Generation of Inhibitory NFκB Complexes and Phosphorylated cAMP Response Element-binding Protein Correlates with the Anti-inflammatory Activity of Complement Protein C1q in Human Monocytes*

Deborah A. Fraser, Meenakshi Arora1, Suzanne S. Bohlson3, Encarnacion Lozano3, and Andrea J. Tenner4

From the Department of Molecular Biology and Biochemistry, Center for Immunology, University of California, Irvine, California 92697

The interaction of C1q with specific cells of the immune system induces activities, such as enhancement of phagocytosis in monocytes and stimulation of superoxide production in neutrophils. In contrast to some other monocyte activators, C1q itself does not induce pro-inflammatory cytokine production, but rather inhibits the lipopolysaccharide (LPS)-stimulated induction of certain pro-inflammatory cytokines and induces expression of interleukin-10. To investigate the molecular mechanism by which C1q exerts this effect on gene expression, the influence of C1q on the activation of transcription factors of the NFκB family and cAMP response element-binding protein (CREB) was assessed. C1q treatment increased kB binding activity in freshly isolated human monocytes in a time-dependent fashion as assessed by electrophoretic mobility shift assays. In antibody supershift experiments, anti-p50 antibody supershifted the C1q-induced NFκB complex, whereas anti-p65 antibody had little effect, suggesting that C1q induced the translocation of NFκB p50p50 homodimers. This is in contrast to the dominant induction of p65 containing complexes in parallel monocyte cultures stimulated with LPS. C1q treatment also induced cAMP response element (CRE)-binding activity as demonstrated by electrophoretic mobility shift assay, increased phosphorylation of CREB, and induction of CRE driven gene expression. In contrast, CREB activation was not detected in LPS-treated monocytes. These results suggest that C1q may modulate the cytokine profile expressed in response to inflammatory stimuli (e.g. LPS), by triggering inhibitory and/or competing signals. Because C1q and other defense collagens have been shown to enhance clearance of apoptotic cells, this regulatory pathway may be beneficial in avoiding autoimmunity and/or resolving inflammation.

The transcription factor complexes of the NFκB family have attracted widespread attention because of their rapid induction, the wide range of genes whose expression is influenced by these complexes, and the apparent consequences of its induction in several diseases (1). The NFκB/Rel family of transcription factors, which include p65, p50, p52, RelB, and c-Rel, form homo- and heterodimers resulting in distinct transcription factor complexes that regulate expression of many different genes (2). In unstimulated cells, NFκB is sequestered in the cytoplasm. Upon stimulation, the inhibitory protein IκBα dissociates from specific pre-existing cytoplasmic NFκB complexes, allowing the transcription factors to translocate to the nucleus and initiate the expression of target genes (1). Lipopolysaccharide (LPS) and other pathogen-associated molecules have long been implicated in the inducible expression of many genes in monocytes, including pro-inflammatory cytokines such as tumor necrosis factor-α, IL-1α and -β, and IL-6 via specific NFκB translocation (3). Prolonged LPS activation can also induce expression of anti-inflammatory IL-10 in monocytes, perhaps an important step in regulation of inflammation (4). Studies suggest that a number of different elements are able to stimulate IL-10 production, including the transcription factors cAMP response element-binding protein (CREB) and ATF-1 (5). The CREB/ATF family of transcription factors are leucine zipper proteins that bind to the CRE consensus sequence, 5’-TGACGTCA-3’. CREB, the most extensively studied CRE-binding protein, is phosphorylated at serine 133, and this leads to transcriptional activation of genes whose promoters contain the CRE sequence, such as IL-10 (4–6). There are a number of different signaling pathways that lead to phosphorylation and activation of CREB (for review, see Ref. 7) such as protein kinase A, calmodulin kinase, the pp90rsk family of serine/threonine kinases, or protein kinase C.

