranslational modifications of the protein.
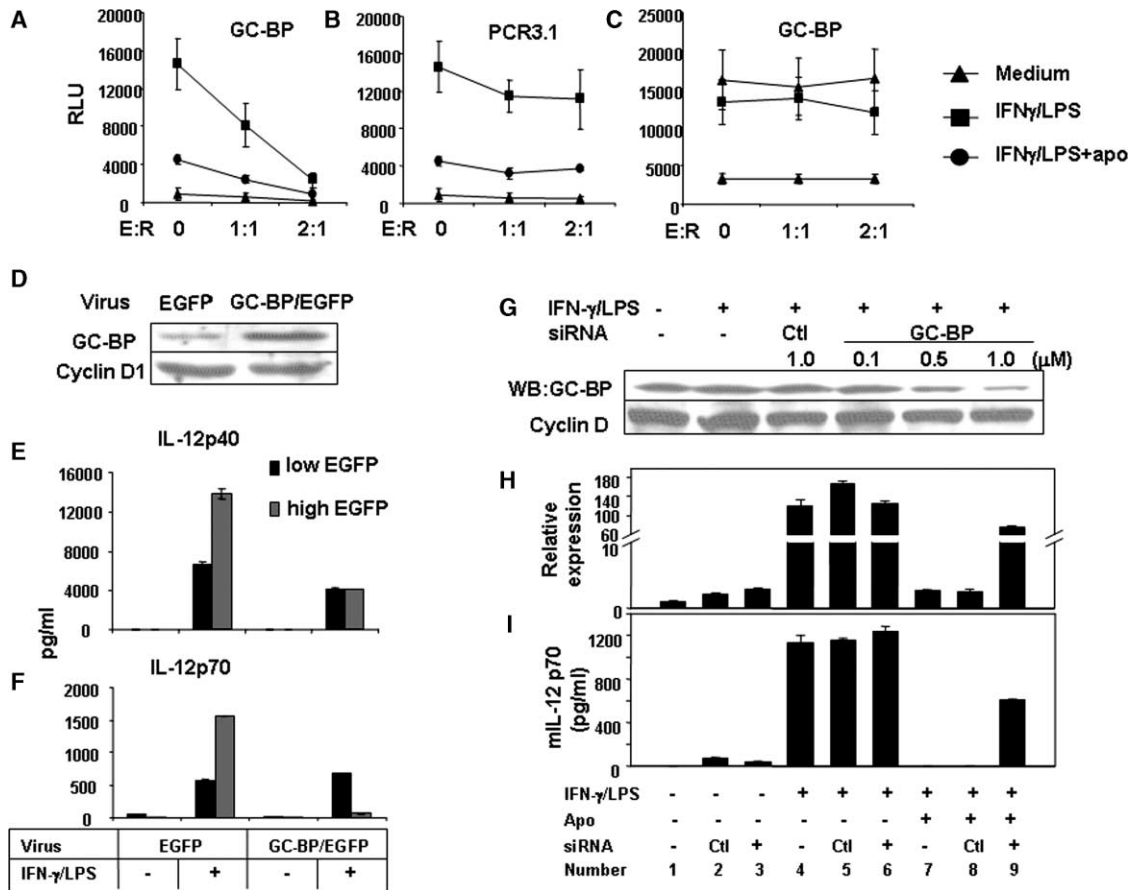
Figure 6. Effects of GC-BP on IL-12 p35 mRNA and Protein Expression

(A–C) Luciferase assay. The (A and B) full-length IL-12 p35 promoter-luciferase construct or the (C) −1082/−4 deletion construct was cotransfected with (A) expression vector GC-BP or the (B) control vector pCR3.1 into RAW264.7 cells by electroporation at molar ratios of 1:1 or 2:1 (effector:reporter). The next day, cells were stimulated by IFN-γ and LPS with (circle) or without (square) apoptotic cells. After 7 hr of LPS stimulation, cells were lysed and luciferase activity was measured. Data represent the mean ± SD of three independent experiments.

(D) GC-BP protein expression in transduced cells. Total cell lysates were isolated from retrovirally transduced macrophages and subjected to Western blot analysis with the polyclonal anti-GC-BP antiserum. The membrane was subsequently stripped and reprobed with an anti-cyclin D1 mAb (Santa Cruz) to confirm equal protein loading.