Human C1q, the recognition component of the classical complement cascade, activates human monocytes from peripheral blood resulting in the enhancement of phagocytosis (8, 9). More recently, it has also been shown that C1q can play a prom-
Transcription Factor Modulation by C1q

Evident role in the clearance of apoptotic cells (10–13). Often concomitant with an activation process, monocytes/macrophages initiate synthesis of pro-inflammatory cytokines. However, we found that C1q does not trigger pro-inflammatory cytokine expression in human monocytes even under the same conditions in which the enhancement of phagocytosis is seen. Furthermore, C1q down-regulates the secretion of the inflammatory cytokines interleukin 1α and β in response to LPS and up-regulates the anti-inflammatory cytokine IL-10 (14). To further evaluate the molecular mechanism by which C1q exerts this effect, an analysis of the influence of C1q on the activation of transcription factors of the NFκB family and CREB was initiated.

EXPERIMENTAL PROCEDURES

Media, Reagents, and Antibodies—Raw264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 100 units/ml penicillin/streptomycin (Invitrogen). THP-1 cells were cultured in RPMI 1640 media (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-alanyl-L-glutamine (Invitrogen), 10 mM HEPES, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 4.5 g/liter d-glucose, 1.5 g/liter sodium bicarbonate, and 100 units/ml penicillin/streptomycin. Monocytes were cultured in, and luciferase assays were carried out in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1% L-alanyl-L-glutamine. The human serum albumin (HSA), produced for the American Red Cross by Baxter Healthcare Corporation (Glendale, CA), was obtained from Sigma. Affinity-purified polyclonal antibodies anti-C/EBP-α, anti-CREB-1 (sc-186X), anti-p65 (sc-372X), and anti-p50 (sc-7178X and sc-1190X) were generously provided by Dr. Nancy Rice (NCI-Frederick, National Institutes of Health). The rabbit sera 1141, 1207, 1267, and 1136 (against a synthetic peptide mapping at the amino terminus of human cRel) were generously provided by Dr. Nancy Rice (NCI-Frederick, National Institutes of Health).

C1q was isolated from plasma-derived human serum by the method of Tenner et al. (15) as modified by Young et al. (16). The preparations used were fully active as determined by hemolytic titration and homogeneous as assessed by SDS-PAGE, and free of endotoxin to below 1 pg/ml in the C1q concentrations used in these studies as assayed by the Limulus Amebocyte Lysate assay (BioWhittaker). Protein concentration was determined using an extinction coefficient (ε380) at 280 nm of 6.82 for C1q (17).

Monocyte Isolation and Stimulation—Human peripheral blood monocytes were isolated by counterflow elutriation using a modification of the technique of Lionetti et al. (18) as described. Blood units were collected into CPDA1 at the University of California, Irvine, Medical Center Blood Bank or General Clinical Research Center (Orange, CA). Usually more than 90% of the cells in each preparation were monocytes according to size analysis on a Coulter Channelizer. For each monocyte preparation, the ability of the cells to respond to C1q with an enhancement of phagocytosis was verified (9). LabTek chambers (Nunc, Naperville, IL) were coated with C1q or HSA (8–16 μg/ml) in coating buffer (0.1 M carbonate, pH 9.6) for 2 h and after washing in phosphate-buffered saline, monocytes in HL-1 media were added, centrifuged at 700 × g for 3 min, and incubated for 30 min at 37 °C. The cells were then stimulated with different concentrations of LPS as described in the text.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts for EMSA were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions, or by following a modification of the procedure of Schreiber et al. (19) and Dignam et al. (20). Briefly, one-well LabTek chambers coated with C1q or HSA were incubated with 5 × 10⁶ monocytes for 30 min. The cells were then stimulated with 30 ng/ml of LPS for the times indicated in the text. Cells were collected by scraping and pelleted in a Sorvall RT6000 centrifuge at 1,000 rpm for 7 min at 4 °C. The cell pellet was resuspended in 1 ml of cold phosphate-buffered saline and spun down at 1,500 × g for 7 min at 4 °C. Phosphate-buffered saline was removed and the pellet was resuspended in 100 μl of cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 10 μg/ml chymostatin, 1 μg/ml apronin, 0.1 μg/ml leupeptin, 0.5 μM phenylmethylsulfonyl fluoride, 3 μg/μl of α1-antitrypsin and 0.05% Nonidet P-40) by gently pipetting. After 5–10 min on ice, the nuclei were pelleted by quick spinning at 16,000 × g. The supernatant (cytoplasmic fraction) was transferred to a new tube and the nuclei were washed with buffer A and then resuspended in 50 μl of ice-cold buffer B (20 mM HEPES, pH 7.9, 20% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 10 μg/ml chymostatin, 1 μg/ml apronin, 0.1 μg/ml leupeptin, 0.5 μM phenylmethylsulfonyl fluoride, and 3 μg/μl of α1-antitrypsin) and lysed on ice for 30 min. The nuclear extract was centrifuged at 16,000 × g for 10 min at 4 °C and the supernatant fractions were frozen in aliquots at −70 °C. Protein concentration was determined by BCA (Pierce) using bovine serum albumin for the standard curve.