(E and F) EGFP- or GC-BP/EGFP-transduced macrophages were sorted based on the expression level of EGFP. A total of 1 × 10⁶ cells of GFP-BP or GC-BP/GFP high- and low-expressing macrophages were stimulated with IFN-γ and LPS as described above. (E) IL-12 p40 and (F) IL-12 p70 were assayed from a cell-free culture supernatant by ELISA. The ELISA results are the mean ± SD from three individual experiments.

(G) A pool of three GC-BP-specific siRNAs or a control siRNA (luciferase) was introduced into J774 cells by Superfect reagent at several concentrations as indicated following IFN-γ treatment, but 2 hr before LPS stimulation. Nuclear extracts from the transfected cells were isolated 18 hr later, and 40 μg each was subjected to Western blot analysis with the anti-GC-BP antibody. The blot was subsequently stripped and reblotted with an anti-cyclin D1 antibody.

(H) IL-12 p35 mRNA expression in siRNA-transfected cells. J774 cells were sequentially stimulated with IFN-γ and LPS for 24 hr in the presence or absence of apoptotic Jurkat cells. The GC-BP-specific siRNA pool or its control (luciferase) was transfected by Superfect reagent at the concentration of 1 μM 2 hr before the addition of LPS. Total RNA was isolated 4 hr after LPS stimulation and analyzed by real-time PCR for mIL-12 p35 mRNA expression. For normalization, mRNA for GAPDH was analyzed. Data are expressed as relative expression to that of unstimulated cells, set as 1.

(I) The production of IL-12 p70 was measured from supernatant of J774 cells following cellular stimulation and siRNA transfection as described in (H). Supernatant was collected 24 hr following LPS stimulation. Results are the mean ± SD of nine individual experiments.

As mentioned before, GC-BP has 16 putative zinc finger motifs throughout the protein and two phosphotyrosine motifs at amino acid 15 and 168, respectively. To localize the responsible region for its IL-12 p35-inhibiting activity, we generated a series of C terminus truncation constructs of GC-BP. Cotransfection of these truncated GC-BP constructs with IL-12 p35 reporter showed that an N-terminal region (the first 198 amino acids) containing the two putative phosphotyrosine motifs retained the inhibitory effects of the full-length GC-BP, whereas a further deletion fragment down to the first 27 amino acids with only one phosphotyrosine motif still retained ~50% of the repressor activity of GC-BP (Figure 7B). This result prompted us to investigate the phosphorylation status of the endogenous GC-BP by coimmunoprecipitation with phosphotyrosine-specific monoclonal antibodies. As shown in Figure 7C, GC-BP was constitutively tyrosine phosphorylated (lane 1), and its level of phosphorylation was enhanced in IFN-γ/LPS-stimulated macrophages (lane 2). The increased phosphorylation
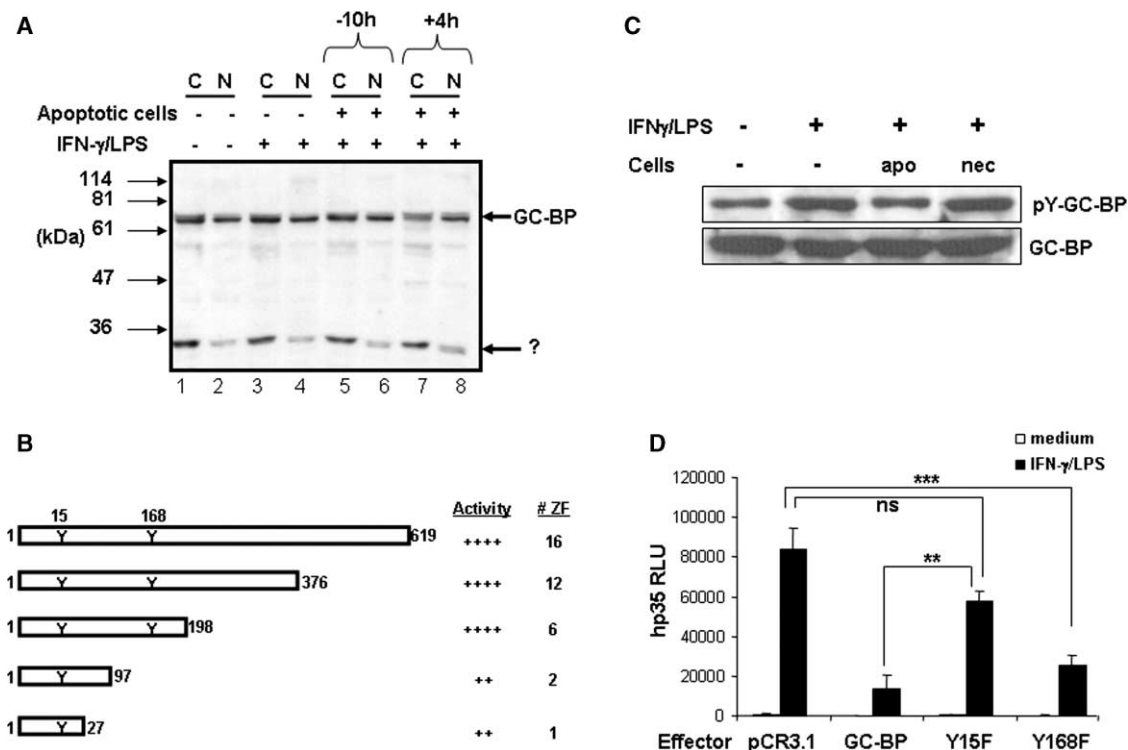
Figure 7. Protein Expression and Tyrosine Phosphorylation of GC-BP

(A) The RAW264.7 cell line was stimulated with IFN-γ and LPS alone or with apoptotic Jurkat cells that were added either 10 hr before (-10 h) or 4 hr after ($+4$h) LPS stimulation. Nuclear and cytoplasmic proteins were prepared. A total of 20 μg of proteins was analyzed on an 8% SDS-PAGE gel and immuoblotted by a polyclonal rabbit antiserum specific for the N terminus of GC-BP. The question mark indicates a major band of unknown nature. Neither of the two bands reacted with the control sera (prebleed) (not shown).

(B) Four C-terminal deletion constructs were generated by PCR from the full-length GC-BP construct (619 amino acids). Their lengths, in the number of amino acids, as well as the number of remaining putative zinc finger (ZF) motifs are marked, as are the positions of the two predicted phosphotyrosine sites at amino acids 15 and 168, respectively. Also indicated are their relative abilities to repress IL-12 p35 promoter activity in transient cotransfections.

(C) Tyrosine phosphorylation of GC-BP in RAW264.7 cells. Nucelar extracts were prepared as described in (A) and were immunoprecipitated with pY-99 AC for 4 hr at 4°C. Then, the immunoprecipitated materials were analyzed by Western blot with the anti-GC-BP antibody. Total GC-BP protein was measured as a control.

(D) The full-length IL-12 p35 promoter-luciferase reporter construct was cotransfected with GC-BP expression vector and its mutants Y15F and Y168F, or with their control vector pCR3.1. Luciferase activity was measured, and the results represent the mean $\pm$ SD of three independent experiments. Statistical analyses were done by Student's t tests with the following: p** = 0.0037, p*** = 0.0086, and ns (not significant) = 0.054.

was reduced by the addition of apoptotic cells (lane 3), but not by necrotic cells (lane 4), to macrophages. To further determine the functional importance of the two putative phosphorylation sites of GC-BP, we generated two GC-BP mutants containing a tyrosine-to-phenylalanine conversion at amino acid residue 15 (Y15F) or 168 (Y168F) and tested their ability to repress IL-12 p35 promoter activity (Figure 7D). Interestingly, the Y15F mutant, but not the Y168F mutant, significantly abrogated the inhibitory effect of GC-BP on IL-12 p35 promoter activity. This is consistent with the observation illustrated in Figure 7B that deleting the region containing Y168 made no functional difference compared to constructs that retained only Y15. These data strongly suggest that the activity of GC-BP may be regulated by modulation of tyrosine phosphorylation during phagocytosis of apoptotic cells.

Discussion

Suppression of the production of inflammatory cytokines such as IL-12 during the clearance of dead cells

by professional phagocytes is a critical mechanism in generating a tolerant state to autoantigens in the immune system (Savill et al., 2002).