Gel mobility shift assays were performed as described previously (21, 22): 10 μg of extracted nuclear protein was incubated with 32P-labeled oligonucleotide containing the consensus CRE sequence (Promega), the consensus binding sequence for NFκB (Promega, Madison, WI), or the kB3 site as described in Baer et al. (23) for 30 min at room temperature in the binding buffer (10 mM HEPES, 50 mM KCl, 0.5 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol, and 0.05% Nonidet P-40) with 1 μg of poly(dI:dC) (Amersham Biosciences). Non-radiolabeled specific and non-specific duplex probe were added to the nuclear extracts in binding assay buffer and incubated for 15 min at room temperature before 32P-probe addition to assess the specificity of the EMSA interaction. Samples were then resolved on a 4% non-denaturing polyacrylamide gel with 0.5× TBE buffer (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA). The gel was dried and visualized using a phosphorimager.
To identify the proteins binding to the radiolabeled probe, specific antibody (1 μl per reaction) was added to nuclear extracts and incubated at room temperature for 1 h. 32P-Probe was then added and the mixture incubated for an additional 30 min before electrophoresis as described above.

Trans-AM NFκB ELISA—The Trans-AM NFκB kit from Active Motif (Carlsbad, CA) was used to determine the levels of p65 and p50 in nuclear extracts as per the manufacturer’s instructions. Briefly, 2 μg of nuclear extract, diluted to 20 μl, was added to the wells coated with oligonucleotides containing the NFκB consensus binding site. The primary antibodies used to detect NFκB recognize an epitope on p65 or p50 that is accessible only when NFκB is activated and bound to its target DNA. Following addition of horseradish peroxidase-conjugated secondary antibodies and substrate, absorbance was read at 450 nm (with a reference wavelength at 650 nm). To monitor the specificity of the assay, competitive binding assays were performed. Wild-type or mutated consensus oligonucleotides were added to the wells containing immobilized oligonucleotides before the addition of nuclear extracts.

Cell Extract and Western Blotting—Monocytes stimulated with C1q or HSA control were harvested into lysis buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1.25 mM sodium orthovanadate) that was supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and incubated on ice for 30 min (for CREB Western blots). Nuclear extracts of C1q or HSA control-stimulated monocytes for NFκB Western blots were prepared as for the Trans-AM NFκB ELISA. 20 (whole cell extract) or 10 μg (nuclear extract) of soluble extract per lane was separated in 10% SDS-PAGE and then transferred to nitrocellulose. The membranes were blocked overnight with blocking buffer containing 3% dry milk in TBST (0.05% Tween 20 in 20 mM Tris, pH 7.4, 150 mM NaCl) before addition of antibodies to the protein of interest. Antibody reactivity on the Western blots was detected using the enhanced chemiluminescent detection system (ECL) (Amer sham Biosciences).