Here, we provide experimental evidence that signals derived from apoptotic cells, probably through the interaction of the externalized PS with its receptors on phagocytes, induce the novel transcription factor GC-BP that selectively represses IL-12 p35 transcription by binding to the GC dinucleotide at $+17/+18$ of the p35 promoter. Apoptotic cells also potently inhibited IL-12 p40 protein production. However, the IL-12 p40 mRNA expression was only moderately inhibited by apoptotic cells (Figure 3). Moreover, overexpression of GC-BP had only a marginal inhibitory effect on IL-12 p40 mRNA expression, and blocking GC-BP by siRNA showed little effect on IL-12 p40 protein synthesis. Although there is a putative GC-BP binding site in the IL-12 p40 promoter region (one nucleotide mismatched with the IL-12 p35 GCF site), overexpression of GC-BP only mildly repressed IL-12 p40 transcription (data not shown). Taken together, these data suggest that GC-BP is not a major transcriptional regulator of the IL-12 p40 gene in re-

sponse to apoptotic cells, which may inhibit IL-12 p40 expression principally by posttranscriptional mechanisms. The inhibition of IL-12 production is direct, rather than through autocrine production of TGF-β1 or IL-10. The mode of action of GC-BP is presently not understood. It is conceivable that, based on the location of the GC-BP binding site immediately downstream of the p35 transcription initiation site (TIS), it may act by impeding the progression or processivity of the RNA polymerase complex that traverses the IL-12 p35 gene during transcription. In addition, the simultaneous loss of the apoptotic cell response and the elevated transcriptional activity following the mutation of the GC dinucleotide in the IL-12 p35 promoter (Figure 4B) is interesting. It is possible that this element binds a protein that is a physiologically relevant repressor of transcription in resting macrophages and in macrophages that are in contact with apoptotic cells. However, it is equally likely that these effects are unique to the transiently transfected promoter-reporter plasmids and are not relevant to the regulation of the endogenous gene. One way to prove the in vivo significance/relevance of the GCF element is to create a "knockout" cell in which this sequence has been deleted from the endogenous chromosomal IL-12 p35 gene.

Our data indicate that both mRNA (not shown) and protein expression of GC-BP are constitutive and not regulated upon cell activation by LPS, by feeding with apoptotic cells, or at the level of nuclear localization; yet, its DNA binding activity is enhanced by apoptotic cells and PS. These results suggest that GC-BP activity is likely controlled by posttranslational modifications at the protein level, or by its complexing with other proteins induced by apoptotic cells. Although GC-BP is predicted to contain 16 C2H2-type zinc finger domains, no more than the first 5 of them within the N-terminal 198 amino acids are critical for inhibitory activity on the IL-12 p35 transcription because deletion of the rest of the molecule does not affect its ability to repress IL-12 p35 transcription (Figure 7B). Numerous potential phosphorylation sites are identified in the GC-BP protein sequence by using the NetPhos 2.0 Prediction program: 28 serine (Ser), 9 threonine (Thr), and 2 tyrosine (Tyr) phosphorylation sites. The two Tyr phosphorylation sites are particularly interesting because they are both located within the "minimal" GC-BP functional protein (the N-terminal 198 amino acids) at amino acid 15 and 168, respectively. We provided clear evidence that GC-BP is modulated by tyrosine phosphorylation in the nucleus (Figure 7C). Furthermore, mutation studies at the two putative tyrosine phosphorylation sites, Y15 and Y16,8 showed that Y15 is functionally critical for the inhibitory activity of GC-BP (Figure 7D).

The characteristics of GC-BP, such as its apparently "ubiquitous" expression (data not shown), suggest that it may have broader activities than the transcriptional repression of IL-12 p35. It will be interesting to identify these additional activities of GC-BP. Discovery of the potential target genes of GC-BP will provide a better and more comprehensive understanding of the pathways and the framework in which GC-BP regulates the functions of various immune cell types.

In summary, the findings presented in this work significantly enhance our understanding of an essential physiological process in which cytokine responses are tightly regulated, with implications on the development and pathogenesis of inflammatory and autoimmune diseases.

Experimental Procedures

Cells and Apoptosis Induction
Human macrophages were isolated from peripheral blood monocytes of normal donors and cultured with RPMI1640 supplemented with 10% heat-inactivated pooled human serum, L-glutamine, and penicillin-streptomycin on 100 mm² dishes for 5 days. On day 5, macrophages were harvested by incubation with 5 mM EDTA in PBS and plated in 24-well plates at 1×10^6 cell/well. Mouse monocytic cell lines RAW264.7 and J774A.1 and human embryonic kidney cell line HEK293 were obtained from ATCC.

Peripheral blood-derived T cells from normal donors or Jurkat cells (human T cell line) were cultured in RPMI1640 supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. Normal T cells were activated by incubation with 2 μg/ml PHA for 24 hr. Nonadherent lymphocytes were collected and washed with medium and then cultured at 10^6 cells/ml for 5 days in medium containing 50 U/ml of recombinant human IL-2 (Chemicon International, Inc.).

Apoptosis of Jurkat T cells or activated peripheral blood-derived T cells was induced by incubation with 0.5 μg/ml staurosporine (Sigma-Aldrich) at 37°C for 6–8 hr. The percentage of early- and late-apoptotic cells was quantified by flow cytometry analysis by using Annexin V and propidium iodide (PI) staining according to the manufacturer's instructions and was routinely 70%–80%. Distinction between apoptotic and necrotic cells was based on exclusion of trypan blue as determined by light microscopy. Necrosis was induced by five cycles of freeze-thaw.

Generation of Bone Marrow Macrophages and Retrovirus Infection
Bone marrow-derived macrophage cultures were prepared as described (Vollmar and Schulz, 1994). To deliver gene expression by retrovirus, 30% of supernatant from the retrovirus-packaging cell line gp2.293 infected with either the retroviral vector pMSCV.EGFP alone or pMSCV.GC-BP/EGFP was added to macrophage culture plates on day 2 and day 4. The efficiency of gene transduction was measured by GFP expression by FACS analysis.

Antibodies and Reagents
Anti-phosphotyrosine, pY99-AC, pY-102, and all other antibodies used in the Western blots and supershift experiments were purchased from Santa Cruz Biotechnology. Anti-HA monoclonal antibody (mAb) was purchased from Covance. Recombinant human and mouse IFN-γ was purchased from Genzyme. Cytochalasin D and LPS from *Escherichia coli* were purchased from Sigma. Synthetic liposomes containing phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine were purchased from Avanti. The T$_N$T Quick coupled Transcription/Translation System was purchased from Promega.

Macrophage Phagocytosis
To quantify macrophage phagocytosis of apoptotic cells, a flow cytometric analysis modified from the method of (Mevorach et al., 1998) was used. In brief, peripheral blood-derived T cells or Jurkat T cells were labeled with the dye TAMRA (5-[and 6-]-carboxy tetramethyl rhodamine succinimidyl ester, Molecular Probes) according to the manufacture's instructions. TAMRA-labeled cells were then incubated with adherent macrophages for 1 hr at 37°C. After extensive washing, macrophages were released with trypsin-EDTA, stained with FITC-anti-CD14 mAb, and analyzed by two-color flow cytometry. Macrophages were gated by forward and side scatter and CD14 positivity. Apoptotic cells that were not internalized were excluded by this gating. The percentage of macrophages that ingested TAMRA-labeled target cells was calculated.

Cytokine Determination
IL-12 p40, p70, IL-10, and TGF-β cytokine secretion was measured by ELISA, according to protocols provided by BD-Pharmingen.

Plasmids

A 1143 bp genomic fragment of the human IL-12 p35 promoter derived from the clone AF050083 corresponding to nucleotide positions 532–1675 was cloned into the pXP2 luciferase vector as a BamHI-PstI fragment. The details are described in (Grazia Cappiello et al., 2001).

Cloning of GC-BP

Total RNA from the 4T1 mammary tumor cell line (Miller, 1983) was isolated and reverse transcribed, and GC-BP cDNA was amplified by RT-PCR. The PCR primers used were: forward primer, 5′-TGGGG AATGGAGTATTGGAA-3′, reverse primer, 5′-CGTCAAGCTGACCCTT CTTC-3′, based on the GenBank sequence BC030314.