Transfection and Luciferase Assay—Induction of NFκB or CREB transcriptional activity was evaluated using a luciferase reporter gene driven by κ enhancer, pNFκB (Clontech, Palo Alto, CA), or CRE (Promega). The IL-10 promoter construct was kindly provided by Dr. A. Kumar, University of Ottawa, Canada (24). RAW 264.7 cells (NFκB) or THP-1 cells (CRE, IL-10) were transfected with the luciferase reporter construct together with pRL-TK Renilla luciferase internal control vector (Promega) using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer’s instructions. After 24 h, cells were replated in HL-1 into two-well Lab-Tek chambers precoated with HSA or C1q and preincubated for 30 min at 37°C prior to addition of LPS for the time stated. Cells were then harvested and the firefly luciferase activity and the Renilla luciferase activity were measured using the Dual Luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity to control for variability in transfection efficiency and to calculate the relative luciferase activity.

RESULTS

C1q Induces NFκB Activity in Human Monocytes—In recent studies, we and others have found that C1q modulates cytokine expression by human monocytes or human peripheral blood-derived dendritic cells (14, 25). In human monocytes, C1q decreased expression of LPS-induced pro-inflammatory cytokines IL-1β and IL-1α, and increased the expression of IL-10 (14). Because LPS stimulates pro-inflammatory cytokine expression through activation of NFκB, we hypothesized that C1q might be inhibiting the NFκB activation by LPS. Nuclear extracts from human monocytes stimulated with C1q ± LPS were analyzed by EMSA for NFκB binding. Although a basal level of nuclear NF-κB binding activity was present, LPS stimulation induced an increase in NFκB binding activity in nuclear extracts, as expected (Fig. 1). Incubation of monocytes with C1q also reproducibly induced an increase in NFκB binding activity in nuclear extracts both in the presence and absence of LPS (Fig. 1). Unlabeled NFκB probe strongly competed for complex formation, whereas the nonspecific SPI probe did not (Fig. 1, right lanes), indicating that the complexes activated by C1q and LPS, detected by EMSAs, are specific for the NFκB binding sequence and do not represent nonspecific binding.

NFκB DNA Binding Activity Stimulated by C1q is due to p50/p50 Homodimers—The opposing effects of C1q and LPS on pro-inflammatory cytokine expression, lead to investigation of the identity of the NF-κB-specific components involved in C1q-induced binding by performing antibody competition EMSAs. As expected, in LPS-stimulated extracts, complex formation with the NFκB probe was either inhibited by anti-p65 or supershifted by anti-p50 (Fig. 2B). In contrast, anti-p50 supershifted C1q-stimulated NFκB complexes, whereas little inhibition of complex formation was seen with anti-p65 suggesting that C1q-stimulated complexes are predominantly p50/p50 homodimers (Fig. 2A). Antibodies to other Rel family members (p52, c-Rel, and Rel-B) did not inhibit or supershift the protein-oligonucleotide complex formation (data not shown). The C1q-induced enhancement in NFκB binding was also seen using oligos corresponding to the κB site of the murine tumor necrosis factor α promoter (data not shown), which is known to preferentially bind p50 homodimers in vitro (23). Finally, whereas C/EBP-α has been detected by others in association with NFκB complexes, antibodies to C/EBP-α did not induce supershift of C1q-induced NFκB complexes (data not shown).
activity upon stimulation with LPS (26). Importantly, RAW 264.7 cells are capable of phagocytosing IgG-opsonized targets and respond to C1q with an enhancement of phagocytosis (data not shown). Incubation of transfected cells with C1q alone did not induce NFκB-driven luciferase activity, whereas incubation with LPS increased luciferase activity in a dose-dependent manner, as expected (Fig. 4A, left). However, cells that adhered to C1q and then stimulated with LPS showed significant inhibition of this LPS-induced transactivation (Fig. 4, A and B, left, n = 3). These data demonstrate that C1q mediates an inhibition of NFκB-stimulated promoter activity. Such inhibition may therefore play a significant role in C1q modulation of pro-inflammatory cytokine expression in phagocytic cells. In contrast, C1q enhanced IL-10 promoter-driven expression in THP-1 cells (Fig. 4A, right) by 2–2.6-fold in the absence of LPS or presence of 10–100 ng/ml LPS (Fig. 4B), supporting the previously reported C1q-induced up-regulation of IL-10 expression in primary monocytes.