Transfections and Luciferase Assay

Transient transfection and luciferase assays were performed as previously described (Grazia Cappiello et al., 2001).

RNase Protection

RNase protection was performed by using the hCK-2 RiboQuant Multiprobe Rnase Protection Assay system from BD-Pharmingen according to the manufacturer's instruction.

Nuclear Extract Preparation

Nuclear extracts for Western blotting and EMSA assays were prepared according to the method of Schreiber et al. (Schreiber et al., 1989).

DNA Affinity Binding Assay and Identification of GC-BP

The DNA affinity binding assay was performed essentially as we described previously (Liu et al., 2003), by using 2 μg biotinylated DNA fragments encompassing the IL-12 p35 GCF element conjugated to 100 μl streptavidin bound magnetic beads (Dynabeads). Bead-eluted proteins were separated by 10% SDS-PAGE gel and visualized by Coomassie blue or silver staining. For mass spectrometry, an LCQ classic ion trap spectrometer with new objective emitters of different sizes was used, and Sonar MS/MS software was used for the protein identification from Rockefeller University core facility. Protein analysis indicated that GC-BP was identical to the hypothetical protein FLJ13479 clone. (GenBank accession number BC030314; Protein ID: AAH30314).

Western Blotting and Immunoprecipitation

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) with 40 μg nuclear extracts or total cell lysates depending on the purpose of the experiments. Phospho-GC-BP was immunoprecipitated from precleared extracts of an equivalent number of viable RAW264.7 cells with either anti-GC-BP polyclonal antibody or agarose-conjugated pY99 monoclonal antibodies. Immunoprecipitates were washed with RIPA buffer before resolving by SDS-PAGE and transfer to PVDF.

Generation of Recombinant GC-BP In Vitro

Recombinant GC-BP protein was generated by using the T_NT Quick coupled Transcription/Translation Systems (Promega) following the manufacturer's protocol. Briefly, the mouse GC-BP cDNA containing the coding region was cloned into the pCMVT$_N$T vector in the correct orientation. A total of 0.5 μg of the GC-BP.pCMVT$_N$T vector or control luciferase.pCMVTNT vector were mixed with TNT Quick Master Mix, 1 mM methionine, and the PCR enhancer, and then incubated at 30°C for 90 min. A total of 5 μl of reaction was used for the analysis of protein expression by luciferase assay or Western blotting. A total of 2 μl were used in EMSA.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) procedure was performed by using an assay kit following the manufacturer's instructions (Upstate Biotechnology), with 1×10^7 RAW264.7 cells/condition. The input DNA was diluted 200 times before PCR. The input and precipitated DNA were PCR amplified with primers encompassing the GCF site in the mouse IL-12 p35 promoter (5′ primer: CGATCGACGCACTTGTCCTT and 3′ primer: AGCGTGATTGACA CATGCTG) and human IL-12 p35 promoter (5′ primer: GCGAACATT

TCGCTTTCATT and 3′ primer: ACTTTCCCGGGACTCTGGT) in a buffer containing 2 mM MgCl$_2$. The samples were amplified for 34 cycles by PCR and were analyzed by electrophoresis on a 1.2% agarose gel.

SiRNA

Chemically synthesized siRNA for GC-BP was designed by Design Center (Dharmacon). The sequences are: GC-BP#1, 5′-GACAAGGU AUCAGCUUCAGdTdT-3′, GC-BP#2, 5′-GCAUUUGCUGAGCGAG CUAdTdT-3′, and GC-BP#3, 5′-ACCUCUUGUGGCUUUGCUAd TdT-3′. A control siRNA for the luciferase gene was purchased from Dharmacon. RNA oligomers in water were annealed at a concentration of 50 mM each with an initial hold at 90°C for 1 min, followed by a time-controlled cooling to room temperature. Transfection of siRNAs for targeting endogenous genes was carried out by using Superfect or Effectene (QIAGEN) and 1 μM siRNA duplex per well. HEK293 or RAW264.7 cell lines were transfected with siRNAs 1 hr before apoptotic cell incubation. Transfection efficiency was determined by RT-PCR or Western blotting for the target gene.

Generation of Polyclonal Anti-GC-BP Antibody

N-terminal GC-BP covering amino acids 1–231 was cloned into the pRSET expression vector, which contains His tag at the C terminus of GC-BP. Cloned GC-BP was transfected into the bacterial cell line, BL21(PE3)pLysS, and then treated with 1 mM IPTG for 4 hr to induce GC-BP expression. The IPTG-treated bacteria was lysed in Guanidinium Lysis buffer, and GC-BP was purified by using a Pro-Bond Purification System (Invitrogen). Recombinant mouse GC-BP was injected into rabbit. Following four injections (one primary plus three boosts), rabbit antiserum was obtained.

Quantitative Real-Time PCR

To determine the level of IL-12 p35 mRNA by quantitative real-time PCR, we used a modified protocol from (Rajeevan et al., 2001). Briefly, cDNA converted from 1 μg of total RNA was diluted to several concentrations. Diluted cDNA was mixed with a pair of primers (10 μM) derived from mouse IL-12 p35 or GAPDH cDNA sequences and SYBR green PCR master mix (Applied Biosystem) in a 15 μl volume. PCR cycling was as followed: 2 min at 50°C, 10 min at 95°C for 1 cycle, followed by 40 cycles at 15 s at 95°C and 1 min at 60°C. The PCR primers used were: forward primer, 5′-AAATGAAGCTCTG CATCCTGC-3′, reverse primer, 5′-TCACCCTGTTGATGGTCACG-3′ for mouse IL-12 p35; forward primer, 5′-AACT TTGGCATTGTG GAAGG-3′, reverse primer, 5′-ACACATTGGGGGTAGGAACA-3′ for mouse GAPCH.

References

Bickerstaff, M.C., Botto, M., Hutchinson, W.L., Herbert, J., Tennent, G.A., Bybee, A., Mitchell, D.A., Cook, H.T., Butler, P.J., Walport, M.J., et al. (1999). Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. Nat. Med. 5, 694–697.

Du Clos, T.W., Zlock, L.T., Hicks, P.S., and Mold, C. (1994). Decreased autoantibody levels and enhanced survival of (NZB × NZW) F1 mice treated with C-reactive protein. Clin. Immunol. Immunopathol. 70, 22–27.

Fadok, V.A., Bratton, D.L., Rose, D.M., Pearson, A., Ezekewitz, R.A., and Henson, P.M. (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405, 85–90.

Grazia Cappiello, M., Sutterwala, F.S., Trinchieri, G., Mosser, D.M.,

and Ma, X. (2001). Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. J. Immunol. *166*, 4498–4506.

Haslett, C., Savill, J.S., Whyte, M.K., Stern, M., Dransfield, I., and Meagher, L.C. (1994). Granulocyte apoptosis and the control of inflammation. Philos. Trans. R. Soc. Lond. B Biol. Sci. *345*, 327–333.

Herrmann, M., Voll, R.E., Zoller, O.M., Hagenhofer, M., Ponner, B.B., and Kalden, J.R. (1998). Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum. *41*, 1241–1250.

Kageyama, R., and Pastan, I. (1989). Molecular cloning and characterization of a human DNA binding factor that represses transcription. Cell *59*, 815–825.

Klenova, E.M., Fagerlie, S., Filippova, G.N., Kretzner, L., Goodwin, G.H., Loring, G., Neiman, P.E., and Lobanenkov, V.V. (1998). Characterization of the chicken CTCF genomic locus, and initial study of the cell cycle-regulated promoter of the gene. J. Biol. Chem. *273*, 26571–26579.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Leonard, J.P., Waldburger, K.E., and Goldman, S.J. (1995). Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. J. Exp. Med. *181*, 381–386.

Leonard, J.P., Waldburger, K.E., Schaub, R.G., Smith, T., Hewson, A.K., Cuzner, M.L., and Goldman, S.J. (1997). Regulation of the inflammatory response in animal models of multiple sclerosis by interleukin-12. Crit. Rev. Immunol. *17*, 545–553.

Liu, J., and Beller, D. (2002). Aberrant production of IL-12 by macrophages from several autoimmune-prone mouse strains is characterized by intrinsic and unique patterns of NF-kappa B expression and binding to the IL-12 p40 promoter. J. Immunol. *169*, 581–586.

Liu, J., Cao, S., Herman, L.M., and Ma, X. (2003). Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. J. Exp. Med. *198*, 1265–1276.