C1q Activates CREB—Activation of the transcription factor CREB is regulated by phosphorylation at serine 133 (7). Western blot analysis of monocyte extracts with an antibody that specifically recognizes phosphorylated CREB showed that C1q induces rapid (within 5 min) phosphorylation of CREB (Fig. 5). No such induction with LPS (30 ng/ml) was observed (data not shown). Activated CREB is known to engage CREB-binding protein (CBP), and thus could compete with NFκB p65p50 for limiting amounts of CBP, a component required for optimal p65p50-dependent transcription (27, 28). This would provide an additional or alternative mechanism for the C1q-mediated inhibition of LPS-triggered pro-inflammatory cytokine production.

Nuclear extracts from cells stimulated with C1q and/or LPS were also analyzed for DNA binding activity by gel shift assay using 32P-labeled oligonucleotides containing the consensus DNA binding sequence for transcription factor CREB. The CRE-DNA binding activity was increased by C1q, whereas LPS did not induce CRE-DNA binding activity (Fig. 6). The C1q-induced CRE binding was inhibited by anti-Creb1 (data not shown). The complex formation was also inhibited in the presence of excess unlabeled CRE-oligonucleotide, but not in the presence of excess unlabeled SP-1 oligonucleotide showing that this complex is specific for CRE-binding protein.

The transcriptional activity of C1q-activated CREB was measured using a luciferase reporter gene assay. Constructs containing a luciferase reporter gene under the control of CRE elements were transfected into THP-1 cells. Adherence of transfected cells to immobilized C1q resulted in enhanced levels of luciferase activity compared with control cells (Fig. 7).

**DISCUSSION**

In this study, we explore the molecular basis for our observation that C1q can inhibit LPS-induced expression of certain pro-inflammatory cytokines and enhance secretion of anti-inflammatory IL-10 (14). Transcriptional regulation of pro-inflammatory cytokine expression by LPS has been shown to involve κB sequence motifs and transcriptional factors from the Rel family (29–31). Members of the Rel family include Rel A (p65), p50, p52, c-Rel, and Rel B. Each member has a conserved Rel homology domain responsible for DNA-binding, dimeriza-

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**FIGURE 2.** Anti-p50 bound the NFκB DNA binding complex stimulated by C1q to a greater extent than anti-p65. Nuclear extracts were prepared from monocytes adhered to C1q or HSA for 1 h without LPS treatment (A) or for 30 min followed by addition of 30 ng/ml of LPS for 2 h (B). Specific antibodies for p65 and p50 were added to the extracts for 1 h before the addition of the 32P-labeled consensus oligonucleotide for NFκB transcription factors. Reactions were then electrophoresed and the retarded/inhibition of protein-oligonucleotide probe complexes assessed. Anti-p50 used in the experiments supershifted the NFκB complexes, whereas anti-p65 inhibited the NFκB complexes.

In both an ELISA-based assay of p65 and p50 nuclear translocation and Western blots, nuclear localization of p50 but not p65 was enhanced by C1q in comparison to the HSA control (Fig. 3A). In the ELISA, nuclear migration of p50 was rapid, and increased by an average of 3-fold after a 1-h treatment of monocytes with C1q relative to HSA both in the absence and presence of LPS (values of -fold enhancement by C1q ranged from 1.6 to 6.2, and 2.2 to 5.4, respectively, n = 3). Relative levels of p50 but not p65 in the nucleus of C1q-treated monocytes were still enhanced above HSA controls at 2 h (1.6–2.4-fold in the presence of LPS), returning to baseline after 18 h. In this assay, the wild-type consensus oligonucleotide, when used as a competitor for NFκB, completely inhibited NFκB binding to the probe immobilized on the plate (as assessed by both detecting antibodies). However, the mutated consensus oligonucleotide had no effect on the binding of NFκB (data not shown) demonstrating that binding of NFκB to the consensus oligonucleotide was specific. These results were also confirmed by Western blot analysis of nuclear extracts using antibodies to p50 and p65 (Fig. 3B). Analysis of band intensities by densitometry showed a greater than 2-fold increase of p50 in the nucleus of monocytes that had been incubated for 2 h on C1q (both with and without LPS) compared with the HSA control cells (Fig. 3C). However, levels of p65 were not greatly altered by incubation on C1q (less than 2-fold). Complete separation of cytoplasmic and nuclear compartments was confirmed by re-probing blots with antibodies to nuclear protein histone-H1 and cytoplasmic protein actin (Fig. 3B).

C1q Inhibits LPS-induced NFκB-mediated Transcription—To further validate the effect of C1q on transcriptional activity of NFκB, a luciferase reporter gene expressed under the control of κB elements was transfected into RAW 264.7 cells, murine macrophage-derived cells that are known to induce NFκB

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**Transcription Factor Modulation by C1q**
tion, and interaction with IκB family members. Dimers of members of this Rel family of proteins constitute the NFκB complex. Each combination has its own transactivating potential (2). Although most of the NFκB protein complexes (p65/p50, p50c-Rel, p65/p65, and p65c-Rel) are transcriptionally active, some combinations such as the p50 homodimer and p52 homodimer are thought to mainly act as inactive or repressive complexes (32–35). The most studied NFκB complex consists of p65/p50 heterodimers, which are complexed to the inhibitor IκB in the cytoplasm of unstimulated cells. Stimuli such as LPS and pro-inflammatory cytokines induce the phosphorylation of IκB, followed by release and subsequent nuclear translocation of the p65/p50 heterodimers, which bind the regulatory sequences in a variety of target genes (36), inducing transcriptional activity.

The studies presented here indicate that C1q alone can induce NFκB translocation to the nucleus, and that this activation is specific. However, whereas anti-p65 and anti-p50 interact with the NFκB complex induced by LPS in monocytes, in C1q-stimulated cells, NFκB DNA binding complexes were predominantly composed of p50p50 homodimers. Both kinds of NFκB complexes were observed in cells stimulated with both C1q and LPS. Western blots and ELISA-based assays for NFκB p50 also indicated a 2–3-fold increase in nuclear p50 levels in monocytes stimulated with C1q, with no increase in levels of p65. These results suggest that the suppressive effect of C1q on the LPS-induced cytokine expression may be mediated by the generation of inhibitory (p50p50) NFκB complexes because this NFκB complex binds to the κB elements but does not promote transcriptional activity of the κB containing promoters. Indeed, here we show in a luciferase reporter assay, C1q-inhibited LPS-induced transactivation of gene expression from an NFκB promoter construct. Overexpression of p50 has been shown previously by others to inhibit transcriptional activation from synthetic promoters consisting of multiple κB motifs (37, 38). Others have shown preferential binding of p50 homodimers to specific NFκB binding sites, which result in suppression of tumor necrosis factor α expression in macrophages (23). Binding of p50 homodimers to the positively acting κB elements of the promoters has been shown to result in inhibition of LPS-induced κB-mediated induction of pro-inflammatory cytokine mRNAs (39).