McIntyre, K.W., Shuster, D.J., Gillooly, K.M., Warrier, R.R., Connaughton, S.E., Hall, L.B., Arp, L.H., Gately, M.K., and Magram, J. (1996). Reduced incidence and severity of collagen-induced arthritis in interleukin-12-deficient mice. Eur. J. Immunol. *26*, 2933–2938.

Mevorach, D., Mascarenhas, J.O., Gershov, D., and Elkon, K.B. (1998). Complement-dependent clearance of apoptotic cells by human macrophages. J. Exp. Med. *188*, 2313–2320.

Miller, F.R., Miller, B.E., and Heppner, G.H. (1983). Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis *33*, 22–31.

Mitchell, D.A., Pickering, M.C., Warren, J., Fossati-Jimack, L., Cortes-Hernandez, J., Cook, H.T., Botto, M., and Walport, M.J. (2002). C1q deficiency and autoimmunity: the effects of genetic background on disease expression. J. Immunol. *168*, 2538–2543.

Murphy, T.L., Cleveland, M.G., Kulesza, P., Magram, J., and Murphy, K.M. (1995). Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol. Cell. Biol. *15*, 5258–5267.

Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H.G., and Moroy, T. (2000). Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat. Genet. *25*, 177–181.

Park, M., Venkatesh, T.V., and Bodmer, R. (1998). Dual role for the zeste-white3/shaggy-encoded kinase in mesoderm and heart development of *Drosophila*. Dev. Genet. *22*, 201–211.

Rajeevan, M.S., Ranamukhaarachchi, D.G., Vernon, S.D., and Unger, E.R. (2001). Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods *25*, 443–451.

Rothe, H., O'Hara, R.M., Jr., Martin, S., and Kolb, H. (1997). Suppression of cyclophosphamide induced diabetes development and pancreatic Th1 reactivity in NOD mice treated with the interleukin (IL)-12 antagonist IL-12(p40)2. Diabetologia *40*, 641–646.

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. Nature *407*, 784–788.

Savill, J., Fadok, V., Henson, P., and Haslett, C. (1993). Phagocyte

recognition of cells undergoing apoptosis. Immunol. Today *14*, 131–136.

Savill, J., Dransfield, I., Gregory, C., and Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. Nat. Rev. Immunol. *2*, 965–975.

Schreiber, E., Matthias, P., Muller, M.M., and Schaffner, W. (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. *17*, 6419.

Simpson, P.B., Mistry, M.S., Maki, R.A., Yang, W., Schwarz, D.A., Johnson, E.B., Lio, F.M., and Alleva, D.G. (2003). Cuttine edge: diabetes-associated quantitative trait locus, Idd4, is responsible for the IL-12p40 overexpression defect in nonobese diabetic (NOD) mice. J. Immunol. *171*, 3333–3337.

Spadaro, A., Scrivo, R., Rinaldi, T., Riccieri, V., Sili Scavalli, A., Taccari, E., and Valesini, G. (2002). The role of interleukin-12 in immune-mediated rheumatic diseases. Reumatismo *54*, 113–121.

Trembleau, S., Germann, T., Gately, M.K., and Adorini, L. (1995). The role of IL-12 in the induction of organ-specific autoimmune diseases. Immunol. Today *16*, 383–386.

Trinchieri, G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. *13*, 251–276.

Voll, R.E., Roth, E.A., Girkontaite, I., Fehr, H., Herrmann, M., Lorenz, H.M., and Kalden, J.R. (1997). Histone-specific Th0 and Th1 clones derived from systemic lupus erythematosus patients induce double-stranded DNA antibody production. Arthritis Rheum. *40*, 2162–2171.

Vollmar, A.M., and Schulz, R. (1994). Gene expression and secretion of atrial natriuretic peptide by murine macrophages. J. Clin. Invest. *94*, 539–545.

Wong, C.K., Ho, C.Y., Li, E.K., and Lam, C.W. (2000). Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus *9*, 589–593.

Yokoi, H., Kato, K., Kezuka, T., Sakai, J., Usui, M., Yagita, H., and Okumura, K. (1997). Prevention of experimental autoimmune uveoretinitis by monoclonal antibody to interleukin-12. Eur. J. Immunol. *27*, 641–646.