The activity of many inducible transcription factors, including NFκB, is regulated through their association with cell-specific coactivators (27, 28, 40–43). Indeed, it has been shown that transcriptionally repressive homodimers of p50 are complexed with the histone deacetylase HDAC-1 in resting cells (44). Conversely, interaction with the coactivator CBP appears to be necessary to optimize the transcriptional activity of NFκB (27, 45). The interaction of the p65 subunit of NFκB with CBP involves the KIX region of CBP, which is the same CBP region responsible for binding the transcriptionally active serine –133-phosphorylated form of CREB (27, 28). Because CBP is present in limiting amounts in the nucleus, competition between NFκB and CREB for binding to CBP has been postulated to be important in regulating the transcriptional activity of these factors. In vitro studies have directly shown such com-

FIGURE 3. Nuclear localization of p50 is enhanced by C1q. Human peripheral blood monocytes adhered to LabTek chamber slides pre-coated with 8 μg/ml of C1q or HSA for 30 min were then stimulated with 30 ng/ml LPS for the time stated and nuclear extracts were prepared. A, the relative amount of p50 and p65 in the nucleus was measured using the Trans AM p50 and p65 kit, respectively. Levels of p65 and p50 expressed as average absorbance ± S.D. of triplicate wells. Data are from one experiment, representative of three. B, Western blot analysis of p65 and p50 levels in control and C1q-treated monocytes. C, band intensities were calculated by densitometry and normalized to histone H1. Data shown are representative of three separate experiments.
petition between p65 and pCREB for limiting amounts of CBP (28). Data presented here demonstrate that C1q, in contrast to LPS, stimulated the phosphorylation of CREB. Thus, the C1q-associated inhibition of the NFκB transcription activity could result in part from the higher amounts of pCREB competing with the LPS-induced p65-containing NFκB for the limiting amount of the coactivator CBP.

Whether suppression of LPS-induced pro-inflammatory cytokine expression by C1q is due to the generation of inhibitory p50p50 homodimers (Fig. 8A), activation of CREB leading to depletion of CBP (Fig. 8B), or the combined effect of both
correlate with the ability of C1q to enhance the ingestion of apoptotic cells. Korb and Ahearn (10) first reported the binding of C1q to apoptotic cells. Subsequently, mice in which the C1q gene had been ablated demonstrated a defect in the kinetics of clearance of apoptotic cells, and, on certain genetic backgrounds, showed accumulation of apoptotic bodies in the kidney and susceptibility to autoimmune disorders (11, 49).

Miura-Shimura et al. (50) recently reported that the presence of a unique insertion polymorphism upstream of the C1q gene in NZB mice leads to development of autoimmune disease. This polymorphism was correlated with a decreased synthesis of
C1q upon stimulation of macrophages (50). In humans, individuals deficient in C1q have long been known to develop lupus-like syndromes (if they live through numerous childhood infections) (51). Taken together, it has been postulated that the increased kinetics of ingestion as a result of C1q binding to the newly exposed membrane constituents of apoptotic cells, may contribute to protection against development of autoimmune disorders. Findings presented here suggest that in addition to kinetically enhancing the clearance of apoptotic cells, the presence of C1q on these cells may also contribute to the suppression of pro-inflammatory cytokine production, which may otherwise promote conditions for inducing immune response against self-antigens. This hypothesis is also consistent with the increasing number of reports that demonstrate novel induction of C1q synthesis as a response to injury, such as in virus infection (52), chemical trauma (53), or a consequence of aging and/or abnormal physiologic events such as occur in Alzheimer disease (54–57). This induced C1q expression may be beneficial in rapidly clearing cellular debris and as demonstrated here, modulating inflammatory cytokines.

The ability to contribute to the modulation of phagocytosis or cytokine production is not limited to C1q. Indeed, of the defense collagens that have been assessed, all possess the ability to enhance phagocytosis both in vitro (58, 59) and in vivo (12, 13), and MBL and SPA have been shown to influence cytokine release (60). There have also been many reports in which these postulated innate pattern recognition molecules influence pro-inflammatory cytokines (14, 60–63).

In conclusion, this study has identified novel mechanisms through which C1q can alter inducible gene expression by LPS and may be responsible for modulating pro-inflammatory cytokine production. As it is clear that cytokines play a significant role in directing the host response to infection, as well as in the control of autoimmunity, inflammation, and tissue repair, these findings provide new candidate targets for beneficial interventions in these processes.

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